Funded by tenovus cancer care gofal canser

Human γδ T-APCs: processing of tumour antigens and induction of anti-tumour immunity

Teja Rus

Thesis presented for the degree of Doctor of Philosophy

March 2021

Department of Infection & Immunity

School of Medicine, Cardiff University

Abstract

γδ T cells play an important role in cancer immune surveillance. *In vitro* expanded Vγ9/Vδ2 T cells retain their cytotoxic potential and functionality as assessed by their cytokine secretion (IFNγ and TNFα) and proliferation responses to phosphoantigens (pAgs). Notably, upon activation, Vγ9/Vδ2 T cells act as professional APCs (γδ T-APCs), capable of processing simple and complex microbial antigens, and inducing responses in CD4 and CD8 αβ T cells. However, their capacity to process tumour antigens and to induce anti-tumour T cell responses remain poorly understood.

Here, the methods for generating tools necessary to study the processing and cross-presentation in Vγ9/Vδ2 T cells are described. Namely, I have optimised a method for purification of recombinant tumour antigen NY-ESO-1 in a single chromatography step and established a protocol for expression and purification of recombinant 5T4 antigen from the bacterial system. Furthermore, I describe a method for the generation of functional 5T4 specific CD8 T cells within 4-6 weeks, which may be used for the generation of other "difficult", self-antigen reactive CD8 T cell clones and lines. Importantly, I discuss a novel protocol for the generation γδ T-APC with an optimal phenotype. My findings demonstrate that briefly stimulated Vγ9/Vδ2 T cells, but not expanded Vγ9/Vδ2 T cells, become robust pro-APCs, capable of cross-presenting tumour antigen and inducing moderate IFNγ responses in cognate CD8 T cells. Results presented here suggest that γδ T-APCs as well as expanded Vγ9/Vδ2 T cells readily take-up parts of cancer cells for processing and successfully cross-present tumour antigen. The data show that zoledronate or γ-radiation-sensitised cancer cells enhanced the APC phenotype and function in expanded Vγ9/Vδ2 T cells. Sensitised cancer cells induced expression of CD36 scavenger receptor involved in endocytosis of extracellular material. Further studies are required to confirm receptors/signalling involved in these processes.

In conclusion, my findings confirm that γδ T-APCs are indeed capable of efficient cross-presentation of tumour antigens (5T4). Further studies are required to expand these findings to other types of tumour antigens (such as NY-ESO-1) and *in vivo* tumour models. As evidenced by my *in vitro* studies and previously reported findings, γδ T-APCs show great potential in cancer immunotherapy. The ease of efficient *ex vivo* expansion, safety, and robust antigen-presenting capability makes Vγ9/Vδ2 T cells a promising alternative to dendritic cells.

Acknowledgments

I want to thank the Tenovus cancer charity who generously funded this research and has made this thesis possible.

To my supervisor, Prof. Bernhard Moser, thank you for giving me the opportunity to enter the field of immunology, undertake this exciting project and for helping me to develop as an independent scientist. Thank you for your encouragement and your critical advice along the journey. Likewise, I would like to thank my co-supervisor, Prof. Matthias Eberl, for helpful suggestions, constructive advice and meticulous attention to detail. Thank you both for taking time and patience in reading and editing my thesis, all the stimulating lab meetings, and feedback during presentations and discussions.

Special thanks to Dr Amanda Tonks for all sincere help and support, encouragement, good offices, and help with the bureaucratic affairs, which massively contributed to bringing this thesis to life.

I would like to acknowledge the advice, help and reagents I received from Dr Garry Dolton, for advice on tumour-specific T cell cloning cultures; Dr Sarah Galloway and Dr Alex Greenshields-Watson for help with ELISpot assays; Dr Katie Thungatt for tetramer staining; Dr Rebecca Bayliss for the reagents and advice with DCs; Dr Wioleta Zelek, Dr Rowan Paul Orme, and Andy Thomas, and Prof. Giulio Spagnoli for all the help and advice with protein purification. I would like to thank Dr Eddy Wong and Dr Virginia-Maria Vlachava for valuable discussion and advice on ICS, Dr Catherine Naseriyan (CBS) for cell sorting, and Dr Ian Brewis and Swee Vickie Nixon (CBS) for mass spectrometry. I would also like to thank Dr Svetlana Hakobyan for letting me “move-in” the 4th-floor labs to complete my protein work.

To all the past and present members of the Moser/Eberl group, thank you for help, discussions, conferences, funny Christmas dos and an odd pint. I would like to thank everyone in my office and all the 4th-floor people at the Henry Welcome Building for making a pleasant working environment.

I would like to express my gratitude to my family for all their support and encouragement over the years. To my friends, thank you for your constant encouragement, making sure I had a life outside the lab too, and keeping me sane whilst preparing this thesis.

And finally, to Federico, thank you for showing a genuine interest, endless understanding and for caring about what I do. You always believed in me and never questioned my path. Thank you for all your support, encouragement and for putting up with my ups and downs, many long nights in the lab and weekends spent reading and writing.

Table of contents

List of figures

List of tables

Abbreviations

2-ME -	β-mercaptoethanol (2-mercaptoethanol)
ANOVA-	analysis of variance
mAb –	monoclonal antibody
ACT -	Adoptive cell transfer
ACPA -	Antigen cross-presentation assay
ADCC -	Antibody-dependent cellular cytotoxicity
AML -	Acute myeloid leukaemia
ATCT –	Adoptive T cell transfer
APC -	Allophycocyanin
APC -	Antigen Presenting Cell
ApppI -	triphosphoric acid 1-adenosin-5′-yl ester 3-(3- methylbut-3 enyl) ester
BCG -	Bacillus Calmette-Guerin
BP -	Bisphosphonate
BrHpp -	Bromohydrine pyrophosphate
BSA -	Bovine Serum Albumin
BTN -	Butyrophilin
CD -	Cluster of Differentiation
CFSE -	5-(and-6-)-carboxyfluorescein succinidimyl ester
CMV -	Cytomegalovirus
CTLA-4 -	Cytotoxic T-lymphocyte-associated protein 4
c-SMAC -	Central-SMAC
CTL -	Cytotoxic T Lymphocytes
DC -	Dendritic cell
DAMPs -	Danger Associated Molecular Patterns
DMAPP -	Dimethylallyl Pyrophosphate
DNA -	Deoxyribonucleic Acid
ECM -	Extracellular matrix
EDTA -	Ethylenediaminetetraacetic Acid
ER -	Endoplasmic Reticulum
Eomes -	Eomesodermin transcription factor
EPCR –	Endothelial protein C receptor
ETP -	Early thymocyte progenitor
FACS -	Fluorescence Activated Cell Sorting
FcR -	Fc Receptor
FCS -	Foetal Calf Serum
FITC -	Fluorescein Isothiocyanate
FOXP3 -	Forkhead Box P3
FPPS -	Farnesyl Pyrophosphate

FSC -	Forward Scatter
FluM1 –	Influenza matrix protein 1
GATA-3 -	GATA binding protein 3
GvHD -	Graft-versus-host-disease
Her2 –	Human epidermal growth factor receptor 2
HIV-	Human immunodeficiency virus
HLA -	Human Leukocyte Antigen
HMB-PP -	(E)-4-hydroxy-3-methyl-2-butenyl Pyrophosphate
ICAM-1 -	Intercellular Adhesion Molecule-1
ICOS -	Inducible Co-stimulator of Signalling
ICOS-L -	Inducible Co-stimulator of Signalling Ligand
iDC -	Immature DC
IFNγ -	Interferon gamma
IFNγ-ICS -	IFNγ -intracellular staining
IFNAR1-	Type I interferon receptor1
Ig -	immunoglobulin
IL –	Interleukin
IPP -	Isopentenyl Pyrophosphate
IS -	Immunological synapse
IV -	intravenous
KIR -	Killer cell immunoglobulin-like receptors
LAG-3-	Lymphocyte-activation gene 3
LAMP-1 -	Lysosomal-associated membrane protein 1
LFA-1 -	Lymphocyte Function-Associated Antigen 1
LN -	Lymph Node
LPS -	Lipopolysaccharide
mAb -	Monoclonal Antibody
MFI -	Median Fluorescence Intensity
Mio -	Million
MHC -	Major Histocompatibility Complex
MLR -	Mixed Lymphocyte Reaction
MMP-	Matrix metalloproteinase
MØ -	Macrophage
Mono -	Monocyte
mo-DCs -	Monocyte-Derived Dendritic Cell
MYD88 -	Myeloid differentiation primary response 88
NCR –	Natural cytotoxicity receptor
NBP –	aminobiphosphonate
NFκB -	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1
NK Cells -	Natural killer cells

NKG2D-	Natural killer group 2, member D
NKp46 -	Natural killer cell p46-related protein
NKR –	Natural killer receptor
NKT cell –	Natural killer T cell
OVA -	Ovalbumin
OX40L -	OX40 ligand
PAMPs -	Pathogen Associated Molecular Patterns
PBMC -	Peripheral Blood Mononuclear Cells
PCR -	Polymerase Chain Reaction
PE –	Phycoerytherin
PDAC –	pancreatic ductal adenocarcinoma
PD-1 -	Programmed cell death protein 1
PD-L1 -	Programmed cell death protein 1 ligand
PGN –	Peptidoglycan
PMA -	Phorbol 12-myristate 13-acetate
PRR -	Pattern Recognition Receptor
qPCR -	Quantitative PCR
RA -	Retinoic Acid
RNA -	Ribonucleic Acid
rpm -	Revolutions Per Minute
RT-PCR -	Real Time PCR
RT -	Room Temperature
SFC -	Spot forming cell
SNP -	Single-nucleotide polymorphisms
SSC -	Side Scatter
Stat1 -	Signal transducer and activator of transcription 1
Tbet -	T-box transcription factor T-box 21
Tc cell -	Cytotoxic T Cell
Tc1 –	Cytotoxic T 1 cell
Tc2 –	Cytotoxic T 2 cell
Tc17 –	Cytotoxic T 17 cell
TCM -	T Central Memory Cell
TCR -	T Cell Receptor
TEM -	T Effector Memory Cell
TEF -	effector T cell
TTE -	Terminally Differentiated effector T cell
TSCM –	Stem cell memory like T cell
TGFβ -	Transforming growth factor beta
Th1 -	T helper 1 cell
Th2 -	T helper 2 cell

Th9 –	T helper 9 cell
Th17 -	T helper 17 cell
TIGIT -	T cell immunoreceptor with Ig and immunoreceptor tyrosine-based inhibition motif domains
TILs -	Tumour-infiltrating lymphocytes
Tim3 -	T cell immunoglobulin- and mucin-domain-containing molecule-3
TLR -	Toll-like receptor
TME -	Tumour microenvironment
TNFα -	Tumour necrosis factor-α
TRAIL –	Tumour necrosis factor-related apoptosis inducing ligand
Treg -	Regulatory T cell
ULBPs -	UL16-binding proteins
WT -	Wild type
ZOL –	zoledronate

Chapter 1 Introduction

1.1 Innate and adaptive arms of the immune system

All multicellular organisms have some form of immunity. One of the well-recognised functions of the immune system is to protect the organism from invasion and damage imposed by pathogens or infectious agents. Another equally important function is the maintenance of the organismal tissue homeostasis, either by tissue repair and/or by removal of dysregulated (e.g. neoplasia) and dying cells. Fundamentally, the biology of the immune system extends beyond classical views of immunity to metabolic homeostasis, tissue remodelling, and dialogue with the nervous system. In fact, 'the danger model' proposed to describe the immune system in the early 1990s presumed that the immune system preferentially responds to damage over foreignness (Matzinger, 1994).

Historically, the immune system has been defined according to binary classification. In vertebrates, the immune system has been divided into two conceptual groups, the innate and the adaptive systems, by Charles Janeway Jr (Janeway Jr. and Medzhitov, 1998). These two immune systems have been characterised by the speed of the response and the level of their specificity. According to traditional definitions, the innate immune system should provide the initial non-specific, rapid defence against a wide range of pathogens and dysregulated cells, some of which can be cleared by the innate immune system alone. Furthermore, it has been generally assumed that repeated exposure to the same challenge does not alter the nature of the subsequent response, and immunological memory does not develop.

In contrast, the adaptive immune system mounts a target-specific response, and it can take days to be initiated, but after a particular target has been encountered by the adaptive immune system, a part of the target-specific cells persist as memory cells. These memory cells can remember the previous response and rapidly proliferate, responding strongly and rapidly to the same subsequent infections or neoplastic transformations. Thus, a long-accepted hallmark of the adaptive immune system is its ability to develop immunological memory. However, with more emerging studies within the past two decades, these traditional assumptions of the immune system have been challenged as we are starting to understand that the cells of both arms of the immune system are rather plastic. With the identification of innate cells that are capable of memory (O'Leary et al., 2006; Pita-López, Pera and Solana, 2016), and subsets of adaptive cells expressing conserved "innate" receptors and functions (Bendelac, Bonneville and Kearney, 2001; Vivier *et al.*, 2011; Lanier, 2013; Godfrey et al., 2015; Liuzzi *et al.*, 2015) we have begun to learn that these systems are overlapping. What is clear is that cells of "adaptive and innate immune systems" act in concert to mount a strong target specific or damage specific response.

For a long time, the immune system has been thought to discriminate between 'self' and 'non-self', with foreign antigens eliciting an immune response and self-antigens being subject to immunological tolerance (Garg and Agostinis, 2017). However, this model has proven to be over-simplistic, as it predicts that only microbial non-self-particles are a source of pathogen recognition receptor (PRR) ligands and thus rules out the existence of endogenous PRR ligands derived from self- cells. New models have since been proposed, termed the pattern recognition receptor model and 'the danger model'. Both

of these models have a common ground and ultimately hypothesise that the immune system responds to 'dangerous' or 'damaging stimuli. The execution of cellular immune response is a complex process involving many different cell types and multiple steps. In that, antigen presenting cells (APCs) seem to play a critical role in establishing immune responses to dangerous/damaging signals. APCs capture, process and present antigens to T cells for their activation. At a basic level of the historical adaptive immunity, the initial response is initiated by APCs migrating from the site of infection/tissue damage to the secondary lymphoid organs to present captured antigens to naive T cells.

On the other hand, naïve T cells continuously circulate between lymph nodes to inspect presented antigens. T cells can then become effector cells to eradicate the infection and/or become memory cells for rapid response to re-exposure to the antigen. These steps of establishing the immune response from the point of view of protein antigen presentation and induction of CD8 T cells response will be described in more detail in this introductory chapter. Moreover, particular attention will be given to the biology and function of CD8 αβT cells and Vγ9/Vδ2 T cells, while the final section will touch on the role of the immune system in cancer and therapy. The description of the immune system in this thesis refers to the human immune system unless otherwise stated.

1.2 Thymic T cell development and selection

Generally, all T cells begin life as lymphoid progenitors, and their development occurs in a specialized organ, the thymus. Thymic development of T cells is a very complex multistep process (Germain, 2002; Zúñiga-Pflücker, 2004; Hayes, Li and Love, 2005; Xiong and Raulet, 2007; Dooley, Linterman and Liston, 2013; Muñoz-Ruiz *et al.*, 2016) that goes beyond of the scope of this thesis, and thus only the key ideas will be briefly described here. T cell development starts with the relocation of the haematopoietic stem cell (HSC)-derived lymphocyte progenitors from the bone marrow (adults) or liver (neonates) into the thymus. As HSCs progenitors enter the cortical region of the thymus, they become early thymocyte progenitors (ETPs) and begin a process of proliferation and maturation. To become T cells, ETPs require a specific, complex microenvironment, the thymic stroma, which structurally supports migrating ETPs and provides guidance through chemokines produced by different stromal cell subtypes (Zúñiga-Pflücker, 2004). Early in development in the cortex, thymocytes are referred to as double-negative (DN), lacking expression of the CD4 and CD8 coreceptors involved in αβTCR signalling. Basically, from an αβT cell perspective, thymic development is divided into three major stages based on the expression of surface markers: (i) the CD4- CD8- double-negative (DN) stage; (ii) the CD4+ CD8 double-positive (DP) stage and (iii) the CD4+ CD8- or CD8 CD4- single-positive (SP) stage. In addition, the DN stage can be further divided into DN1, DN2, DN3 and DN4, in which T cell lineage fate takes place. During DN1 stages, chemokine signalling provided by thymic stromal cells and CCR9/CCR7 expression in ETPs initiates genetic programs, directing their development into T cell lineage via Notch signalling (Taniuchi, 2018). In DN1, thymocytes still have the potential to become non-T lymphoid cells, including NK cells, dendritic cells, macrophages, and B cells. Soon after commitment to the T cell lineage, thymocytes undergo a binary cell fate decision with the choice to become either an αβ or a γδ T cell. The αβ/γδ lineage commitment occurs in DN2-DN4, where there are two pre-TCR isoforms expressed on the surface of immature DN thymocytes; a TCRβ chain paired with

an invariant pre-TCRα (pTα) chain, and the γδ TCR. While the mechanism behind αβ/γδ lineage decision is a complex topic, what is clear is that the TCR plays a critical role in both αβ and γδ T cell development. A few models of T cell development into αβ – γδ linages have been proposed (Turchinovich and Pennington, 2011). A 'signal-strength' model proposed by Hayes and colleagues (Hayes, Shores and Love, 2003; Hayes, Li and Love, 2005) was best supported by data suggesting TCR signal strength is the determining factor in αβ/γδ lineage commitment. In this model, under certain circumstances, depending on strong vs. weak signalling, pre-, αβ-, and γδ TCRs are all capable of delivering signals to direct the fate of thymocytes to either γδ TCR or αβTCR lineages. Strong signalling through a γδ TCR promotes the adoption of the γδ lineage, whereas weaker signals lead to the adoption of the αβ lineage, irrespective of the TCR isotype from which those signals originate. Conversely, weak αβTCR signals also promote the adoption of the γδ lineage.

1.2.1 "Conventional" αβT cell development

fter the αβT cell linage commitment, further αβT cell development happens around the transition from DN to DP stage. αβTCR cells then migrate between cortical thymic epithelial cells and dendritic cells, auditioning for the ability to recognise self-pMHC. First, in a process called positive selection, cells with TCR conformations that are non-responsive to self-peptide–MHC complexes (self-pMHC) are eliminated. Then these positively-selected thymocytes begin a journey into the medulla for negative selection, a process that eliminates overly reactive cells to self-pMHCs so to prevent autoimmunity. As they move through the outer capsule of the thymus into the medulla, they complete negative selection through interactions with medullary thymic epithelial cells and dendritic cells. There is evidence that DP cells with non-functional TCR can undergo repeated TCRα rearrangements (Petrie *et al.*, 1993) to re-audition. The αβTCR thymocytes, which recognise self-peptides presented by MHC I or MHC II with acceptable affinity/reactivity, will develop into either the CD8 or CD4 SP T cells, respectively (Zúñiga-Pflücker, 2004; **Figure 1.1).** To add an extra level of complexity to this process, thymic differentiation crucially relies on the microRNA network from the earliest throughout the last stages of development (Dooley, Linterman and Liston, 2013). Finally, most SP T cells will be exported into the peripheral blood as naive T cells. Interestingly, some CD8 SP thymocytes may, however, already acquire a memory phenotype in the thymus under the influence of IL-4 production, presumably in the absence of classical antigen-mediated differentiation (Weinreich *et al.*, 2010).

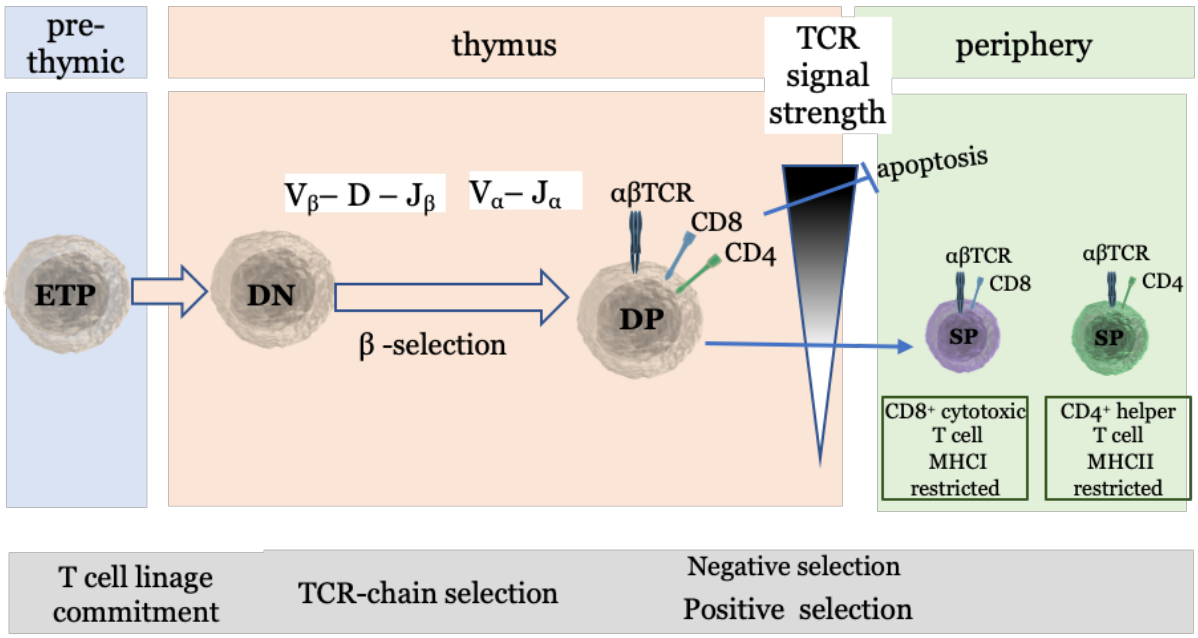


Figure 1.1. A Simplified illustration of thymic development of αβT cells.
Several stages of αβ thymocyte differentiation can be distinguished based on the expression of CD4 and CD8 coreceptors. In the thymus, CD4-CD8 double-negative (DN) thymocytes transition into the double-positive (DP) stage. αβ T cell repertoire is shaped by a process known as a positive selection during the DP to single-positive (SP) transition. At this stage, DP thymocytes that express a high-affinity T-cell receptor (TCR) for self-peptides are potentially harmful (autoreactive) thus are deleted by negative selection. DP thymocytes that express TCRs that do not recognise self-peptide–MHC complexes are deleted in a process known as 'death by neglect' (not shown). Positive selection correlates with a commitment to the CD4+ or CD8+ T cell subsets. Thymic fate decision checkpoints in the thymus are shown in blue in the bottom panel.

1.2.2 γδ T cell development

Unlike the development of αβT cells, the development of human γδ T cells has only just now been better understood. This can account for experimental difficulties and limited access to tissues; there was no accepted approach for stage-wise assessment of thymic γδ T cell development. While studies have analysed Vγ usage, effector potential capabilities, gene transcription, and surface marker expression, none of the study approaches combines all parameters. Most of the γδ T cells in the thymus and spleen do not express either CD4 or CD8 (Tonegawa *et al.*, 1989). The studies in mouse models showed that γδ T thymocytes that have passed through γδ-selection, unlike their αβ-counterparts, appear not to undergo classical ligand driven positive/negative selection in the thymus. Thymic epithelial cells are thought to provide ligands for γδ TCRs, which shape some of the γδ T cell compartments. However, the role of ligands in shaping the γδ TCR repertoire remains poorly understood and largely controversial. This may be partly due to the lack of known potential selecting ligands that could be used to address this question and (until recently) due to the lack of known antigen-presenting molecule(s), and in dissimilarities between human and mouse models. However, there is some evidence for agonist selection of several γδ T cell subsets (Wencker *et al.*, 2014; Fahl *et al.*, 2018).

In human, butyrophilin (*BTN)* genes may provide a universal mechanism by which blood γδ T cell compartments get shaped and regulated. In fact, BTN3A1 and BTN2A1 are critical for "presentation" of phosphoantigens to Vγ9/Vδ2 T cells and their activation. While it seems that the development of γδ T cell repertoire does not call for MHC molecules nor CD1 molecules, they may potentially provide clone-specific ligands (Hayday and Vantourout, 2013; Vantourout and Hayday, 2013). Similarly, the development of several tissue-specific γδ T cells (γδIELs) compartments depends on the expression of butyrophilin-like (Btnl) (mouse) or BTNL (human) genes in tissue-specific epithelial cells, keratinocytes and enterocytes. These epithelial cells thus provide ligands and selectively regulate the development of γδIELs (Di Marco Barros *et al.*, 2016; Melandri *et al.*, 2018). For example, BTNL3 and BTNL8 expressed by human gut epithelial cells jointly determine selective TCR-dependent responses and the biological activity of human colonic Vγ4T cells (Di Marco Barros *et al.*, 2016). Stress antigens have been regarded potential ligands, shaping γδIEL cell compartments against dysregulated tissues, including cancer cells. What is clear is that a conserved extrathymic mechanism for shaping local γδ T cell compartments exist and can be provided by organ-specific epithelia. And this suggest a broader utilization of *BTN*/*BTNL* genes in γδ biology.

1.2.3 TCR

1.2.3.1 Structure of TCR

TCRs are heterodimers built of two protein chains: TCRα + TCRβ, and TCRγ + TCRδ. Each of these chains is a type-I transmembrane spanning glycoprotein, and the pairs are linked together with disulphide bonds. Like other activating immune receptors, each heterodimer has an extracellular, transmembrane, and intracellular region. Moreover, each heterodimer is composed of an individual variable domain (ligand-binding) and constant domain (signalling units), joined by a hinge region with an intra-chain disulphide bond. (Rudolph, Stanfield and Wilson, 2006). The constant domains are linked to the transmembrane region, while the variable domains (V) attach to the constant domain at the C terminus of constant domains. Three hypervariable hairpin loops referred to as the complementarity determining regions (CDRs; CDR1-3) – form at the C-terminal end of each variable domain (Vα, Vβ, Vγ, Vδ) and are responsible for the binding of the TCR to its cognate peptide -MHC complexes (ligands). Thus, each TCR binds the ligand via 6 CDR loops (**Figure 1.2A**). These TCR-ligand interactions are flexible as TCRs undergo large conformational changes to accommodate binding to their ligands (Attaf *et al.*, 2015). Because a well-defined contact with peptide-MHC complexes is essential, the length of the CDR3s of both α and β chains is restricted. In contrast, CDR3δ are longer and more variable than CDR3γ (Rock *et al.*, 1994). The CDRs form loops in the γδ TCR structure to provide a highly variable antigen-binding domain at the membrane-distal end of the molecule. This greater variability may allow for the recognition of both proteins and smaller molecules. Because of that, the γδ TCR resembles the BCR more than αβTCR..

1.2.3.2 TCR repertoire diversity

The construction of the TCR is a crucial stage of T cell development and occurs in DN2-DN4 stages of thymic development through a stochastic process of gene rearrangement, such that each T cell generally expresses a single type of TCR (Tonegawa *et al.*, 1989). In the blood of an adult human, there are about 4 x 10^{11} T cells. A group of cells with the same TCR defines a T cell clonotype, so groups of T cells with different TCRs in the body can be considered a repertoire of clonotypes. T cell repertoires with extensive antigen specificity arise from clonotypic rearrangement of the gene segments that encode the TCR-CD3 complexes (Davis and Bjorkman, 1988). The rearrangement of the TCR β-, δ- and γ-chain gene segments occurs at the DN2 to DN3 transition, while the TCR α-chain gene rearrangement occurs later. Briefly, the constant (C) and hinge (H) region of each TCR chain are encoded by distinct exons of the C gene, whereas the transmembrane and cytoplasmic domains are encoded by a single exon of the C gene. The variable domains (ligand-binding domain) of each TCR chain are assembled following rearrangement of the variable (V), diversity (D), and joining (J) gene segments in a multistep process called V(D)J recombination (**Figure 1.2 B**).

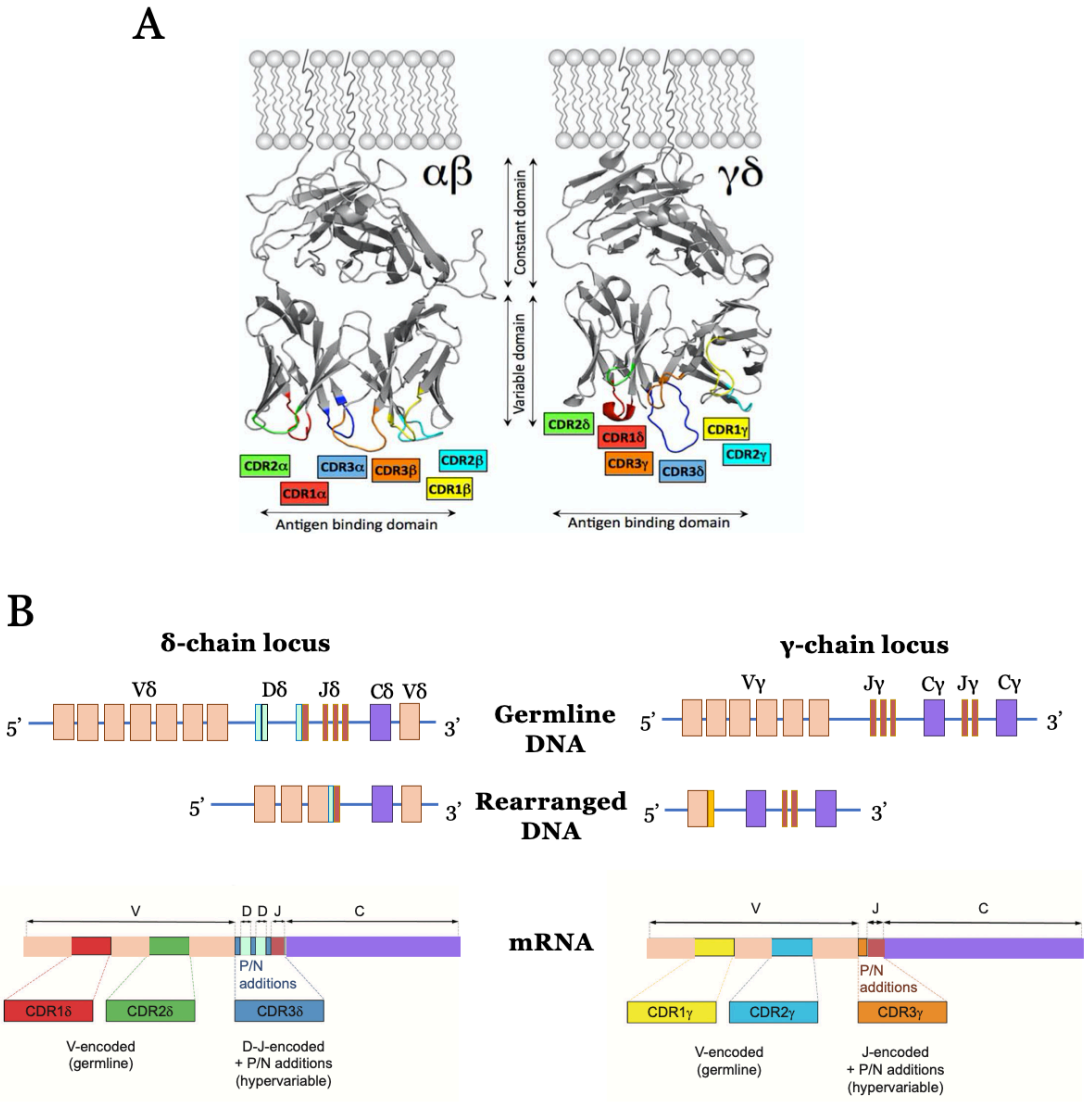


Figure 1.2. TCR structure and V(D)J gene rearrangement.

(**A**) αβ and γδ TCR structures. (Protein Data Bank (PDB): 3HG1) (PDB: 1HXM). Both TCRs show similar tertiary structures, with complementarity-determining regions (CDR) loops located at the distal end. The six CDR loops (CDR 1-3 of each α, β, or γ, δ chains) form the antigen-binding site and differ between the two TCRs. The grey coloured structures represent the constant and variable domains of the TCRs (without CDR loops) (**B**) V(D)J gene rearrangement and expression of TCR γ – and δ -chains. Only the functional gene segments are shown. The TCRγ chain is produced using only single V-J recombination, with P/N additions occurring at the V-J junction. The TCRδ chain is produced using V-D-J rearrangement that can involve either 2 or 3 D segments, leading to the creation of up to 4 N diversity regions. CDR are areas of hypervariability as a result of the addition of P/N nucleotides. CDR1, CDR2 and CDR3 are highlighted in red/yellow, green/turquoise, blue/orange, respectively, on the schematic representation of V(D)J recombination and ribbon structure of a TCR in (A). Adapted from Attaf *et al.*, 2015.

RAG1 and RAG2 enzymes are selective for recombination signal sequences that flank the V, D, and J segments. First, the N-terminal of the variable TCR domain is formed by the recombination of one of many V segments and one J segment; an additional D segment is added in the case of the TCRβ- and TCRδ chain. The TCR diversity arises in the hypervariable CDR loops that frame the central contact

points with their ligands. The CDR1 and CDR2 are encoded within the germline V gene segments of the TCR, whereas the hypervariable CDR3 loops are formed by the random addition or deletion of the template and non-template nucleotides in the junction of V and J (in TCRα or γ), or V, D and J (in TCRβ and δ) by the segments DNA-repair machinery. Thus, the TCR diversity happens not only because of the paring of different TCR chains but also because of the junctional diversity created by the inclusion of random nucleotides during recombination of the coding nicked gene segments (Davis and Bjorkman, 1988).

The theoretical number of possible TCRδ gene rearrangements is of the order of 10^{13}, and the hypothetical total γδ TCR diversity is ~10^{17} (counting the additional diversity of >10^4 pairings of TCRγ genes) (Sebestyen *et al.*, 2020). Regardless of this potential huge variety in the γδ TCR repertoire, there is only a limited number of different γδ T cell populations, which are typically delineated by the type of the specific Vδ segments used for TCRδ rearrangements in humans. The preferential pairing of particular Vγ with Vδ chains is noted in different peripheral tissues in both mice and human γδ T cells (Hayday, 2000; Bonneville, O'Brien and Born, 2010; Silva-Santos, Serre and Norell, 2015) (**Table 1.1**). This additional restriction of specific V segments in different tissues greatly reduces the V domain diversity within γδ T cell populations (Kazen and Adams, 2011).

Table 1.1. Repertoire of γδ T cells in humans

Peripheral location	Predominant usage (and pairing, if applicable) of Vγ and Vδ gene	V(D)J diversity	Key cytokines produced[#]
Blood	Vγ9/Vδ2	Intermediate	IFNγ, TNFα, IL17
Thymus	Vδ1	Not defined	IFNγ and IL17
Spleen	Vδ1	High	IFNγ, TNF, IL17
Liver	Vδ1 and Vδ3	High	Not defined
Gut Epithelia	Vδ1 and Vδ3	High	Not defined
Dermis	Vδ1	High	IFNγ and TNF, IL17

Adapted from Hayday, 2000; Bonneville *et al* 2010; Silva-Santos *et al* 2015

*The nomenclature used for human γδ T cell receptor genes is based on the Lefranc and Rabbits' system. IFNγ, interferon-γ; IL, interleukin; TNF, tumour necrosis factor.

[#] depending on the inflammatory condition

However, overall marked differences between the γ and δ chain usage are noted. Most of the γδ T cells in the circulation are oligoclonal and use the same Vγ and Vδ gene segments, —Vγ9 and Vδ2 — with limited junctional diversity (Casorati *et al.*, 1989). Recently it has been reconfirmed that combination of the Vγ9 and Vδ2 TCR chains, with a high frequency of the Vγ9–Jγ1.2 Vδ2 usage, dominate the adult human blood γδ TCR repertoire (De Rosa *et al.*, 2004; Gu *et al.*, 2018). Interestingly, a great proportion of these Vγ9/Vδ2 T cells use identical germline TCRγ gene rearrangement of Vγ9 and JγP without the classic addition of nucleotides at the V–J junction. Most of the γδ T cells that carry such Vγ9/Vδ2 TCRs

composed of such canonical and similar Vγ9JγP variants are generated before birth and expand during the first years of life (Dimova *et al.*, 2015). Strikingly, this rearrangement is shared between all humans, thus is "public", and takes over most of the TCRγ sequences in blood. In contrast, TCRδ genes show greater clonal diversity in both cord blood and adult samples. Consistent with that, TCRδ chain repertoires are mostly "private", particularly in the Vδ2 negative T cell compartment; whereas shared or "public" TCRγ chain sequences (Vγ9JP sequences in adults) are found within unrelated donors (both, cord blood and adult). The preference for Vγ9/Vδ2 rearrangement is likely driven by postnatal proliferation in response to environmental and pathogen-derived ligands Furthermore, the γδ T cell repertoire may also be influenced nutritionally by various plant-derived molecules, such as alkylamines and polyphenols (Bukowski, Morita and Brenner, 1999; Holderness *et al.*, 2007) (**Figure 1.3**). Eventually, Vγ9/Vδ2 T cells display a memory phenotype and become the dominant circulating γδ T cell population in adult blood (Caccamo *et al.*, 2006).

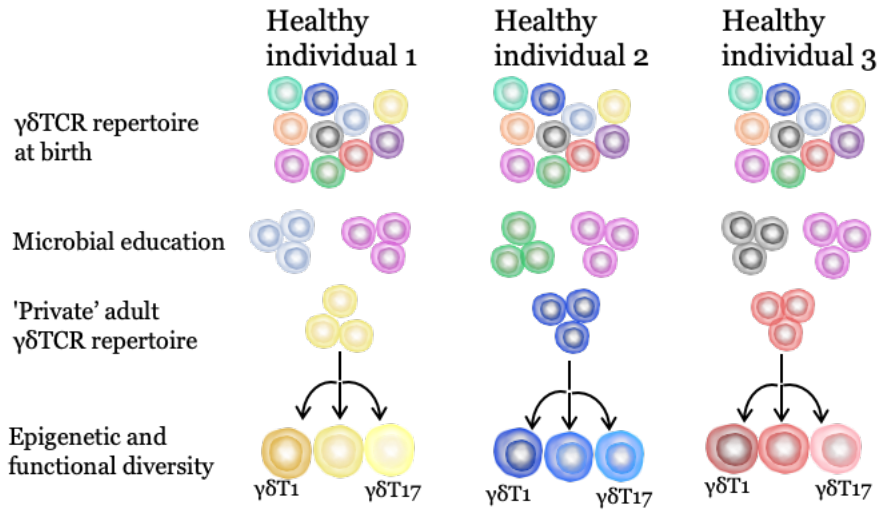


Figure 1.3. A Simplified illustration of γδ T cell repertoire shaping in healthy individuals. γδ T cell repertoire shapes as an individual encounters microorganisms throughout their childhood (microbial education). Environmental factors will stimulate the expansion of some γδ T cell clonotypes to form "public" repertoire (e.g. Vγ9/Vδ2 TCR; pink cells), while each adult also has their unique 'private' γδ T cell repertoire in terms of γδ TCR diversity and epigenetic and functional diversity. Different cell colours indicate expression of defined γδ TCRs. Different shades of the same colour indicate γδ T cells with different functional characteristics (e.g. but not limited to γδ T1, γδ T17 cells.

1.3 "Conventional" CD8 αβT cells

1.3.1 Antigen recognition by CD8 T cells

Each individual T cell can be thought of as a computer node with modular biosensors continuously scanning the environment regarding the quantity and quality of antigens displayed on the surfaces of other cells. The information obtained determines the T cell fate decisions and whether a naive T cell will replicate, and it directs the differentiation path of daughter cells and the strength of the overall response. Naïve T cells constantly circulate between the blood and secondary lymphoid organs such as LNs, Peyer's patches (PPs), and the spleen. When lymphocytes enter the T-cell zones, they move randomly over dense networks of DCs and fibroblastic reticular cells. The first challenge for a T cell entering the T cell area of secondary lymphoid tissues is to find its cognate antigen on the surface of an APC. The chemokine signals guide the migration and positioning of T cells through the strategic distribution of APCs. This migration is driven by CCR7-binding chemokines. In addition to CCL21, other chemokine fluxes in lymph nodes may coordinate specific encounters between cells. It is generally accepted that after a TCR contact with a cognate antigen presented by MHC, naïve T cells are activated, proliferate rapidly, and acquire effector functions after a few days. To note, T cell priming does not necessarily have to occur in lymph nodes. Interestingly, activation of T cells by pro-APCs can also occur extranodal in ectopic lymphoid tissues (aka tertiary lymphoid tissues (TLOs) at the site of infection, within chronically inflamed tissues and in the tumour microenvironment (TME) (Chen *et al.*, 2013). For example, Peyer's patches (PPs) also represent important antigen sampling and immune inductive sites.

1.3.2 CD8 T Cell activation & response

The mode of CD8 T cell recognition of the pMHC complexes directly impacts its fate, including activation or anergy and lineage differentiation. The outcomes are influenced and directed by the interactions of T cells with the target cell and by the biological condition they find themselves in. During T cell activation, the information exchange within the immune synapse is crucial for the T cell fate.

T cells require three distinct signals for their optimal activation and responses. TCR -pMHC complex interactions (signal 1) and co-stimulation between a variety of co-stimulatory receptors and ligands expressed by the T cell and APC, respectively, such as CD28 -CD80/CD86 and CD27-CD70 interactions (signal 2) are crucial signals involved in initial priming of naive CD8 T cells. The co-stimulatory interactions provide a critical secondary signal for T cell activation. The third signal is provided by cytokines and is crucially responsible for full activation of T cells, directing T cell differentiation and appropriate functional responses (signal 3). **Figure 1.4** illustrates the receptors and ligands that constitute the three signals. The mode of each of the three signals in CD8 T cell activation is described in more detail below.

1.3.2.1 Signal 1

The duration of TCR stimulation, governed by TCR-peptide affinities, represents the driving force for T cell activation, differentiation, and ultimately death. However, TCR-pMHC interactions alone are not sufficient to provide this activation signal to the T cell. Instead, the CD8 coreceptor is involved in binding to the pMHC-complex and plays a fundamental role in initiating downstream signalling. Thus, the CD8 coreceptor provides the most important signal for the full activation of T cells, growth and production of regulatory cytokines such as IL-2 (Lopez de Castro, 1989; Park *et al.*, 2007; Laugel *et al.*, 2011; Stone *et al.*, 2011). On the other hand, TCR affinities play a crucial role in dictating the responsiveness and differentiation of CD8 T cells, and marked differences in the TCR affinities are observed among CD8 T cells. Typically, though, when a T cell interacts with an APC, it is exposed to a diverse set of pMHC complexes, most of which are non-stimulatory. Furthermore, it has been shown that affinity and strength of CD8 coreceptor for MHC/TCR complex is plastic and can be tailored by the cell to fine-tune its responsiveness (Lopez de Castro, 1989; Alexander-Miller *et al.*, 1996; Park *et al.*, 2007; Stone *et al.*, 2011; Anikeeva *et al.*, 2012; Harland *et al.*, 2014).

1.3.2.2 Signal 2

CD8 T cells that receive a signal via antigen (Ag) and receptor co-stimulation (signal 2) undergo programmed cell division. Signal 2 can be provided many co-stimulatory as well as coinhibitory receptors (**Figure 1.4**). Two of the critical signals required for activation of naïve T cells are provided by CD28- and CD27- receptors, which belong to the immunoglobulin (Ig) superfamily (IgSF) and tumour necrosis factor receptor (TNFR) superfamily, respectively. Most of the other co-signalling molecules are members of these two big receptor superfamilies; inducible T-cell costimulator (ICOS, CD278) also belong to IgSF, while 4-1BBL (CD137), GITR (CD357), OX40 (CD134), CD40 belong to TNFRS. The expression of these co-stimulatory receptors is absent but inducible on conventional T cells in the first days following initial activation (Pollok *et al.*, 1993).

In contrast, CD27 and CD28 are constitutively expressed on naïve T cells and get transiently downregulated as T cells develop towards TTE cells. The ligand for CD27, CD70, is expressed by T cells, B cells and DCs, and its expression is primarily controlled by antigen receptor and TLR stimulation in humans and mice. Stimulation by CD70 on APCs supports CD8 T cell cross-priming, differentiation and anti-tumour immunity (Buchan *et al.*, 2018). Alongside TCR ligation, CD28 is a vital costimulatory molecule required for the activation, growth and survival and is required for maintenance of late T cell proliferation (Schwartz, 1992; Lucas *et al.*, 1995). In αβT-cells, CD28 lowers the threshold for T-cell activation via TCR and enhances proliferation and differentiation.

Co-signalling receptors/ligands have been identified on almost every cell type, and they provide important signals during T cell priming, proliferation, and memory/effector formation. However, the expression of their ligands has been best described on pro-APCs. On one hand, as protection from immune response to harmless environmental antigens, the activation of mature T cells without signal 2 stimulation results in anergy and clonal deletion. Thus, should a naïve T cell only encounter cognate

antigen on APCs, without co-stimulation, it either goes into a nonresponsive state (anergy) or dies via apoptosis (Parish and Kaech, 2009).

Programmed cell death 1 ligand 1 (PDL1) and PDL2 expressed on other cells inhibit the function and proliferation of PD1-expressing activated T cells. CTLA4 binds with greater affinity than CD28 to CD80 and CD86 on APCs, thus preventing costimulatory signalling and T cell activation(Kon and Benhar, 2019). CTLA4 binds with greater affinity than CD28 to CD80 and CD86 on APCs, thus preventing costimulatory signalling and T cell activation (Rowshanravan, Halliday and Sansom, 2018). Some of these inhibitory checkpoint receptors will be discussed further in the context of cancer immunotherapy in section 1.7.1.4. T cell checkpoint inhibitors

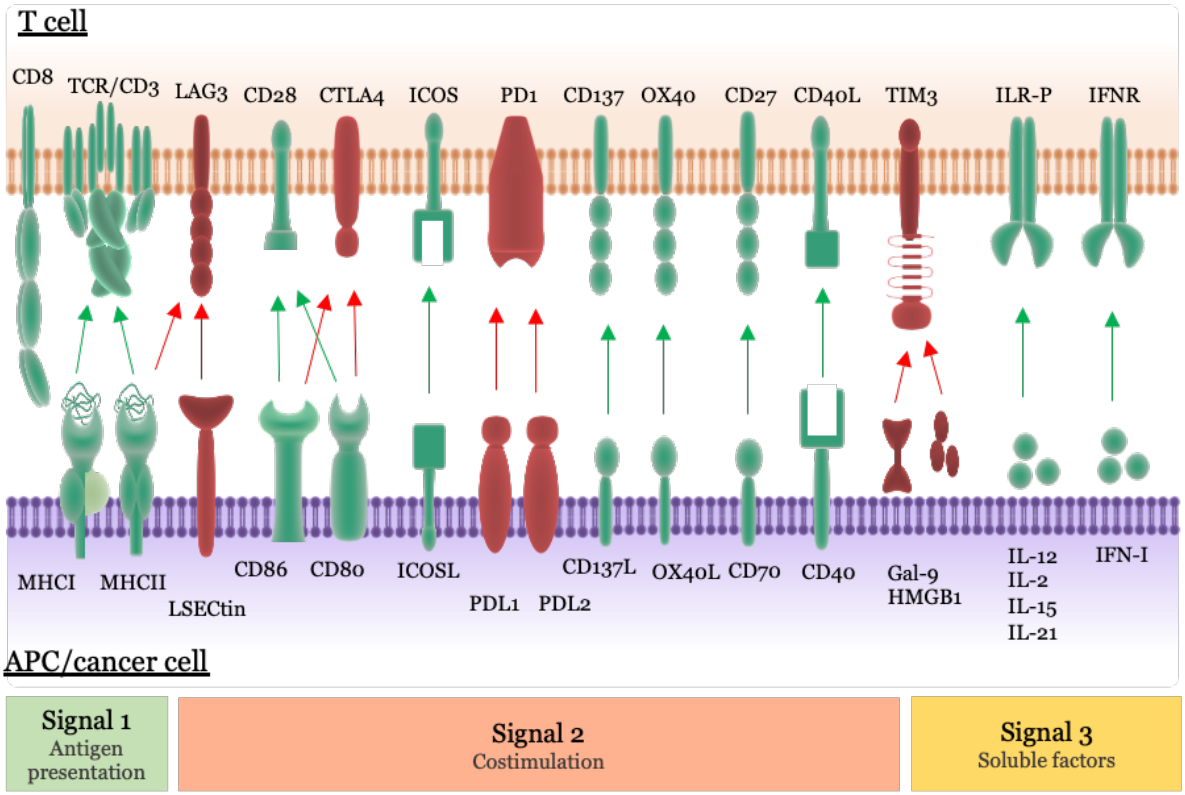


Figure 1.4. Three signal mode in activation of αβT cells.
Key receptors involved in the induction of T cell ¬mediated immunity or tolerance are shown. pro-APCs can present tumour-associated antigens on MHC I and MHC II molecules for recognition by αβT cells. However, this is insufficient to prime effective (antitumour) immunity, which requires further positive co-signalling (green arrows and receptors). Costimulatory molecules (including B7 and tumour necrosis factor (TNF) superfamilies) and soluble factors, including IL-12 and type I interferons (IFN-I), constitute signals 2 and 3, respectively. Inhibitory receptors (red arrows and receptors) can prevent T cell activation. CTLA4, cytotoxic T lymphocyte antigen 4; ICOS, inducible T cell costimulator; Gal-9, galectin-9; HMGB1, high mobility group box 1; IL-R, Interleukin receptors; IFNR, interferon receptors; LSECtin, liver and lymph node sinusoidal endothelial cell C-type lectin; PD1, programmed cell death protein 1; PDL1, programmed cell death 1 ligand 1; TCR, T cell receptor; TIM3, T cell immunoglobulin and mucin domain-containing protein 3.

1.3.2.3 Signal 3

The first two signals alone are still insufficient to drive functional responses and survival of T cells. Cytokines have a fundamental role in fully activating T cell responses and determine whether the response of naïve T cells to Ag and co-stimulation results in full activation, effector or/and memory differentiation, or tolerance in their absence. Thus, cytokines are responsible for directing the naive T cell differentiation towards the appropriate functional quality while also providing survival signals. Inflammatory cytokines such as IL-12, type I IFNs (IFN-α/β) and IFNγ appear to be the major signal 3 cytokines that support the induction of effector responses in naïve CD8 T cells against pathogens, transplants, tumours, and adjuvants (Curtsinger *et al.*, 2007, 2012; Curtsinger and Mescher, 2010; Obar and Lefrancois, 2010; Cox, Harrington and Zajac, 2011; Keppler *et al.*, 2012). Only recently, the requirements for signal 3 cytokines in CD8 T cell differentiation attracted more attention. The third signal provided by homeostatic cytokines is essential for clonal expansion and differentiation into various memory/effector populations (section 1.4.3), acquisition of cytolytic effector functions, and memory formation (Cieri *et al.*, 2013; White, Cross and Kedl, 2017; Callender *et al.*, 2018; Costa del Amo *et al.*, 2018; Lugli *et al.*, 2020). The signals such as IL-12, IL-15, IL-21, IL-6 and IL-2 provided by the APC, helper CD4 T cells, and also from location and context-dependent sources, bring a crucial contribution to directing the type of immune response generated and to shaping the fates of CD8 T cell differentiation (Rufer *et al.*, 2003; Sallusto, Geginat and Lanzavecchia, 2004; Cui and Kaech, 2010; Cox, Kahan and Zajac, 2013) (**Figure 1.5**). Thus, signals 2 and 3 play a crucial role in 'fine-tuning' the magnitude and quality of the T cell response (Homero Sepulveda, Adelheid Cerwenka, 1999; Croft, 2003; Borst, Hendriks and Xiao, 2005; Chen and Flies, 2013; Imanishi and Saito, 2020).

Despite this, during the course of an immune response, not all T cells will see antigen, costimulation, and cytokines at the same time or in the same order. *In vitro* experiments utilising limited stimulatory conditions found that T cells could be expanded without inducing their full differentiation. Interestingly, under appropriate conditions, it is possible to prime non-polarised T cells in the absence of polarising cytokines, and as well with limited duration of TCR stimulation in the absence of polarising cytokines (Iezzi, Scheidegger and Lanzavecchia, 2001; Gérard *et al.*, 2013; Enamorado *et al.*, 2018). Thus, timing of CD8 T cell exposure to these signals can also influence the shaping of their responses and differentiation (Curtsinger *et al.*, 2012; Keppler *et al.*, 2012; Hosking, Flynn and Whitton, 2014; Urban and Welsh, 2014; Urban, Berg and Welsh, 2016).

Taken together, recognition of pMHC complexes triggers TCR signalling, but it is co-receptors and their ligands (referred to as co-signalling receptor/ligand system for simplicity) together with cytokine milieu that direct T cell fate and determine T cell function.

1.3.3 The shades of mature CD8 T cell subsets

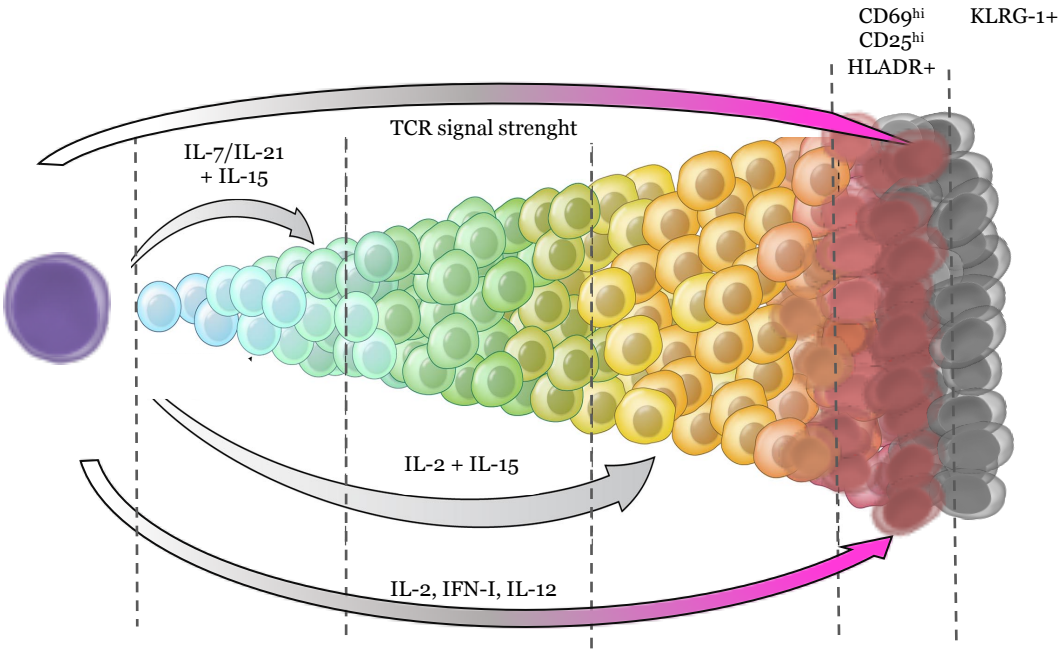
After positive/negative selection in the thymus, reactive mature naïve T (TN) cells are shaped into different memory and effector subsets by specific interactions with the antigen and cytokine signals in the periphery. Upon contact with foreign antigen in a stimulatory and inflammatory context such as

acute infection, naïve CD8 T cells initiate a cell-intrinsic program that drives them to expand and differentiate into cytotoxic effector cells (expansion phase) to eliminate the pathogen and clear the antigen. These cytotoxic effector T (TEF) cells are distinguished by their ability to secrete high concentrations of effector cytokines such as IFNγ and TNFα and produce cytolytic molecules such as granzymes and perforin. As the pathogen is cleared at the peak of the response, most (~95%) of TEF die (the contraction phase), and only a small proportion will differentiate into memory T (TM) cells. Each of these naive, effector, and memory T cell differentiation states have unique gene signatures that drive their functional and phenotypic properties (Parish and Kaech, 2009; Obar and Lefrancois, 2010; Buchholz, Schumacher and Busch, 2016). However, with the exception of T_N cells that show a "clean" distinguishable phenotype, there are many different shades of T_M and T_{EF} cells.

1.3.3.1 Post-thymic phenotype characterisation of CD8 T cell memory states

In the late 1980s, the characterisation of human TN and TM cells was proposed on the basis of differential expression of surface molecules, including CD45RA or CD45RO, and adhesion molecules LFA-3 (CD58) and LFA-1 (Sanders, Makgoba and Shaw, 1988). CD45 is a transmembrane tyrosine phosphatase that is involved in the regulation of TCR signalling, but its ligands and mechanisms of action are not clear. CD45RA and CD45RO are two splice variants of the CD45 gene that result in high and low molecular weight protein products, respectively, and mark leukocyte cell lineages. CD45RO is preferentially expressed on memory cells, while CD45RA is usually found expressed on naïve T cells but also when T cells reach terminal differentiation (van den Broek, Borghans and van Wijk, 2018). Later the analysis of homing receptors showed that T cells have different capacities to localise to peripheral tissues. T_N cells express high levels of the lymph node homing receptor CD62L (L-selectin), while memory cells were divided into CD62L+ and CD62L– subsets (Picker *et al.*, 1993). Initially, the classification of memory subsets was based on the expression of CD62L as well C-C chemokine receptor 7 (CCR7), and later also on receptors CD27 and CD28 with a combination of CD45RA to phenotypically define different T cell subsets. T_N cells are defined by high expression of CD45RA, while it lacks in T_M cells. In addition, high-level expression of CD27, CD28, CCR7 and CD62L marks T_N cells, and while their expression is absent in T_{EF} cells, these markers are differentially expressed among the spectra of T_M cells (Romero *et al.*, 2007) (**Figure 1.5).**

	T_N	T_{SCM}	T_{CM}	Early-late T_{EM}	T_{EF}	T_{TE}/ exhausted
CD45RA	+	+/-	-	-	-	+
CD45RO	-	+/-	+	+	+	-
CD27	++	++	++/+	+/-	-	-
CD28	++	++	+	+/-	-	-
CD62L	++	++	+	-	-	-
CCR7	++	++	+	+/-	-	-
CD95	-	+	+	+	-	-
IL-7R	++	++	+	-	-	-



IL-2	hi	hi	hi	low	low	no
IFNγ TNFα	no	rapid	low	yes	hi	low
perforin, granzyme A, B	no	yes	no	low	hi	low

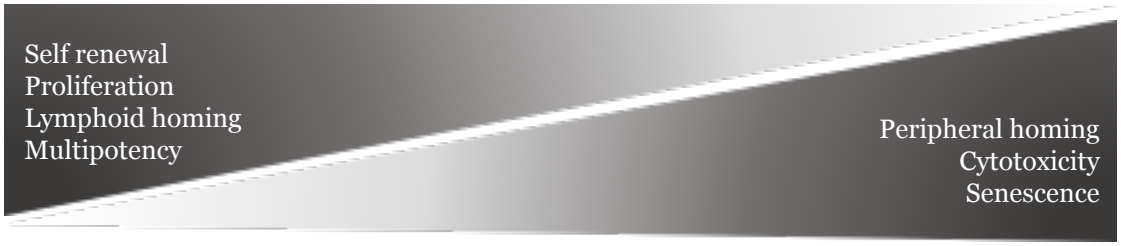


Figure 1.5. Shades of CD8 T-cell memory compartments.
Multiple naïve and memory T cell subsets can be identified in the peripheral blood according to differential expression of CD45RA/RO, CCR7, CD28, CD27, IL-7R, CD62L, and CD95. Together with activation markers CD25 and CD65 and senescence marker KLRG-1, six major states of T cells can be determined. Memory T cells lose or acquire specific functions while differentiating (through activation) into either T_{SCM}, T_{CM}, $T_{EM,}$ and T_{EF} and T_{TE} cells. Following an encounter with Ag, quiescent T cells develop into effectors with a highly dynamic and largely unpredictable phenotype. When the antigen is cleared, effector T cells that survive the

return to a quiescent memory state. The signals that control fate decisions, i.e. the acquisition of a given phenotype, are largely unknown. Current understanding of the signals that control fate decision include TCR signal strength/duration together with cytokines: IL7, IL-21 and IL-15 driving differentiation intoT_{SCM} cells, IL-2 and IL-15 into memory cells, high levels of type I IFNγ, IL-12 and IL-2 into T_{EF} and T_{TE} cells. As shown at the bottom, various cellular functions are preferentially, but not uniquely, expressed by these subsets. Adapted from Gattinoni *et al.*, 2013.

The memory T cell compartment is divided into three main subsets: the well-established central memory (T_{CM}) and effector memory (T_{EM}) T-cell subsets, and more recently recognised stem cell-like memory cells (T_{SCM}) (**Figure 1. 5**) Memory T cell subsets as a whole are often defined by possession of self-renewal capacity and do not present with as immediate rapid effector functions like T_{EF} cells. T_{CM} cells are early differentiated progenitors defined by high-level expression of all above markers CD27, CD28, CCR7 and CD62L, but lack CD45RA. TCM cells can self-renew and simultaneously generate more differentiated offspring. As T cells shift through different states, they sequentially downregulate CCR7, CD27 and CD28 expression as they differentiate into many T_{EM} stages to become terminally differentiated T_{EF} cells (T_{TE} cells) (**Figure 1.5**). With activation, T cells are guided by co-signalling and cytokine milieu to transit towards effector or memory states, and their phenotypes remain unpredictable by virtue of the large number of genes that are (transiently) up-or downregulated (Gattinoni, Lugli, Ji, Pos, Chrystal M Paulos, *et al.*, 2011; Mahnke *et al.*, 2013). It is clear that they appear to go through a CCR7- CD27+ CD28+ to a CCR7- CD27- CD28- stage while upregulating the expression of cytolytic molecules (Romero *et al.*, 2007). The T_{EF} phenotype considerably overlaps with that of quiescent T_{EM} cells; however, the difference is that effectors also overexpress the activation markers CD69 and CD25, in addition to CD38 and HLA-DR (Mahnke *et al.*, 2013). Phenotypically, T_{TE} cells are defined by (re)expression of CD45RA, but a lack of all previously mentioned markers, thus are CCR7– CD62L– CD27- CD28-, and display the shortest telomeres among T cells (Romero *et al.*, 2007). In addition, T_{TE} cells express senescence maker KLRG-1 (Henson and Akbar, 2009) and show different epigenetic landscape and chromatin rearrangements, which renders them with low proliferative and functional capacity (Gorochov *et al.*, 1997). (**Figure 1.5**)

Relatively recently discovered/described members of the memory T cell compartment are naïve-like T_{SCM} cells - that are CD45RA+, CD45RO–, and show high expression levels of CCR7, CD62L, CD27, CD28 and IL-7Rα (Gattinoni, Lugli, Ji, Pos, Chrystal M Paulos, *et al.*, 2011). Unlike T_N cells, T_{SCM} cells are clonally expanded and express the memory markers CD95, CD122, and LFA-1. These T_{SCM} cells have attracted great attention due to their intrinsically enhanced homeostatic self-renewal capacity and proliferation driven by WNT signalling (Gattinoni *et al.*, 2009). They are capable of multipotent production of differentiated progeny, including T_{CM} and T_{EM} *in vitro* and upon adoptive transfer into humans and xenogeneic mouse models (Cieri *et al.*, 2013)(Lugli *et al.*, 2013)(Biasco *et al.*, 2015; Roberto *et al.*, 2015).

Further characterisation of different shades of CD8 T cell subsets also includes their effector functions. The functional hallmark characteristic of memory T cells is the production of cytokines but lack of immediate effector killing activity (**Figure 1.5**). T_{SCM} cells can retain the expression of core T_N cell genes while they promptly produce cytokines (such as IFNγ) upon TCR stimulation (Buchholz, Schumacher

and Busch, 2016). Additionally, epigenetic profiling has identified several epigenetically preserved effector loci in TSCM cells, with limited transcriptional activity and great potential to promptly recall an effector response (Abdelsamed *et al.*, 2017). These qualities make TSCM attractive for application in anti-cancer adoptive immune-cell therapies since they might overcome current limitations, including inefficient T-cell engraftment, poor persistence, and inability to mediate a prolonged immune attack(Gattinoni and Restifo, 2013). TCM cells produce low IL-2 and IFNγ and lack cytolytic molecules such as granzyme B and perforin, thus lacking immediate killing activity (Sallusto *et al.*, 1999; Romero *et al.*, 2007). Early TEM cells produce high amounts of IL-2 and low levels of other effector cytokines (such as IFNγ, TNFα) and cytolytic molecules. In contrast, the late TEM and TEF cells produce high levels of IFNγ and TNFα but not IL-2 and hold perforin and granzyme granules for immediate attack (Hamann *et al.*, 1997; Sallusto *et al.*, 1999; Romero *et al.*, 2007). Interestingly, perforin expression shows an inverse correlation with cell surface expression of CD27 (Tomiyama, Matsuda and Takiguchi, 2002) and CD28 as well as a positive correlation with the senescence marker CD57, which can be used as a surrogate marker for T cells with high cytolytic potential (Chattopadhyay *et al.*, 2009).

T_{EM} cells are found in the blood and have a potential to home to peripheral lymphoid tissues and show low expression of molecules involved in long-term survival and high level of transcription factors (TFs) mediating terminal differentiation - and, thus, are considered phenotypically more rigid with decreased self-renewal and multipotent capacity compared with T_{CM} cells (Hamann *et al.*, 1997; Hamann, Roos and van Lier, 1999; Cui and Kaech, 2010). Furthermore, T_{CM} cells can recirculate between blood and secondary lymphoid tissues, and they possess longer telomeres than T_{EM} cells. Initially, T_{CM} cells were thought to constitute the stem-cell-like memory precursor population since they have a capacity of becoming/generating T_{EM} and T_{EF} cells. However, seeing that T_{CM} cells can migrate into inflamed peripheral tissues while CCR7 negative cells are capable of entering lymphoid organs at a steady-state (Steinbach, Vincenti and Merkler, 2018) and during inflammation (Guarda *et al.*, 2007; Jameson and Masopust, 2018), the definitions of T cell memory differentiation states need further attention.

1.4 γδ T cells

γδ T cells are distinguished by their γδ TCRs. They represent around 1-5% of blood lymphocytes in healthy individuals, while they are generally a prevalent population among epithelial tissues such as the lung and epidermis and in the mucosa of the digestive and reproductive tracts. Since their discovery in the mid-1980s it was shown that γδ T cells are involved in several diseases in addition to infection - including cancer, neurological and autoimmune diseases. The γδ T cells function at the edge of innate and adaptive immunity and contain a range of subsets with diverse and controlled TCR repertoires (Willcox, Davey and Willcox, 2018)(Pauza and Cairo, 2015; Di Lorenzo, Déchanet-Merville and Silva-Santos, 2017) that respond to a vast range of ligands most of which are yet to be identified (Vermijlen et al., 2018).

γδ T cells are often classified into different subtypes based on their specific TCR arrangements, each with specific phenotypic and functional characteristics. In humans, they are broadly divided into δ2 and

δ2-negative subgroups and show district tissue and subtype-specific localisations as well as subtype-specific responses driven by various ligands (Born, Kemal Aydintug and O'Brien, 2013; Vermijlen *et al.*, 2018). Understanding the biology of γδ T cells is somewhat opaque, but what is known is that they provide pleiotropic functions. γδ T cells can present with both specific adaptive features, yet they are not MHC-restricted, while they also possess innate-like functions of NK cells and myeloid cells. Thus, γδ T cells are uniquely equipped with various functions that allow them to effectively lyse infected or malignant cells via their TCR and/or NKG2D receptor; modulate immune responses by providing help to B cells, DCs, monocytes and neutrophils (He *et al.*, 2014). In addition, they can also boost adaptive αβT cell responses by modulating the immunogenicity of transformed cells (Chen *et al.*, 2017); or act as regulatory cells suppressing immune responses (Paul and Lal, 2016). Importantly, γδ T cells can professionally present peptide antigens to αβT cells, either polarising helper CD4 T cells or initiating cytotoxic CD8 T cell responses (Brandes, Willimann and Moser, 2005; Marlène Brandes *et al.*, 2009; Tyler *et al.*, 2017). Thus, their function can be related to tissue integrity and maintenance of homeostasis **(Figure 1.6).**

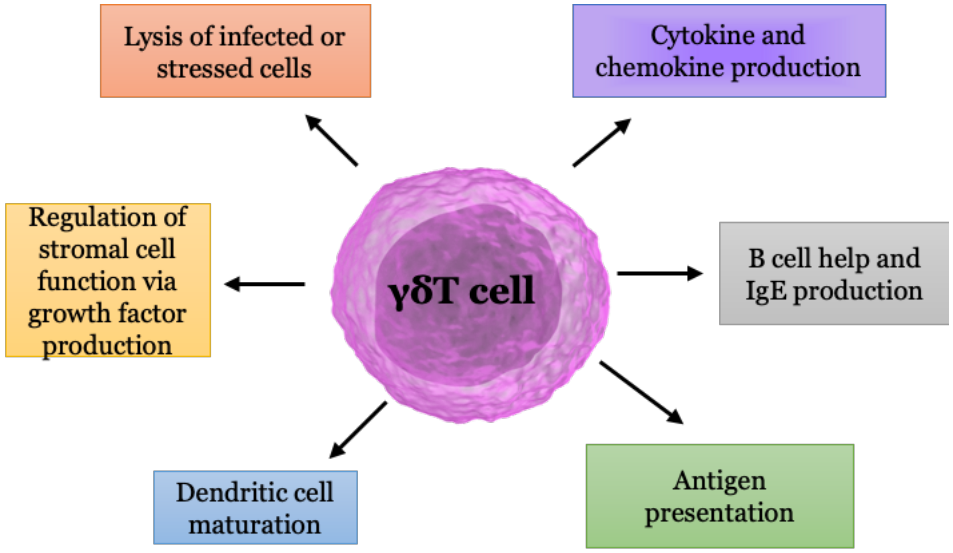


Figure 1.6. Various functions of γδ T cells.

The functional characterisation of γδ T cells has been based around their Vδ (Di Lorenzo, Déchanet-Merville and Silva-Santos, 2017). As mentioned previously, γδ T cells are primarily defined by their Vδ chain usage and are broadly grouped into Vδ2 and Vδ2-negative subtypes, of which Vδ1 subtype is predominant among the Vg2 negative cells. Vδ1 cells predominate in the thymus and peripheral tissues, mostly mucosal surfaces, while Vδ2 cells constitute the majority of blood-circulating γδ T-cells. As noted in an earlier section (T cell development), the extensive degree of junctional and CDR3 diversity privileges γδ T cells to recognise a broad range of ligands. The diversity of the molecules that γδ T cells respond to encompasses chemical and conformational structures associated with infection, stressed tissues and tumours as well as with the health status (Vermijlen *et al.*, 2018)**.** Thus, whereas αβT cells are limited to recognition of antigens presented in the context of MHC and CD1, γδ T cell subsets can recognise a far more diverse repertoire of antigens on the surface of target cells whose expression

increases upon stress, infection or transformation. On the other hand, particular γδ T cell subsets found in specific tissues locations show a favourable usage of certain TCR V gene segments and can express invariant TCRs, with identical junctional sequences (Hayday, 2000; Shires, Theodoridis and Hayday, 2001; Bonneville, O'Brien and Born, 2010). Due to their rapid innate-like responses, γδ T cells have an advantage for preventing the spread of infected or malignant cells while sustaining the tissue integrity and regulating the nature and magnitude of downstream responses (Vantourout and Hayday, 2013).

1.4.1 Ligand recognition by γδ T cells

In TCR-dependent manner, the γδ T cell response is noted against metabolites of isoprenoid synthesis pathway associated with BTN, metabolites/molecules in the context of MR1 presentation, lipid antigens presented by CD1 molecules, endothelial protein C receptor (EPCR), and Annexin A (Vermijlen *et al.*, 2018). Interestingly, some ligands drive the activation of γδ T cells due to their molecular context, i.e. they create a multi-molecular stress signature together with other factors which drive the activation of γδ T cells, such as in the case of CMV infection. For example, EPCR, the ligand for a γδ T cell clone expanded in CMV transplant recipient, is upregulated on the plasma membrane upon CMV infection (Weekes *et al.*, 2014). However, the EPCR and upregulation of other co-stimulatory factors appear to drive the responses via TCR engagement (Willcox *et al.*, 2012). Yet, these molecules constitute only a tiny fraction of the ligands recognised by γδ T cells. Besides the TCR, γδ T cells can express and recognise ligands via other activating receptors such as NKG2D and CD16, (Ribeiro, Ribot and Silva-Santos, 2015) and TLRs (Wesch *et al.*, 2011), which may complement or even replace TCR signalling in different contexts. Many of the ligands recognised by γδ T cells are still unidentified, and new studies are after identification of new ligands of γδ T cells. Thus, also the immunological function of most γδ T cell subsets remains unclear.

Unlike with αβT cells, human γδ T cells do not directly compare with those identified in mice – either regarding their functionality, anatomical location, TCR structure, or ligand recognition – thus, it is difficult to make direct correlation/translation between these species. This is particularly evident for the Vγ9/Vδ2 T cells, which do not exist in mice. As opposed to the clearly defined MHC restricted presentation of peptide antigens to αβT cells, except for Vγ9/Vδ2 T cells, there is no consensus as to the class of antigens γδ T cells recognise, and how such antigens are presented. The growing evidence suggests that the antigens are not 'presented' to γδ T cells in the 'conventional' sense.

1.4.2 Vδ2 negative subsets

Vδ1 T cells are paired with several different Vγ chains. While Vδ1 T cells are present in the blood at only <1% of CD3 cells, they are enriched in the peripheral tissues, the mucosa and epithelial tissues of skin and intestine, and solid tumours (Silva-Santos, Serre and Norell, 2015). Due to the diverse usage of Vγ chains expressed with Vδ1 TCR, this subset can detect various ligands. Despite this, only a few ligands have been identified for Vδ1+ T cells; so far, they are mainly lipid ligands (phospholipids and

glycolipids) presented by MHC-like antigen-presenting molecules, the CD1 molecules (Vermijlen *et al.*, 2018). CD1c can present various endogenous and exogenous lipids and is recognised by a significant proportion of Vδ1 T cells. A high proportion of Vδ1+ cells are also reactive to ligands presented by CD1d molecules, such as phosphatidylethanolamine, sulfatide and α-galactosylceramide, and ligands presented by CD1a molecules. Despite this, most CD1-restricted γδ T cells also respond with lower affinities to 'empty' CD1 molecules, which may or may not present yet unknown self-lipids (Vermijlen *et al.*, 2018). Thus, some human Vδ1 T cells are restricted to CD1 molecules; however, a large proportion do not exhibit this same ligand recognition characteristics, but instead show reactivity to tumour cells, CMV/HIV-infected cells, and certain bacterial and fungal species, via yet unknown molecules (Willcox *et al.*, 2012)(Poupot, Pont and Fournié, 2005; Schneiders *et al.*, 2014). A recent study has identified MR1-restricted Vδ1 and Vδ3 T cells combined with a broad range of Vγ chains, in human blood (up to 5% of γδ cells) and tissues (such as liver, stomach, lung, and duodenum) of healthy subjects, while these cells were also found highly enriched within a tumour infiltrate from a Merkel cell carcinoma and in association with celiac disease (Le Nours *et al.*, 2019).

Furthermore, intestinal epithelium Vδ1 cells can recognise and respond to stress-induced whole protein antigens and MHC class I-related molecules independent of Ag processing. From a functional perspective, upon activation, Vδ1 T cells rapidly secrete cytokines and cytotoxic molecules against many cellular targets while can also activate DCs for antigen presentation (Hua *et al.*, 2013). In addition, Vδ1 T cells have been found to exhibit regulatory characteristics to suppress immunity; while also capable of IL17 production and playing a role in autoimmunity and neurological disorders (Papotto, Ribot and Silva-Santos, 2017).

1.4.3 Vγ9/Vδ2T cells

1.4.3.1 Function of Vγ9/Vδ2 T Cells

While in many mammals γδ T cells constitute a substantial fraction of T cells in the blood or peripheral tissues, Vγ9/Vδ2 T cells with similar characteristics and antigen responsiveness have so far only been found in humans, primates, armadillos and alpacas (Fichtner *et al.*, 2018). However, they do not seem to be functional in armadillos.

Vγ9/Vδ2 T cells have diverse receptor equipment (see below), which gives them broad functional plasticity. Thus, Vγ9/Vδ2 T cells possess innate-like functions combined with multiple adaptive effector functions. The discovery of the Vγ9/Vδ2 TCR ligands has provided opportunities for studying the roles of Vγ9/Vδ2 T cells in human immunity. Vγ9/Vδ2 T cells are rapidly activated upon TCR ligation and play a critical role in early inflammation and producing various cytokines and chemokines, and they are involved in stress surveillance, tissue homeostasis and wound repair. While γδ T cells interact with other immune cells and promote their activation, differentiation and maturation, they can also lyse infected, transformed/stressed cells. It was shown that upon activation, Vγ9/Vδ2 T cells promote DC maturation (Ismaili *et al.*, 2002), B-cell activation (Brandes *et al.*, 2003) and polarize adaptive immunity toward particular immune responses (Tyler *et al.*, 2017).). Thus, this highlights the capacity of Vγ9/Vδ2 T cells

to influence the nature of the immune response to different challenges. The most exciting feature of Vγ9/Vδ2 T cells is their ability to act as cytotoxic cells and professional antigen-presenting cells. The following sections describe how the acquisition of these functions is driven through assorted receptors and various stimuli.

1.4.3.2 Activation and response of Vγ9/Vδ2 T cells

As noted in the section

1.2.3.2 TCR repertoire diversity, the postnatal circulating γδ TCR repertoire seem to be shaped in the periphery, most likely driven by proliferation in response to continuous exposure to environmental and pathogen-derived phosphoantigens, which leads to preferential use and to amplification of Vγ9-JP/Vδ2 T cells (De Rosa *et al.*, 2004; Gu *et al.*, 2015; Ravens *et al.*, 2020). In contrast to the mode of activation of αβT cells, the primary activation of Vγ9/Vδ2 cells occurs by signals received via different classes of surface receptors. In αβT cells, CD4 and CD8 coreceptors play a major role in initiating functional responses, and while γδ T cells can express CD8, it is unclear how signal 1 (TCR/coreceptor) occurs in γδ T cells. While similarly to αβT cells, Vγ9/Vδ2 cells also require stimulation via co-stimulatory receptors (CD27, CD28), but unlike with αβT cells, co-stimulatory receptor signalling can highly impact γδ T cell effector phenotype. In Vγ9/Vδ2 cells, functional responses are initiated upon recognising non-peptide antigens, which are sensed by either TCRs or NK receptors (particularly NKG2D), while some ligands are also particularly reactive toll-like receptor (TLR) agonists. When activated through their TCR, Vγ9/Vδ2 T cells show cytotoxic responses by releasing granzymes, perforin, granulysin and IFN-γ. Like in CD8 αβT cells, cytokine signalling also drives homeostasis, functional differentiation and expansion of Vγ9/Vδ2 T cells (see section 1.5.3.2.4). The notion that some γδ TCRs might be specific for self-encoded proteins while others show clonal restriction has given rise to the view that both innate and adaptive γδ T cells exist (Kisielow *et al.*, 2011).

Ligands other than isoprenoid metabolites have also been described in the induction of Vγ9/Vδ2 T cell responses. Vγ9/Vδ2 T cells express the NK receptor NKG2D alongside other NK receptors that allow detection of infection- or stress-associated ligands such as MICA/B and UL16-binding proteins (ULBPs) on transformed cells(Gomes *et al.*, 2010)(Silva-Santos and Strid, 2018). Whether Vγ9/Vδ2 T cells can recognise targets via NKG2D independently of TCR stimulation is a hot topic. Interaction of Vγ9/Vδ2 T cell with tumour target cells promotes the formation of an immune synapse where clustering of both surface molecules TCR and NKG2D occurs alongside a range of other receptors and molecules. Whereas TCR ligation induces ZAP70-mediated activation of the AKT pathway, NKG2D ligation causes DAP10-mediated PKC signalling. The likely scenario is that these two pathways work hand in hand to promote cytokine release and cytotoxic responses towards target cells (Nedellec, Bonneville and Scotet, 2010).

Vγ9/Vδ2 T cell activation is closely controlled also by inhibitory receptors such as PD-1, CTLA4, B- and T-lymphocyte attenuator (BTLA), LAG-3, TIM-3 that are specific for constitutively expressed or stress-induced ligands, but except for PD-1, their physiological relevance for γδ T cell biology remains opaque, and will not be described here. It was shown that PD-1 could drive inhibition of Vγ9/Vδ2 T cells in TME

and in inflammation, where they can further inhibit other PD-1 positive immune cells via PDL-1 expression (Peters *et al.*, 2014)(Hoeres *et al.*, 2019). A great variety of other receptors contribute to the stimulation and inhibition of activation and responses of Vγ9Vδ2+ cells. This section will describe the influence of stimulatory signals via TCR, NKG2D and some co-stimulatory receptors on activation and responses of Vγ9/Vδ2 T cells; while inhibitory receptors also play an important role, they will not be described here.

1.4.3.2.1 TCR Activation by Phosphoantigen ligands

In terms of antigen selectivity and mode of antigen recognition, human Vγ9/Vδ2 T cells differ fundamentally not only from αβT cells but also from their γδ T cell counterparts .Vγ9/Vδ2 cells use their TCR to recognise small pyrophosphate molecules known as phosphoantigens (pAgs), which do not trigger responses in any other lymphocytes. Cipriani and colleagues showed that upon Vγ9/Vδ2 TCR ligation, rapid and persistent PKC-dependent phosphorylation of ERK1/2, p38 MAPK, and JNK is induced, which results in NF-κB and AP-1 activation, as well as the release of MIP1α, MIP1β, IFNγ, and TNFα pAgs, are intermediates of the non-mevalonate (found in bacteria) and mevalonate metabolic (in eukaryotes) pathways. Thus, pAg ligands can be found naturally in mammalian cells or produced by infectious agents (Cipriani *et al.*, 2000). The best-characterised metabolite agonist of the non-mevalonate pathway is (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMB-PP), which was discovered to selectively stimulate Vγ9/Vδ2 T in a non-MHC restricted manner (Eberl *et al.*, 2003). It is not found in humans but in plant chloroplasts, the majority of Gram-negative bacteria and many Gram-positive species, as well as apicomplexan protozoa such as *Plasmodium falciparum* and *Toxoplasma gondii*. Likewise, the mevalonate pathway plays an important role in eukaryotes, which is essential for cholesterol biosynthesis and protein prenylation. The best-characterised pyrophosphate of the mevalonate pathway is isopentenyl pyrophosphate (IPP), but the non-mevalonate pathway can also produce it via conversion of HMBPP. IPP is used for the downstream synthesis of cholesterol, prenylated proteins, and ubiquinone. During normal physiological conditions, mammalian cells contain a low concentration of IPP intracellularly and thus do not activate Vγ9/Vδ2 cells. However, cells will start accumulating IPP in case of cellular distress and consequently dysregulated mevalonate pathway and metabolism in tumour cells. It is thought that the higher IPP concentrations will cause changes that are sensed by Vγ9/Vδ2 cells via their TCR, leading to their rapid activation and effector response, ultimately resulting in cancer cell killing. Thus, IPP accumulation represents a DAMP and HMB-PP a PAMP for Vγ9/Vδ2 T cells. Compared to IPP, HMBPP is 10,000 times more potent *in vitro* (Eberl *et al.*, 2003; Puan *et al.*, 2007). Other metabolites of the mevalonate pathway that activate Vγ9/Vδ2 T cells (although at much higher concentration) include the dimethylallyl pyrophosphate (DMAPP) and by-products such as ApppI (APM conjugate of IPP) (Morita *et al.*, 2007; Riganti *et al.*, 2012).

Additional Vγ9/Vδ2 TCR stimulating compounds are natural and synthetic primary alkylamines and polyphenols. These antigenic alkylamines and phenols are secreted to millimolar concentrations in bacterial supernatants and are found in certain edible plants (Bukowski, Morita and Brenner, 1999; Thompson, Rojas-Navea and Rogers, 2006; Holderness *et al.*, 2007).

Moreover, synthetic activating "phosphoantigen" molecules such as monoethyl phosphate, bromohydrin pyrophosphate (Phosphostim; BrHPP) and a pyrophosphonate analogue of HMB-PP (Picostim) have been developed (Vermijlen *et al.*, 2018). In addition, phosphoantigen over-expression can also be induced chemically with amino bisphosphonates (NBPs), such as zoledronate (ZOL), pamidronate, and alendronate. NBPs actively inhibit the farnesyl pyrophosphate synthase (FPPS) enzyme, an IPP-downstream enzyme of the mevalonate pathway, leading to a significant intracellular accumulation of various pyrophosphate molecules including IPP, DMAPP and 'by-products' (Vantourout *et al.*, 2009; Moulin *et al.*, 2017). Thus, administration of NBP in patients elevates numbers of Vγ9/Vδ2 T cells (Kunzmann, Bauer and Wilhelm, 1999; Naoe *et al.*, 2010). Besides this, NBPs prevent bone resorption and are commonly used to treat tumour-associated bone diseases and osteoporosis (Roelofs *et al.*, 2009; Zhang *et al.*, 2010).

Although many peripheral blood leukocytes can take up ZOL, it was shown that it is monocytes and DCs who produce high levels of IPP, and are therefore essential accessory cells for Vγ9/Vδ2 T-cell activation by NBPs (Roelofs *et al.*, 2009; Kamigaki *et al.*, 2013; Fowler *et al.*, 2014). Besides its intracellular accumulation, zoledronate treated DCs can also release IPP into the microenvironment where it becomes available for uptake by other cells and/or activation of Vγ9/Vδ2 T cells (Riganti *et al.*, 2012; Castella *et al.*, 2017). In that, ATP-binding cassette transporter A1 (ABCA1) plays a major role in the extracellular release of IPP from ZOL-treated DCs. Likewise, monocytes are also superior APCs for pAgs such as HMBPP and BrHPP and thus potent for Vγ9/Vδ2 T cell activation. In contrast, neutrophils also efficiently uptake zoledronate but instead induce unfavourable effects, such as inhibition of T cell proliferation due to hydrogen peroxide production (Kalyan *et al.*, 2013).

In addition to phosphoantigens, an increase of mevalonate metabolite ApppI (triphosphoric acid 1-adenosine-5′-yl ester 3-(3- methylbut-3 enyl) ester) is also noted in stressed cells (Mönkkönen *et al.*, 2008). ApppI is synthesised from IPP and ATP in a reaction likely to be catalysed by aminoacyl-tRNA synthetases. ApppI functions as a proapoptotic agent by inhibiting the mitochondrial ADP/ATP translocase in osteoclasts, macrophages, and tumour cells (Mönkkönen *et al.*, 2008). The molecular similarity of ApppI to the nucleoside conjugates of HMBPP, IPP, and DMAPP were identified without a context for Vγ9/Vδ2 T cell interaction (Constant *et al.*, 1994; Tanaka *et al.*, 1995; Eberl *et al.*, 2003). Exogenous ApppI can sufficiently expand Vγ9/Vδ2 T cells from PBMCs but also requires the presence of accessory cells, likely due to cell surface-associated nucleoside pyrophosphatases cleaving ApppI into AMP and free IPP (Champagne, 2011). ApppI can be detected in untreated tumour cells such as Daudi cells and may represent a natural "reservoir" of IPP, or it may be an intermediate in phosphoantigen processing/presentation pathway (Champagne, 2011). The role of mitochondrial ecto-F1-ATPase has been portrayed as responsible for shuttling of intracellular ApppI to the cell surface and presentation to Vγ9/Vδ2 T cells (Champagne, 2011). Importantly, cells that do not express surface F1-ATPase do not activate Vγ9/Vδ2 T cells (Mookerjee-Basu *et al.*, 2010). Like HMBPP presented by BTN molecules, ApppI activates Vγ9/Vδ2 T cells at nanomolar concentrations when presented by F1-ATPase, representing a significantly lower concentration than required when applied as a soluble compound (Mookerjee-Basu *et al.*, 2010). This signifies the importance of specific and directed presentation to Vγ9/Vδ2 T cells in concert with appropriate co-stimulatory signalling (Ribot *et al.* 2011).

The mechanisms by which soluble pAgs induce Vγ9/Vδ2 T-cell activation still remain to be fully elucidated. It seems that exogenously added pAg also require binding to the intracellular domain of BTN proteins to activate Vδ2 T cells, but how exogenous pAgs enter cells has yet to be described.

1.4.3.2.1.1 Presentation of pAg ligands by butyrophilins

With increasing knowledge about pAgs that could be recognised by Vγ9/Vδ2 T cells, the question that had to be answered was what molecules and which mechanism were responsible for pAgs presentation and recognition. Initial studies showed the importance of the cell-cell contact for activation of Vγ9/Vδ2 cells in response to pAgs, which paved the notion that an antigen-presenting molecule is involved in this (Morita *et al.*, 1995). Research into pAg presentation demonstrated that cells lacking β2-microglobulin are still efficiently presenting IPP, suggesting they are presented by alternative antigen-presenting molecules. Finding that Skint1 was involved in the presentation of antigen to mouse Vγ5Vδ1 cells stimulated the search for a human Vγ9/Vδ2 T cell antigen-presenting molecule on human Skint homologs. This led to the identification of BTN3A proteins and BTN2A1 as molecules responsible for the pAg driven reactivity of Vγ9/Vδ2 cells.

Butyrophilins ("butter-loving") received their name after the founding member BTN1A1 which encodes a milk fat micelle-associated protein (Franke *et al.*, 1981). Nevertheless, their more recently discovered function of several BTN/BTNL gene products in immune regulation contrasts this initial implication (Rhodes *et al.*, 2015). BNT and BTNL proteins belong to the B7 superfamily. In humans, all three BTN3A isoforms, BTN3A1, BTN3A2, and BTN3A3, possess extracellular domains structurally similar to CD80 and CD86. Hence, BNT/BTNL proteins could potentially also regulate Vγ9/Vδ2 T cells via coreceptors. On the other hand, a pAg binding site was identified on the cytoplasmic tail of the BTN3A1, which is essential for Vγ9/Vδ2 T cell activation (Sandstrom *et al.*, 2014). Such intracellular recognition of pAgs shows the similarity of innate responses through proteins such as TRIM5α and TRIM21 (Rhodes, de Bono and Trowsdale, 2005; D'Cruz *et al.*, 2013) thereby adding BTN3A1 to the rapidly growing number of innate PRRs. Nevertheless, because of their strict association with Vγ9/Vδ2 TCR usage, BTN/BNTL activity seems to be exerted directly via the TCR.

Initially two models of pAg presentation were proposed: (i) a **direct antigen presentation model**, where pAg binds to the extracellular domain of BTN3A1, thus being available for immediate recognition by Vγ9/Vδ2 TCR (24); (ii) and **inside out signalling model**, where pAg bind to the intracellular part of the BTN3A1, causing conformational changes of the molecule and potentially interacts with other molecules, which is sensed by Vγ9/Vδ2 T cells (Sandstrom *et al.*, 2014). In fact, Vγ9/Vδ2 TCR–mediated cytolytic responses to HMBPP and IPP directly rely on butyrophilin 3A1 (BTN3A1) and BTN3A2 (Harly *et al.*, 2012; Vantourout *et al.*, 2018), while the GTPase RhoB is essential for BTN3A1 driven Vγ9/Vδ2 cell activation (Sebestyen *et al.*, 2016). Furthermore, RhoB, in association with BTN3A1, induces cytoskeletal changes, which result in decreased membrane mobility of BTN3A1 and thus support pAg induced conformational changes in the molecule. With this, an addition to the inside out signalling model was introduced, whereby complex and coordinated molecular rearrangements are

required for sensing and stimulation of Vγ9/Vδ2 T cells while BTN3A1 protein stability and trafficking represents a checkpoint for regulation of Vγ9/Vδ2 cell activation (Rhodes *et al.*, 2018).

The most recent evidence also suggests a crucial role of the BTN2A molecule required for pAg-mediated activation of Vγ9/Vδ2 T cells (Karunakaran *et al.*, 2020; Rigau *et al.*, 2020). Rigau and colleagues **proposed a new model** of pAg recognition by Vγ9//Vδ2 T cells whereby BTN2A1 and BTN3A1 co-bind the Vγ9/Vδ2 TCR in response to pAg (**Figure 1.7**). This model suggests that as pAg binds intracellular BTN3A1 domain, the BTN2A1–BTN3A1 complex interacts with the Vγ9/Vδ2 TCR via two separate binding sites. BTN2A1 binds to Vγ9 framework regions, while BTN3A1 (or another ligand) binds to the γ-chain CDR3 as well as Vδ2 CDR2 loops. Thus, together BTN2A1–BTN3A1 complex appears to deliver pAg signal for recognition and activation of Vγ9/Vδ2 T cells (Karunakaran *et al.*, 2014; Eberl, 2020; Rigau *et al.*, 2020). It was demonstrated that without BTN2A1, neither bacterial nor mammalian pAgs stimulated Vγ9/Vδ2 cells (Rigau *et al.*, 2020).

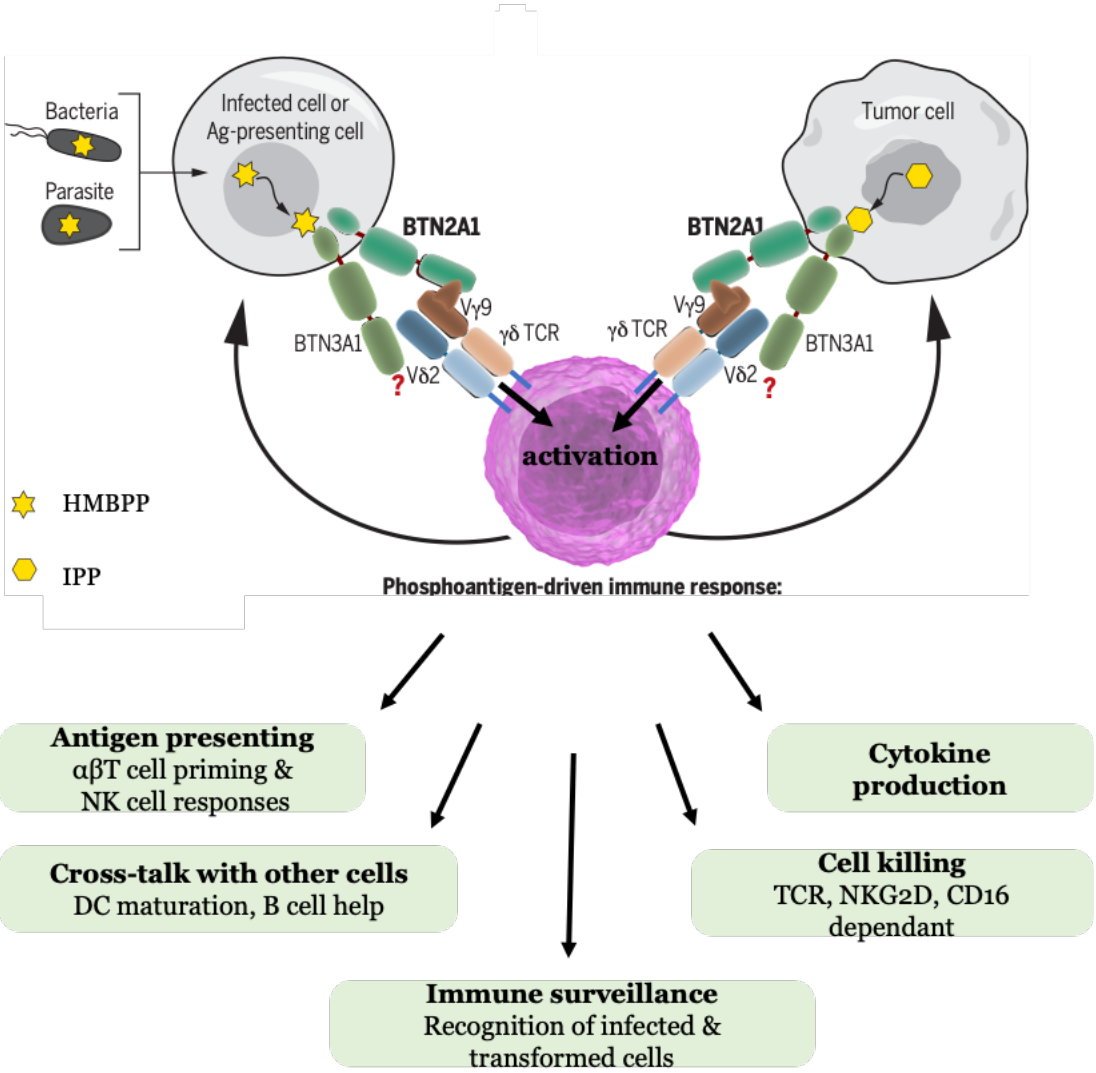


Figure 1.7. Mechanism of phosphoantigen driven Vγ9/Vδ2 T cell activation and functional response.
Butyrophilins (BTN) play a critical role in phosphoantigen recognition by human Vγ9/Vδ2T cells. Adapted from Rigau *et al* 2020

1.4.3.2.2 TCR-independent and -synergistic activation of Vγ9/Vδ2 T cells

In addition to stimulation by pAgs, Vγ9/Vδ2 T cells also sense infected or stressed cells via NK receptors and a selection of PPRs and innate-like receptors (Hudspeth, Silva-Santos and Mavilio, 2013). This section will describe a few receptors that are currently best described and are used by Vγ9/Vδ2 T cells to exert their functions upon sensing dangerous material in their surroundings.

1.4.3.2.2.1 NK receptors

In addition to TCR-dependent sensing of pAg, another mechanism by which Vγ9/Vδ2 T cells detect transformed/dysfunctional cells involves NKG2D, and often together with other NK receptors including NKp30, NKp44 or NKp46 (Hudspeth, Silva-Santos and Mavilio, 2013). However, whether Vγ9/Vδ2 T cells can recognise targets via NKG2D independently or in the presence of TCR stimulation remains controversial. Some studies have demonstrated the induction of effector function in Vγ9/Vδ2 T cells through NKG2D stimulation alone (similarly to NK cells) (Das *et al.*, 2001; Rincon-Orozco *et al.*, 2005; Wrobel *et al.*, 2007). However, others have failed to show NKG2D-induced activation without synergistic TCR stimulation. In this synergy, the NKG2D function in Vγ9/Vδ2 T cells is suggested to be a co-stimulatory receptor to the TCR. Work by Silva-Santos' group proposed a model whereby TCR recognition is sufficient to activate Vγ9/Vδ2 T cells, but ligation of NKG2D is required to promote their targeted cytotoxic functions. The engagement of NKG2D activates cytolytic responses in human Vγ9/Vδ2 T cells (Bauer, 1999) while the killing of tumour cell lines by Vγ9/Vδ2 T cell relies on the expression of NKG2D ligands like ULBPs and MICA and B on tumour cells (Gomes *et al.*, 2010).

Mouse carcinogenesis models have highlighted the power of NKG2D for γδ T cell-mediated immune surveillance (Girardi *et al.*, 2001) and cytotoxicity against human cancer cells (Corvaisier *et al.*, 2005; Nedellec *et al.*, 2010). Strikingly, *in vitro* experimental blockade of NKG2D diminishes the ability of pAgs-activated Vγ9/Vδ2 T cells to kill leukemic cells by ~ 50% (Lança *et al.*, 2010). Furthermore, it was shown that NKG2D plays a key role in tumour cell recognition by murine intraepithelial γδ T cells (Girardi, 2001; Strid *et al.*, 2008) as well as human peripheral blood (Bauer, 1999; Lança *et al.*, 2010) and tumour-infiltrating γδ T cells (Groh *et al.*, 1999).

As noted before, NKG2D recognises and binds MICA and B and ULBP1-6 (Silva-Santos and Strid, 2018). This binding triggers cytotoxicity and cytokine secretion in γδ T cells (Groh *et al.*, 1998; Corvaisier *et al.*, 2005; Wrobel *et al.*, 2007; Kong *et al.*, 2009; Xu *et al.*, 2011). In fact, expression of ULBP4 in ovarian and colon carcinomas (Kong *et al.*, 2009) and ULBP3 in B-cell chronic lymphocytic leukaemia (CLL) (Poggi *et al.*, 2004) are the major factors responsible for tumour cell targeting by γδ T cells. Furthermore, the impaired killing of leukaemia/lymphoma cells was noted when ULBP1 is downregulated, whereas its overexpression enhanced Vγ9/Vδ2 T cell-mediated killing (Bauer, 1999). Similarly, longer overall survival of gastric cancer patients was associated with the expression of ULBP1 and NKG2D (Kamei *et al.*, 2018).

However, the NKG2D ligand shedding can cause an opposite effect by inducing immune evasion mechanism (Chitadze *et al.*, 2013). Along these lines, γδ T cells also express NKG2A and immunoglobulin-like transcript 2 (ILT2), which acts in an inhibitory way upon recognition of transformed cells with defective MHC I expression (Nedellec *et al.*, 2010a). This action greatly complements the frequent failure of MHC-restricted surveillance mediated by cytotoxic CD8 T cells. Thus, overexpression of NKG2D ligands may have important therapeutic potential and can be achieved by bortezomib or temozolomide treatment in multiple myeloma (Niu *et al.*, 2017) and glioblastoma multiforme (Chitadze *et al.*, 2013) cells, respectively.

Taken together, the relative significance of TCR vs NKG2D stimulation of human Vγ9/Vδ2 T cells is still a topic of debate. In addition, further research is also required to establish whether independent usage of NKG2D also varies between different γδ T cell subtypes.

1.4.3.2.2.2 TLRs

TLR expression has been noted in γδ T cells (Pietschmann *et al.*, 2009). Certain TLR ligands can modulate T-cell activation either directly or indirectly *via* accessory cells (Oberg *et al.*, 2011; Reynolds and Dong, 2013). (Co)stimulation of Vγ9/Vδ2 T cells by TLR1/TLR2, TLR2/TLR6, TLR7/8, TLR3, and TLR5 ligands have been shown to result in enhanced activation and production of cytokines and chemokines (Wesch *et al.*, 2011). Table 1.2 shows currently known TLR mediated stimulatory effects and TLR expression in Vγ9/Vδ2 T cells. In line with the notion that not all activation signalling is directed solely through the Vγ9/Vδ2 TCR, TLRs alone or coupled with other receptors can act alone, or synergistically with TCR signalling to drive particular functional responses, such as phagocytosis driven by TLR-scavenger receptor dimers.

Table 1.2. Currently known TLR-mediated responses in Vγ9/Vδ2 T cells.

TLR signalling	Currently known functional response	reference
TLR2	enhanced IFNγ and CD107a expression; in synergy with MTDs induction of sterile inflammation (IL-1β, IL-6, IL-10, RANTES,VEGF production) and tissue/cellular repair; activation following burn injury	(Mokuno *et al.*, 2000; Deetz *et al.*, 2006; Schwacha and Daniel, 2008; Martin *et al.*, 2009; Cai *et al.*, 2011; Schwacha *et al.*, 2013)
TLR3 (TLR3/7)	TCR-dependent enhanced IFNγ production & proliferation; enhanced tumour cell lysis; cells differentiation status likely determines response rate	(Hedges, Lubick and Jutila, 2005; Wesch *et al.*, 2006; Zhang *et al.*, 2007; Gibbons *et al.*, 2009; Shojaei *et al.*, 2009)
TLR4	LPS recognition; proliferation, IFNγ release, enhanced cytotoxic potential; activation following burn injury; indirect IL17 secretion in liver inflammation; indirect activation by BCG stimulated DCs & CD4 helper cells	(Cairns *et al.*, 2006; Cui *et al.*, 2009; Reynolds *et al.*, 2012; Paleja *et al.*, 2013)

TLR7/9	Upregulated upon poly I:C costimulation; IL17 production; Indirect enhanced cytotoxic activity against tumour cells	(Paulos *et al.*, 2007; Peng *et al.*, 2007; Pietschmann *et al.*, 2009; Cai *et al.*, 2011; Paleja *et al.*, 2013)
TLR7/8	Indirect, monocyte-dependent stimulation and production of IFNγ; enhanced cytotoxicity against tumours; indirect inhibition of proliferation by TLR7/8 - ZOL/HMBPP stimulated monocytes	(Peng *et al.*, 2005, 2007; Paulos *et al.*, 2007; Serrano, Wesch and Kabelitz, 2020)
Adapted from (Wesch *et al.*, 2011; Dar, Patil and Chiplunkar, 2014)		

TLR signalling may seem a great pair to the anti-tumour synergy of amino bisphosphonates and activated Vγ9/Vδ2 T cells. However, paradoxically, TLR agonists are double-faced. On one side, enhancing immune response (Paulos *et al.*, 2007) can also promote tumour cell invasion (Conroy, Marshall and Mills, 2008; Smits *et al.*, 2008; Yu and Chen, 2008; Ridnour *et al.*, 2013). Hence, care must be taken when considering the tripartite cooperation of tumour cells, TLRs, and γδ T cells in cancer treatment. A study reported enhanced tumour cell lysis by γδ T cells stimulated by TLR3/7 ligands (Shojaei *et al.*, 2009). In this, Poly(I: C) treatment resulted in overexpression of CD54 in pancreatic adenocarcinoma cells, which led to an enhanced effector function of γδ T cells triggered by CD54-CD11a/CD18 ligation (Shojaei *et al.*, 2009). In addition, treatment with TLR7 surrogate ligand-induced MHC I downregulation on tumour cells and resulted in the enhanced cytotoxic activity of γδ T cells possible via reduced affinity for inhibitory receptor NKG2A expressed on γδ T cells (Shojaei *et al.*, 2009). In contrast, indirect stimulation of Vγ9/Vδ2 T cells by TLR7/8 but not TLR7 ligands can result in enhanced activation and production of IFNγ as well as inhibition of proliferation in a monocyte-dependent manner (Serrano, Wesch and Kabelitz, 2020). Thus, TLR8 ligands may be useful for boosting anti-tumour activity in γδ T cells when applied locally into the tumour microenvironment, as proposed in clinical studies (Shayan *et al.*, 2018). To date, no studies have examined how/if TLR signalling influences induction/enhancement of APC functions in Vγ9/Vδ2 T cells.

1.4.3.2.2.3 Costimulatory receptors

As noted in the previous section describing CD8 T cells, a range of co-stimulatory receptors functions to induce qualitative and quantitative changes that lower activation thresholds, prevent anergy and enhance T cell functions. Alongside their MHC-independent activation, Vγ9/Vδ2 T cells lack CD4 expression. While they can express CD8, it remains unclear what functional purpose CD8 serves on Vγ9/Vδ2 T cells.

While αβT cells highly count on CD28 co-stimulation for their responses, the effect of CD28 co-stimulation on γδ T cells is paradoxical (Ribot, deBarros and Silva-Santos, 2011). The constitutive expression of CD28 was noted on some lymphoid γδ T cells, where it promotes survival and proliferation via IL-2 production. In this, CD28 receptor agonists boosted, whereas blocking anti-CD80/CD86 antibodies inhibited γδ T cell expansion in mice and humans (Ribot *et al.*, 2012). In contrast, experiments in CD28-deficient mice reported lower numbers of total or activated γδ T cells

upon *Plasmodium berghei* infection, while there was no expansion of IFNγ and IL-17 producing subsets (Ribot *et al.*, 2012), indicating CD28 is important for normal γδ T cell expansion. On the other hand, another mouse study reported that both functional γδ T cell subsets differentiated and expanded normally in a *Listeria* model (Laird *et al.*, 2013). Thus, this suggests that additional co-signalling receptors or cytokine signalling may be involved, depending on the indirect differential responses of different infectious agents. It would be interesting to know how variable is the dependence on CD28 co-stimulation for γδ T cell responses in different pathological conditions.

CD27 plays a pivotal role in the selective generation of IFNγ producing γδ T cells in the mouse thymus. Beyond this, CD27 plays a vital role in the expansion of IFNγ-producing γδ T cells in the periphery. This is supported by the evidence in mouse models with herpes viruses or malaria parasites infection (Ribot *et al.*, 2010), as well as *in vitro* experiments demonstrating that that co-stimulation via CD27/CD70 enhances Vγ9/Vδ2 T cell expansion while blocking either receptor/ligand with antibodies greatly reduces their proliferation (deBarros *et al.*, 2010). Moreover, CD27/CD70 ligation induces a non-canonical NF-κB signalling pathway and enhances the expression of anti-apoptotic and cell cycle-related genes, thus promoting murine γδ T-cell survival and proliferation (Ribot *et al.*, 2010). Activated Vγ9/Vδ2 T cells also express high levels of CD70 (Brandes *et al.*, 2003); thus, it could also contribute to their activation due to its known reverse signalling ability.

Other TNFR superfamily members that get upregulated upon human γδ T cell activation are CD30 (Ferrarini *et al.*, 2008) and 4-1BB (Lee *et al.*, 2013). Co-stimulation via CD30 potentiated calcium fluxes induced by TCR activation and enhances pro-inflammatory cytokine production (Biswas *et al.*, 2000). High levels of 4-1BBL on activated Vγ9/Vδ2 T cells (Lee *et al.*, 2013) may also contribute to their activation (Shao and Schwarz, 2010). A study in mice and human cells demonstrated that co-stimulation via 4-1BB induces activation, expansion, and effector functions of γδ T cells. In addition, it was shown that 4-1BB activated human γδ T cells protected NOD/SCID mice against *Listeria* infection (Lee *et al.*, 2013).

1.4.3.2.3 Co-stimulation by cytokines drives Vγ9/Vδ2 functional phenotype

As in other T cells, cytokines play a pivotal role in survival, proliferation, and driving differentiation and functional responses. Vγ9/Vδ2 T cells can be directed towards specific effector functions, in a similar way, and beyond the emerging plasticity of CD4 T cells. Thus, depending on the biological context, Vγ9/Vδ2 T cells can show features similar to different helper T cells. So far, four functional γδ T cell phenotypes have been defined according to their cytokine production, cytotoxic effector type 1 γδ T cells (γδ T1; producing IFNγ), type 2 (γδ T2), type 17 γδ T cells (γδ T17; producing IL17) as well as regulatory γδ T cells (γδ Treg). In addition, a subset of Vγ9/Vδ2 T cells defined by their professional APC function called γδ T-APCs have been described (Brandes, Willimann and Moser, 2005; Lafont *et al.*, 2014; Coffelt *et al.*, 2015). In αβT cells, IL-7, IL-15, and IL-2 are essential for lymphocyte development, homeostasis and proliferation, and IL-1β, IL-12, IL-18, IL-21, and IL-23 influence T-cell functions upon

inflammation. Here, the main influences of homeostatic and inflammatory cytokines specifically to Vγ9/Vδ2 T cell physiology and differentiation will be described, and it is summarised in **Figure 1.8.**

IL-7 is produced mainly by epithelial and stromal cells (Mazzucchelli and Durum, 2007). There is very little information available on the function of IL-7 on human Vγ9/Vδ2 T cells. Relatively recent studies suggest that IL-7 selectively promotes the expansion and homeostasis of IL17-producing Vγ9/Vδ2 T cells (Nakamura *et al.*, 2015). Even more unanticipated is a finding that IL-7 may potentially contribute to an indirect promotion of inhibitory functions. Following IL-7 treatment, Vγ9/Vδ2 T cells have been found to upregulate B- and T-lymphocyte attenuator (BTLA), which upon engagement with its ligand inhibits late-phase immune reactions, reduces activation, proliferation and anti-lymphoma response (Bekiaris *et al.*, 2013; Gertner-Dardenne *et al.*, 2013; Domae *et al.*, 2017). However, further research is required to provide more clarity on the function of IL-7 signalling in human mature γδ T cells.

IL-2 and IL-15 are closely related cytokines and mediate their effects through a heterotrimeric receptor complex consisting of a cytokine specific α-chain (IL-2Rα [CD25] or IL-15Rα), the shared IL-2/15Rβ-chain (CD122), and the γc-chain (Ring *et al.*, 2012). The experiments with paediatric thymic tissue conducted by Ribot and colleagues have shown that extrathymic IL-2 or IL-15 signals alone, in the absence of TCR stimulation/coreceptor signalling, are sufficient for functional differentiation of immature human γδ thymocytes into γδ T1 cells with tumour-killing functions (Ribot *et al.*, 2014). However, naturally, it cannot be assessed if human γδ thymocytes have already experienced TCR signals *in vivo*. Similarly, IL-2 and IL-15 are essential for the peripheral expansion of Vγ9/Vδ2 T cells stimulated with pAgs or NBPs. Interestingly, such stimulation results in IFNγ production that is as compared to IFNγ production induced in stimulated αβT cells (Chen *et al.*, 2007), which emphasises the importance of Vγ9/Vδ2 T cells in driving γδ T1- cytokine-induced immune response. Moreover, expansion of Vγ9/Vδ2 T cells from healthy donor PBMC in the presence of IPP and IL-2 results in 30–70% of expanded Vγ9/Vδ2 T cells expressing CD56 (Wesch, Glatzel and Kabelitz, 2001; Alexander *et al.*, 2008). Interestingly, both CD56+ and CD56– Vγ9/Vδ2 T cells display similar expression levels of receptors involved in the regulation their cytotoxicity (such as NKG2D and CD94). However, only CD56+ Vγ9/Vδ2 T cells were shown to kill solid tumour cell lines, including squamous cell carcinoma (Alexander *et al.*, 2008). Differentiation into γδ T1 cells can be further enhanced by IL-18 (Li *et al.*, 2010; Tsuda *et al.*, 2011) or IL-12 (Thedrez *et al.*, 2009). Interestingly, in addition to cytotoxic/cytolytic function acquired *via* pAg/IL-2/IL-15 stimulation, the same stimulation cocktail also induces pro-APCs function in Vγ9/Vδ2 T cells (Brandes, Willimann and Moser, 2005; Khan *et al.*, 2014). The combination of pAg and IL-2 and/or IL-15 stimulation generates γδ T-APCs that can process different antigens for presentation and to stimulate other immune cells (Brandes, Willimann and Moser, 2005; Marlène Brandes *et al.*, 2009; Chen *et al.*, 2017; Tyler *et al.*, 2017). Furthermore, IL-15 upregulates the low-affinity type III Fcγ receptor CD16, which is involved in phagocytosis, ADCC, and the acquisition of potent APC function (Himoudi *et al.*, 2012). Contrasting the studies above, expansion of blood Vγ9/Vδ2 T cells in the presence of IPP and low concentration of IL-2 generates T_{EM} - like cells that start to secrete high levels of IL17 in addition to IFNγ at ~14 days of culture (Hu *et al.*, 2012)

Inflammatory cytokines also play a huge role in effector differentiation of γδ T cells. Particularly, IL-12 and IL-18 typically promote IFNγ production; and IL-1β and IL-23 mostly drive IL17 production.

On the other hand, differentiation of adult Vγ9/Vδ2 T cells into γδ T17 cells requires pAg stimulation and cytokines IL-1β, TGFβ, IL-6 and IL-23 (Ness-Schwickerath, Jin and Morita, 2010; Caccamo *et al.*, 2011), while only IL-23 is required for γδ T17 cells in neonates (Moens *et al.*, 2011). These γδ T17 cells also express CCR6, granzyme B, TRAIL, FasL, and CD161 but do not produce IFNγ. Such γδ T17 cells can exert pro-tumour effects (see section 1.4.3.1) or may exhibit features of Treg cells. Studies reported that expansion of peripheral Vγ9/Vδ2 T cells in the presence of pAg and TGF-β alone or together with IL-15 induces a regulatory-like phenotype in Vγ9/δ2 T cells (Peters *et al.*, 2016) with enhanced expression of FOXP3 (Casetti *et al.*, 2009). Furthermore, these γδ T17 cells negatively impacted CD8+ and CD4+ T cell proliferation when co-cultured with antigen-stimulated PBMCs. While further studies using γδ T cell - cancer cell/tissue co-culture system mimicking TME are required to clarify the influence of TME on the physiology of γδ T cells, these findings should be taken into consideration when designing anti-cancer immunotherapeutics using γδ T cells.

Vγ9/Vδ2 T cell stimulation in the presence of IL-21 increased the production of CXCL13, and its function was associated with a T follicular helper cell-like phenotype (Vermijlen *et al.*, 2007). This IL-21 induced functional attribution of Vγ9/Vδ2 T cells may influence the generation of high-affinity antibodies against microbial infection (Bansal *et al.*, 2012) or in an early host defence mechanism protecting against cancer as described in mice (Crawford *et al.*, 2018). Another study reported that IL-21 enhances pro-inflammatory response and anti-tumour cytolytic activity of pAg-induced Vγ9/Vδ2 T cells in a dose-dependent manner (Thedrez *et al.*, 2009).

Taken together, Vγ9/Vδ2 T cell activation and differentiation into potent effectors are crucial events necessary for effective immunity against infection or tumours. An essential role in these processes represent surface receptors that capture the key extracellular signals and communicate downstream intracellular messages that regulate γδ T cell physiology. Thus, understanding how environmental signals are integrated by γδ T cells is critical for their use in clinical settings.

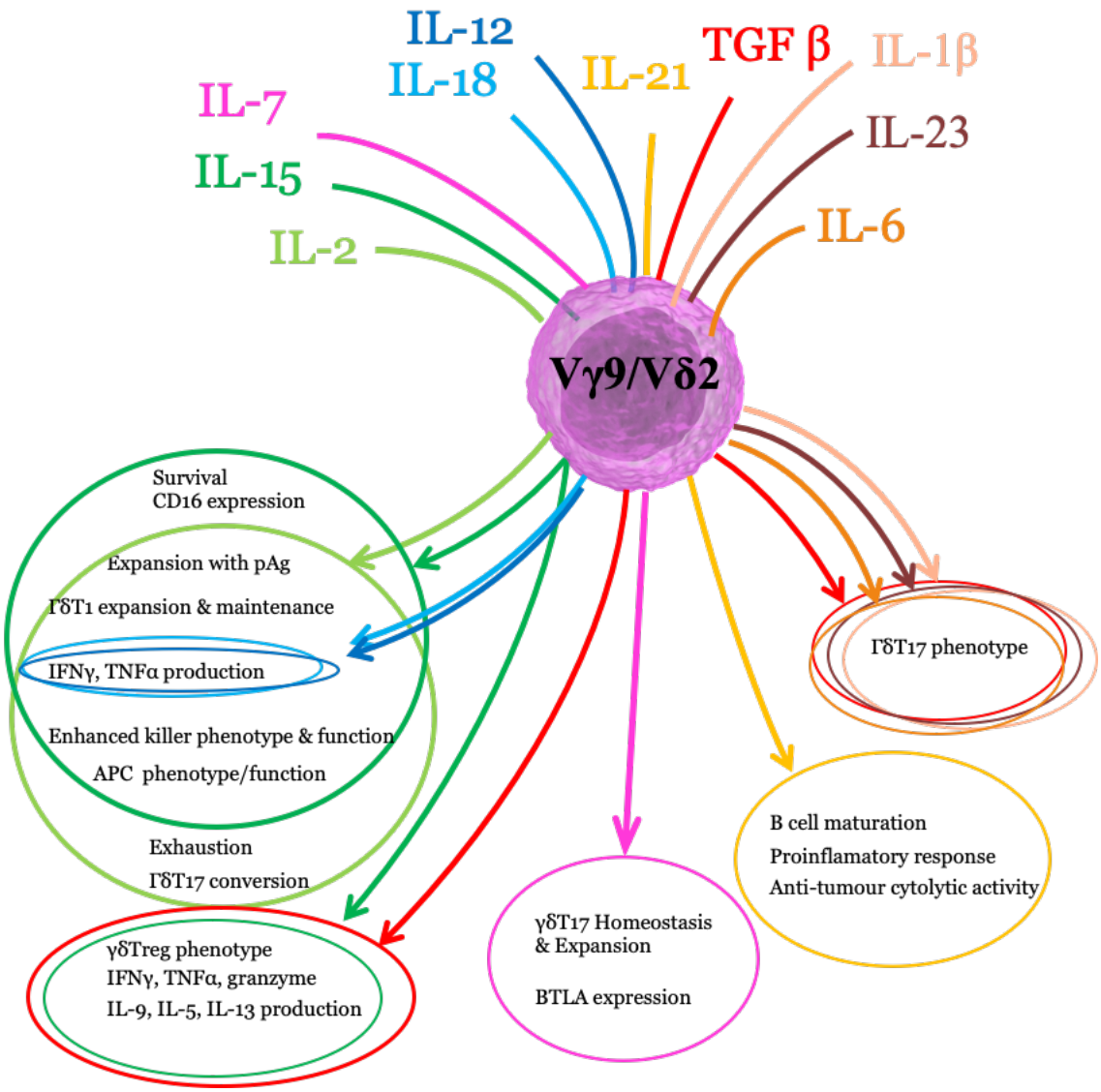


Figure 1.8. The influence of homeostatic and inflammatory cytokines on Vγ9/Vδ2T cell physiology and differentiation.
Abbreviations. BTLA, B- and T-lymphocyte attenuator; IFN-γ, interferon-γ; IL, interleukin; PAg, phosphoantigen; TNFα, tumour necrosis factor α; γδ Treg, regulatory-like γδ T cell; γδ T17, helper type 17-like γδ T cell.

1.4.3.3 Post-thymic "memory" differentiation fates of Vγ9/Vδ2 T cells

Like αβT cells, Vγ9/Vδ2 T cells can generate long-lived memory populations, in addition to their innate-like functions (Lalor and McLoughlin, 2016). Thus attempts have been made to define naïve, memory and effector subsets based on some of the αβT cell markers, particularly on CD45RA and CD27 expression or the lack of (Dieli *et al.*, 2003). However, such terminology for γδ T cells is rather vague and has to be considered with caution. For example, TEM cells and TCM cells describe subsets of memory αβT cells present in peripheral blood and were initially defined based on the expression of

lymph node-homing markers (CD62L, CCR7). In contrast, a combination of CD45RA and CD45RO expression with CD27 and/or CD28 define memory subsets, while they lack the expression of LN homing markers. However, Vγ9/Vδ2 T cells do not rely on activation by DCs and do not need to migrate to secondary lymphoid/tertiary tissues for the same purpose as αβT cells do - to receive appropriate activation/differentiation signals.

Cytotoxic γδ T_{TE} cells show unexpected persistence *in vivo* and are largely enriched in inflammatory tissues (Dieli *et al.*, 2003). This suggests fundamental mechanistic differences in controlling phenotype direction fate between these two classes of T cells. This suggests fundamental mechanistic differences in controlling phenotype direction fate between these two classes of T cells. While with years of research, many different shades of αβT cell memory states have been defined by a spectrum of different markers, the definition of γδ T cell memory states has not changed (Ryan *et al.*, 2016). It was suggested that upon stimulation, Vγ9/Vδ2 T cells differentiate linearly from T_N to T_{EM} and finally T_{TE} cells. These different populations of Vγ9/Vδ2 T cells show distinct responses to stimulation by phosphoantigens (Dieli *et al.*, 2003; Angelini *et al.*, 2004; Battistini *et al.*, 2005). As noted in previous sections, CD70-CD27 interactions greatly contribute to Vγ9/Vδ2 T cell activation and provide important survival and proliferative signals (deBarros *et al.*, 2010). Expression of CD27 is confined to naïve and T_{CM} Vγ9/Vδ2 T cells which tend to proliferate more and produce higher levels of cytokines compared to T_{EM} and T_{TE} Vγ9/Vδ2 T cells which do not express CD27 and seem to be more cytotoxic (Dieli *et al.*, 2003). T_{CM} -like γδ T cells are considered an antigen-primed subset, predominantly found in peripheral blood and showing rapid responses to re-stimulation. The cytotoxic capacity and the production of IFNγ (and other cytokines) increase with the differentiation stage of Vγ9/Vδ2 T cells, with γδ T_{EM} cells producing the highest concentrations of IFNγ compared to other memory subsets, whereas γδ T_{TE} cells produce low levels of IFNγ, highest levels of cytotoxic molecules, while they proliferate poorly (Dieli *et al.*, 2003). The broad expression of NCRs by T_{TE} cells leads to the speculation that this subset differentiates to acquire a wider "cytotoxic spectrum" for recognising targets at inflammatory sites. Interestingly, T_{EM} – like γδ T cells infiltrate peripheral inflammatory sites, and they lack NCR expression, which suggests that they are mainly activated through the TCR (Dieli *et al.*, 2003).

Similarly, as with αβT cells, depending on the cancer type and stage, the population of "memory" γδ T cells will differ in those patients. In patients with chronic lymphocytic leukaemia, poor proliferative response to zoledronate was associated with an even more pronounced bias toward terminally differentiated γδ T_{TE} cells (Coscia *et al.*, 2012). Whereas γδ T_{CM} - like cells (CD45-CD27+) predominate in PBMC of pancreatic ductal adenocarcinoma (PDAC) patients, PDAC-infiltrating γδ T cells were mostly T_{EM} -like cells. Despite this, 50% of PDA-infiltrating γδ T cells also showed high expression of PDL-1 and downregulation of CD62L, which is indicative of an activated and exhausted phenotype (Daley *et al.*, 2016). Predominant generation of T_{EM} -like Vγ9/Vδ2 T cells (CD45RA–CD27–) in expansion with NBP and IL-2 (Meraviglia *et al.*, 2010), together with great persistence of T_{TE} γδ T cells observed *in vivo* has to make Vγ9/Vδ2 T cells attractive for the potential for application in anti-cancer adoptive immune-cell therapies, since they may mediate a prolonged and effective immune attack (Dieli *et al.*, 2007; Santini *et al.*, 2009; Meraviglia *et al.*, 2010; Welton *et al.*, 2013a).

1.5 Vγ9/Vδ2 T cells as professional APCs

1.5.1. Professional & non-professional antigen presenting cells

Although there are many types of antigen-presenting molecules and thus many modes of antigen presentation, historically, everything is centred around "conventional" T cells, and so is the definition of APCs. Experiments showing that virtually any cell type expressing cell surface MHC II molecules was able to engage with "conventional" αβT cells in an antigen-specific manner opened the door to a field of studies where surrogate APCs and model protein antigens have been used to understand the process of antigen presentation under defined conditions (Trombetta and Mellman, 2005). With the description of pathways for the loading of peptides onto MHC I and MHC II molecules (described in the following sections), we have learned that different cell types can process and present antigens with greatly different efficiencies. This finding has brought a classification of certain cells into "professional" APCs (pro-APCs). The selection criteria for a cell to be called pro-APC is based on the possession of essential functional and phenotypic characteristics, such as upregulation and cell surface expression of MHC II together with a repertoire of co-stimulatory molecules such as CD80, CD86, CD40, and CD70. These phenotypic characteristics, of course, have to be coupled with the crucial ability to take up, process and present fragmented protein on MHC I and MHC II and induce functional activation of T cells. Thus, the critical feature considered for classification into pro-APCs is the ability to deliver the three signals for T cell activation (peptide-MHC-TCR engagement, co-stimulation, and cytokines) required for stimulation of naïve CD4+ and CD8+ αβT cells in the process of generating adaptive immunity.

Furthermore, the expression of PRRs provides a switch for activation and phagocytic function, which is important for their APC function. Since it is thought that the naïve T cell priming only happens in lymph nodes, the ability to migrate to the secondary lymphoid organs upon activation also add important value. Professional APCs present antigens to naïve T cells and can initiate specific immunity against a particular antigen or tolerate them to prevent autoimmunity. The long-time considered prototype members of this group of cells are dendritic cells (DCs), macrophages (MØ), and B cells (Pozzi, Maciaszek and Rock, 2005; Kashem, Haniffa and Kaplan, 2017). However, accumulating evidence from more than 15 years strongly portrays a subset of Vγ9/Vδ2 T cells featuring all professional APC characteristics (Landmeier *et al.*, 2009; Wu *et al.*, 2009; Meuter, Eberl and Moser, 2010; Himoudi *et al.*, 2012; Khan *et al.*, 2014; Howard *et al.*, 2017). Thus, Vγ9/Vδ2 T cells can be considered the newest member of pro-APCs.

On the other hand, a vast range of other cells categorised as non-professional or atypical APCs (Kambayashi and Laufer, 2014) and non-immune cells are also capable of MHC II presentation, often in response to infections or inflammation. Cells of both haematopoietic and non-haematopoietic origin are capable of MHC II expression and antigen presentation. These include monocytes, mast cells, basophils, eosinophils, neutrophils, ILCs, lymph node stromal cells, and CD4 αβT cells (Sprent, 1995; Schuijs, Hammad and Lambrecht, 2019); as well as epithelial cells, mesenchymal stromal cells, fibroblasts and endothelial cells. MHC II expression and antigen presentation were shown in epithelial cells and enteric glial cells during Crohn's disease and eosinophilic oesophagitis, in keratinocytes in

psoriasis, and in airway epithelial cells during viral infection, chronic bronchitis, asthma, idiopathic pulmonary fibrosis or lung transplant rejection, but not bacterial infection (Wosen *et al.*, 2018). Interestingly, *Mycobacterium bovis* strain Bacillus Calmette Guerin (BCG) can induce the expression of the MHC II on urothelial cells, indicating that these cells may also have a role as APCs (Pettenati and Ingersoll, 2018). Although these non-professional APCs and non-immune cells are normally capable of MHC II expression and antigen presentation, they lack one of the essential characteristics of professional APCs – they cannot stimulate and initiate responses in naïve αβT cells and cannot migrate to lymph nodes. Thus, for an immune response to be generated, pro-APCs are essential, while the non-professional APCs function as modulators of already initiated responses, and it seems that they can orchestrate immune responses depending on the pathobiological context.

1.5.2 MHC molecules

Recognition and activation of αβT cells are restricted to short peptide ligands bound to antigen-presenting molecule MHC, also known as Human Leukocyte Antigen (HLA) at the cell surface. MHCs are encoded within the most polymorphic region of the human genome - the HLA locus (Potts and Slev, 1995). The HLA locus spans a region of about four million bp on the short arm of chromosome 6 and encodes more than 7,000 allelic variants. Still, some variants are found at considerable frequencies across the population (Robinson and Marsh, 2003). The HLA genes encode two types of homologous proteins, MHC class I (MHC I) and class II (MHC II).

At the surface, every cell of each individual expresses 6 different MHC I (two of each, HLA-A, HLA-B, and HLA- C), and up to 6 different MHC II (two of each, HLA-DR, HLA- DQ and HLA-DP), giving rise to their individual tissue type or MHC restriction.

MHCs are massively polymorphic molecules, each potentially being able to present thousands of different peptides. Overall, it is estimated that the order of 10^{15} different pMHCs can be expressed (Wooldridge *et al.*, 2012) while a single cell may express up to 10^{6} MHC I molecules at its cell surface (Yewdell, Reits and Neefjes, 2003). Although some of these MHCs may present the same peptides, most of them will differ. Thus, each cell will have a unique, complex cell surface milieu for the immune system inspection and identification of abnormal or infected cells. Several cytokines can regulate the expression of MHC molecules; especially interferons are known to upregulate both MHC I and MHC II expression (Propper *et al.*, 2003) and PAMP/DAMP signals (Reinicke *et al.*, 2019). In addition, MHC II expression can be induced by IFNγ and other stimuli in non-APCs, but also in the absence of co-stimulation to maintain peripheral tolerance (Yewdell, Reits and Neefjes, 2003). Finally, MHC molecules are crucial for αβT cell recognition. However, the coreceptors play a fundamental role in their function and aid in identifying dysfunctional/infected cells at the protein level.

1.5.2.1 MHC I molecules

MHC I molecules are generated from the HLA-A, -B or -C loci and each bind different peptide ligands. They are expressed by almost all nucleated cells and platelets (Johnson, 2000), but their expression

level varies between tissues and different cells types. For example, brain and kidney cells express lower levels, whereas lymphocytes show high expression of MHC I, but testicular cells do not. Thus, all nucleated cells are able to present peptides on MHC I. MHC I molecules present short peptide antigens (8-13 amino acids). T cells that are able to recognise peptide-MHC I complexes express the CD8 coreceptor, which plays a crucial role in the recognition of pMHC I complexes and will be discussed in later sections. Under normal homeostatic conditions, these MHC I peptides are mostly derived from endogenously processed intracellular proteins. Therefore, a healthy immune system will be nonreactive to these due to self-tolerance. In case of intracellular viral infection or if the cell produces mutant proteins due to dysfunction/carcinogenesis or is from an allogeneic transplant, this results in the presentation of "foreign" pMHC I and will stimulate cytotoxic T cells (CTL) to eliminate the affected cell.

MHC I is a heterodimeric glycoprotein that consist of a 44 kDa heavy α chain and a 12 kDa β2-microglobulin (β2m) chain (**Figure 1.9**). The heavy a chain is composed of three domains called α1, α2 and α3. The α1 and α2 are polymorphic with many allelic variants across the population (Neefjes *et al.*, 2011), whereas the α3 domain is anchored to the membrane with transmembrane helices and covalently bonds to the β2m. The cytosolic tail possesses a conserved tyrosine residue, which is required for MHC internalization from the cell surface and is important upon DC maturation. Interestingly, the β2m gene is encoded outside the HLA loci on the chromosome 15. The peptide-binding grove is closed-ended and is formed cranially by α1 and α2 chains. This conformation of the α1α2 binding groove restricts the length of presented peptides to 8-13 aa. The type of interactions of individual peptide side-chains with the MHC depends on the geometry, charge distribution, and hydrophobicity of the binding groove (Wieczorek *et al.*, 2017).

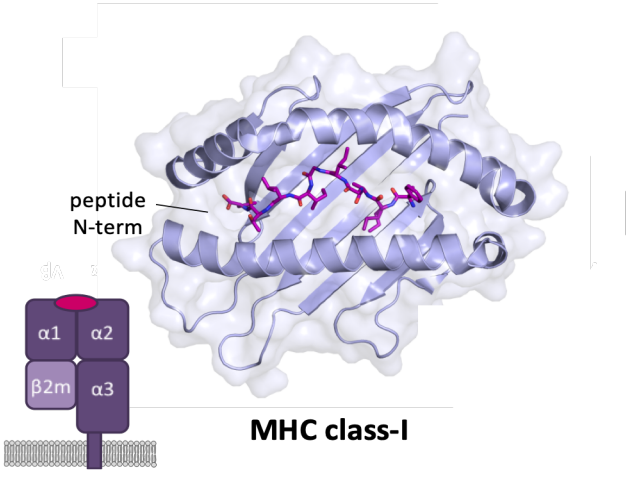


Figure 1.9. MHC molecules.
Schematic representation of MHC class I domains are shown as well as ribbon representation (PDB-code: 4WJ5) together with surface presentation of the peptide in the binding groove. The peptide is depicted in pink.

1.5.3 Classical Antigen processing and presentation pathways

The immune system provides continuous surveillance against viral infections and cancers by monitoring whether cells synthesise foreign or mutant proteins. In this process, distinctive intracellular pathways for protein antigen processing are known that combine peptide ligands with MHC I or MHC II and deliver the peptide-MHC (pMHC) complexes to the cell surface for their presentation to αβ T cells. Moreover, in professional APCs, this process is coupled with the different routes of antigen uptake from the extracellular microenvironment, which can influence the processing pathway and thus a mode of antigen presentation.

1.5.3.1 Antigen presentation by MHC I

For classical MHC I presentation of intracellular self and foreign antigens, peptides are largely generated via the ubiquitin-proteasome degradation pathway of intracellular proteins, which requires ATP (Rock and Goldberg, 1999). The intracellular events of antigen processing and presentation by MHC I can be described in six major steps: (i), acquisition of antigenic peptides; (i), tagging of the antigenic peptide by ubiquitylation to be deconstructed by; (iii), proteolysis; (iv), delivery of peptides from the cytosol to the endoplasmic reticulum (ER); (v), binding of peptides to newly generated MHC class I molecules; and (vi), transport to and display of peptide–MHC class I complexes on the cell surface (**Figure 1.10).**

The proteasome is a large proteolytic complex that can be found in the cytosol in two forms. Under normal homeostatic conditions, cells contain a standard proteasome, which includes constitutive catalytic subunits β1, β2, and β5. Upon inflammation/infection/stress, the proteasome catalytic machinery is replaced by interferon-γ (IFNγ) – inducible subunits β1i, β2i, and β5i to form the immunoproteasome, which has different cleaving specificities (Rock and Goldberg, 1999; Kelly and Trowsdale, 2019). Some tumour tissues exclusively use intermediate proteasomes composed of a blend of catalytic and inflammatory subunits (Guillaume *et al.*, 2010). In the cytosol, proteasomal peptide products can be further trimmed or destroyed by cytosolic proteases (Trombetta and Mellman, 2005). For MHC I presentation, selected cytosolic peptides are then translocated to the ER lumen by a transporter associated with antigen processing (TAP). In the ER, the peptide fragments can be further trimmed at the N-terminus by endoplasmic reticulum aminopeptidases ERAP1 to adjust their length for optimal binding to MHC I. Newly synthesised α and β heterodimers of the MHC I are assembled with the help of chaperons calnexin, calreticulin, ERp57 and tapasin (Rock and Goldberg, 1999). These new MHC I molecules are then combined with the peptide to form a stable pMHC complex. Finally, the pMHC I and empty MHC I molecules exit the ER and are transported to the cell surface for presentation to CD8 T cells (Harding and Unanue, 1990; Pamer and Cresswell, 1998) **(Figure 1.10).** Some viral antigens are presented on the cell surface very rapidly after infection, which can be several hours before functional viral proteins are first detected inside the cell – and this surface presentation is more rapid than the half-life of the viral peptide would last (Neefjes *et al.*, 2011) (Schubert *et al.*, 2000; Vyas, Van Der Veen and Ploegh, 2008).

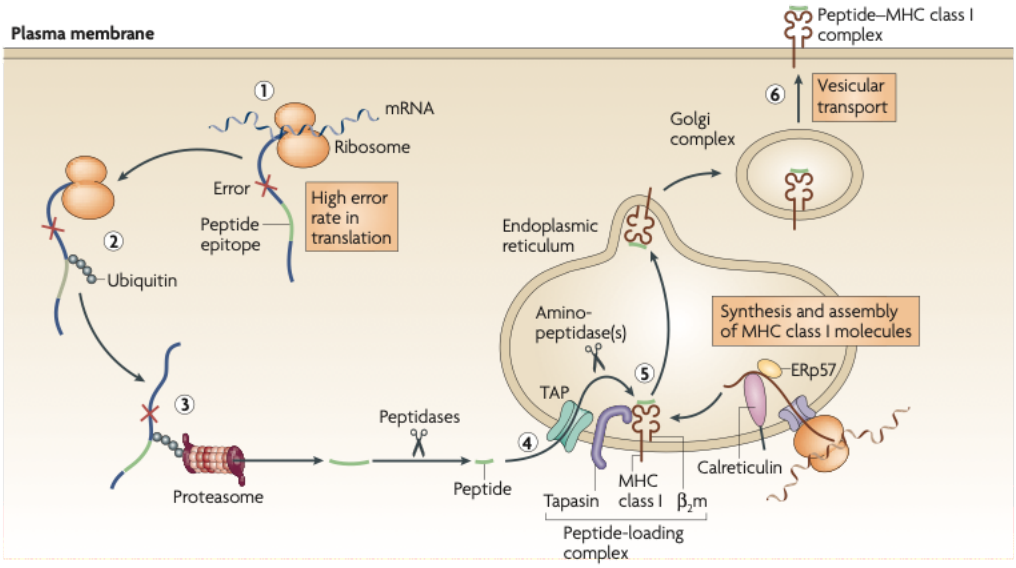


Figure 1.10. Antigen processing for peptide presentation by MHC I molecules at the cell surface.
Antigen processing and presentation can be arranged into six steps. 1) acquisition of intracellular proteins. 2) misfolded proteins are tagged with ubiquitin (U) for degradation. 3) the proteasome chops these U-tagged proteins into peptides. 4) which are then transported to the endoplasmic reticulum (ER) by the transporter associated with antigen processing (TAP) complex. 5) peptide is loaded onto nascent MHC I molecules, facilitated by members of the peptide-loading complex, e.g. Tapasin & housekeeping ER proteins calreticulin and ERp57. Finally, 6) peptide-loaded MHC I molecules are transported via Golgi onto the cell surface. Adapted from Vyas *et al.*, 2008.

1.5.4 Alternative antigen presentation pathways

For a long time, the pathways for the generation of peptides for both MHC I and MHC II presentation have been viewed as exclusively driven by the proteasome and lysosomal-associated proteases, respectively. Furthermore, antigens captured from extracellular sources were considered an exclusive aspect of MHC II presentation. However, then the question arose as to how naïve CD8 T cells recognise viral and tumour antigens. For the past 20 years, it has been clear that such antigens can also be processed and presented on MHC I via a cross-presentation process. Equally, the presentation of antigens taken from the nucleo-cytoplasmic space was considered the feature of MHC I, but it is now clear these antigens add a substantial proportion of the peptide repertoire presented by MHC II. Alternative routes of antigen processing and intracellular trafficking coupled with specific antigen uptake mode allow antigens to be presented in an "unconventional" fashion.

1.5.4.1 MHC I Antigen cross-presentation

MHC I cross-presentation is a process that enables exogenous Ag to "cross" into the MHC class I pathway for activation of CD8 T cells. Such alternative presentation routes involve specific endosomal organelles and complex intracellular trafficking pathways, different from those involved in classical MHC I presentation. In addition, self-antigens can be presented via these pathway for induction of

tolerance in a process termed cross-tolerance. A few different immune cell types can use cross-presentation pathways (Giodini, Rahner and Cresswell, 2009), although it has been best described in DCs (Joffre *et al.*, 2012). Nevertheless, not all types of DCs can cross-present antigens. For example, it was shown that only CD1c+ blood DCs and monocyte-derived DCs (moDCs) could cross-present peptides of the full-length tumour antigen NY-ESO-1 on MHC I (Schnurr *et al.*, 2005). Any phagocytic cell can likely cross-present at some level, given that this activity has been seen in neutrophil, myeloid cells, osteoclasts, Kupffer cells, microglia, and even cells of nonhematopoietic lineage, such as endothelial cells, FcR- transfected 293T cells and thymic epithelial cells. B cells can cross-present in a situation when antigen is internalised by receptor-mediated endocytosis and located into endocytic compartments (Rodríguez-Pinto, 2005).

Cross-presentation pathways can vary depending on the antigen, route of uptake (phagocytosis/endocytosis/macropinocytosis), triggering of PRR signalling pathways, and cell type. For example, in DCs, the mode of DC activation and the antigen delivery strategy are critical factors for MHC I cross-presentation and efficient stimulation of tumour-specific CD8 T cells (Schnurr *et al.*, 2005; Mintern, Macri and Villadangos, 2015; Dasari *et al.*, 2016; Van Kaer *et al.*, 2019). In addition, a variety of intracellular processes, vesicle trafficking and autophagy play critical roles in antigen processing pathways for MHC I presentation (Mintern *et al.*, 2015; Dasari *et al.*, 2016; Van Kaer *et al.*, 2019). Nevertheless, many of the proteins involved in cross-presentation remain unidentified and/or can differ in pro-APCs versus other cells. Despite intensive investigations, overall, there does not appear to be one straightforward model for cross-presentation which would encompass all of the data published in a clear picture (Grotzke *et al.*, 2017). The routes by which exogenous antigens access newly formed MHC class I molecules also remain opaque.

In order to be cross-presented on MHC I molecules, it is generally acknowledged that the internalised protein material requires proteasomal processing and trimming of peptides; thus, it should reach cellular compartments containing proteasome, although it can also occur in a proteasome-independent manner. In DCs, two intracellular routes for antigen processing have been described: the "cytosolic" and the "vacuolar" pathways (Joffre *et al.*, 2012) **(Figure 1.11).**

The hallmark of the cytosolic pathway of MHC I cross-presentation is that the internalised antigens are transported from the endosomes into the cytosol (Zehner *et al.*, 2015) where they are digested by the cytosolic proteasome (Ackerman *et al.*, 2003). This was backed-up by experiments that showed that cross-presentation was inhibited by proteasome inhibitors (Falo *et al.*, 1995) thus suggesting that internalised proteins *must* enter cytosol for processing. Proteasome-generated peptides can be then transported by TAPs either (i) back into endocytic compartments and are loaded onto newly formed MHC I molecules (**cytosolic pathway with endocytic loading**); or into (ii) the ER for the same purpose as in the classical MHC I pathway manner therein (**cytosolic pathway with ER loading**). However, where peptide processing and MHC I loading occurs is still a matter of debate. (Ackerman *et al.*, 2003; Zehner *et al.*, 2015)(Guermonprez *et al.*, 2003)(Houde *et al.*, 2003).

In contrast, **the vacuolar pathway** pathway is generally considered minor. It overlaps with the MHC II Ag processing pathway and does not require internalised antigen to exit the endosomal compartment;

antigen processing and loading on MHC class I molecules occur in endocytic compartments thus are generally TAP- independent. The internalised exogenous antigens are degraded within endosomal compartments by lysosomal proteases (cathepsin S inhibitors), and derived peptides are trimmed and loaded onto recycling MHC I molecules *in situ* (Shen *et al.*, 2006; Joffre *et al.*, 2012) **(Figure 1.11).**

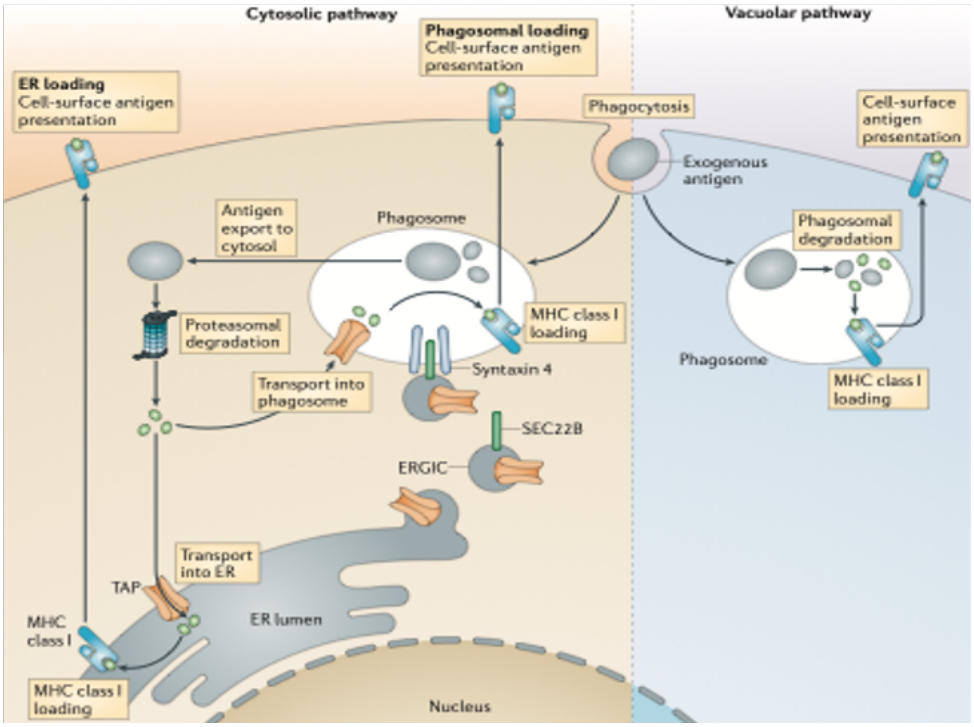


Figure 1.11. A Simplified illustration of cytosolic and vacuolear intracellular pathways for cross-presentation.
As extracellular antigens get taken up into a phagosome, they can be exported into the cytosol, where they are processed into peptides by the proteasome. These peptides can then be loaded on MHC I molecules either in the endoplasmic reticulum (ER) (the cytosolic pathway with ER loading) or transported back into the phagosome to be loaded on MHC I molecules (cytosolic pathway with phagosomal loading). Alternatively, extracellular antigens can be degraded into peptides in the phagosome, where they can be loaded onto MHC I molecules in situ (vacuolar pathway). Adapted from Joffre *et al.*, 2012.

In cross-presentation specialised DCs, the crucial requirement for the cross-presentation pathway to occur is their distinctive ability to maintain a higher pH in endosomal compartments, as compared with non-specialised DCs or macrophages. A higher endosomal pH delays lysosomal antigen protein degradation since lysosomes require acidic conditions for optimal performance. This delayed acidification in prelysosomal or lysosomal compartments allows protein export to the cytosol and its loading onto recycled MHC-I molecules in the endosomes (Joffre *et al.*, 2012).

Many studies suggest that phagosomes play an important role in antigen cross-presentation, but whether the cross-presentation preferentially occurs via the cytosolic or vacuolar pathways is not easy to determine. That is because MHC class I molecules that are retained in the ER are unstable and/or are rare in endosomes in both TAP-deficient cells and proteasome inhibitor-treated cells (Kurts and Wagner, 2011; Joffre *et al.*, 2012; Mintern *et al.*, 2015). The bottom line from all these studies is that

our understanding of the intracellular mechanisms used for trafficking and sorting cross-presenting components from the plasma membrane, ER, and ERGIC are rather opaque and incompletely described.

1.5.5 APC markers and phenotype characteristics in Vγ9/Vδ2 T cells

The finding that Vγ9/Vδ2 T cells switch their migratory profile in terms of chemokine expression shortly after activation led to the notion that human Vγ9/Vδ2 T cells could act as APCs (Brandes *et al.*, 2003). In their steady-state, circulating Vγ9/Vδ2 T cells express chemokine receptors, including CCR5, CCR2 and CXCR3, which allow them to infiltrate into sites of inflamed tissues producing corresponding chemokines CCL5/RANTES, CCL2/MCP-1 and CXCL11/I-TAC, respectively. By contrast, these resting Vγ9/Vδ2 T cells do not express lymph node homing receptor CCR7 receptor, which indicates their role in immune surveillance at inflammatory sites. However, as Vγ9/Vδ2 T cells receive activation signals, a rapid downregulation of CCR5 (inflammatory site-homing) accompanied with upregulation of CCR7 and other homeostatic chemokine receptors such as CCR4 and CXCR4 occurs within 48 hours of stimulation (Brandes *et al.*, 2003). In addition, other changes in relation to a response to chemokines also occur, such as upregulation of L-selectin or CD62L (Brandes *et al.*, 2003). As such, Vγ9Vδ2T cell regulation of chemokine receptors expression is opposite to that in CD4 and CD8 αβ T cells, and rather this migratory behaviour is remarkably similar to DCs, who rapidly upregulate CCR7 in response to PAMPs such as LPS. Accordingly, human Vγ9/Vδ2 T cells were identified in secondary lymphoid tissues such as those from gastrointestinal sites, tonsils, and spleen; they could be found in both the T cell and B cell zones (Brandes *et al.*, 2003). Thus, the finding that Vγ9/Vδ2 T cells acquire lymph node-homing phenotype suggested their potential role as professional APCs, potentially being able to prime naïve CD4 and CD8 T cells responses or being involved in regulating adaptive immunity.

In addition, Vγ9/Vδ2 T cells also rapidly acquire a professional APC phenotype in terms of surface expression of the prototype antigen-presenting molecules MHC II (HLA-DR) and MHC I, in combination with a large repertoire of co-stimulatory and adhesion molecules (Brandes, Willimann and Moser, 2005). They express or upregulate CD80, CD86 and CD70, which is also accompanied by an expression of CD83 and CD40, while these molecules are not detectable on resting Vγ9/Vδ2 T cells. In addition, adhesion molecules including CD11a, CD18, CD50, and CD54 are also expressed by γδ T-APCs (Khan *et al.*, 2014), suggesting that tight interactions can be formed with other immune cells. This repertoire of APC markers is almost indistinguishable from that displayed by mo-DCs (Khan *et al.*, 2014), and is consistent across a range of Vγ9/Vδ2 T cell stimulants. Remarkably, unlike with DCs where MHC II is recycled from the cell membrane, the expression of HLA-DR in γδ T-APCs is the result of *de novo* production. The functional relevance of this HLA-DR expression is to execute an immediate innate-like rapid response to inflammatory stimuli. This HLA-DR expression, together with subsequent phenotype and function alteration in Vγ9/Vδ2 T cells and their ability to migrate to the lymph nodes, supports the notion of their role in directing adaptive responses. In addition, γδ T-APCs can endocytose and process protein antigen for presentation on MHC class II to CD4 T cells. Remarkably, there is a

high morphological similarity between γδ T-APCs and DCs, presenting numerous dendrite-like protrusions (Brandes, Willimann and Moser, 2005; Welton *et al.*, 2013). The identification that Vγ9/Vδ2 T cells acquire the ability to migrate to the lymph nodes shortly after activation and develop a professional APC phenotype *in vitro* suggests that these cells play a role in establishing adaptive immune responses and interact with B and T cells in the lymph nodes.

1.5.6 Uptake and processing of protein antigens by γδ T-APCs

In addition to their pro-APC phenotype and ability to migrate to lymph nodes following their activation, Vγ9/Vδ2 T cells have also shown the ability to take up, process and present antigens on MHC molecules. For example, it has been demonstrated that Vγ9/Vδ2 T cells can take up smaller particulate antigen and cellular debris by macropinocytosis (Meuter, Eberl and Moser, 2010). An earlier study has suggested that γδ T cells use trogocytosis as a way to sample and acquire molecules from other cells for their immunosurveillance (Poupot, Pont and Fournié, 2005). While immature DCs exhibit great ability to endocytose and phagocytose, which decrease upon their maturation, it was suggested that Vγ9/Vδ2 T cells appear to be less efficient in this process, showing diminished function more similar to that shown by B cells and monocytes (Hu *et al.*, 2012). Nevertheless, a few studies have described that under specific experimental conditions, Vγ9/Vδ2 T cells are capable of phagocytosis. A study by Gustafsson lab showed that Vγ9/Vδ2 T cells could take up larger particles such as 1 μm synthetic beads and even *E. coli* (Wu *et al.*, 2009). This phagocytic ability relies on the expression of the Fc receptor CD16. Lastly, it was shown that Vγ9/Vδ2 T cells could kill and take up fragments of tumour cells for the presentation of tumour associated antigens in an antibody-opsonisation dependent manner, by a mechanism the authors termed licensing (Himoudi *et al.*, 2012). The latest exciting study has shown that activated Vγ9/Vδ2 T cells can detect, phagocytose and degrade *Plasmodium falciparum*-infected red blood cells in CD16 dependent manner (Junqueira *et al.*, 2021).

Like DCs, γδ T-APCs are capable of processing antigens via lysosomal and proteasomal pathways. It was shown that γδ T-APC take up and process antigens such as the influenza virus matrix protein M1 (fluM1), complex protein mixture of Mycobacterium tuberculosis-purified protein derivative (PPD), and even debris from influenza-infected cells for presentation to classical αβ CD8 T cells (Brandes, Willimann and Moser, 2005; M. Brandes *et al.*, 2009; Meuter, Eberl and Moser, 2010).

1.5.7 Induction of αβT cell responses

The stimulation of CD8 T cells by professional APCs allows for the generation of cytotoxic T lymphocyte responses. The initial studies demonstrated that IPP stimulated Vγ9/Vδ2 T cells can induce proliferation in alloreactive T cells as well as their differentiation into cytotoxic effector cells in mixed lymphocyte reactions (Brandes, Willimann and Moser, 2005). Importantly, these activated and antigen-loaded γδ T-APCs induced CD8 T cell responses comparable to responses induced by LPS-matured moDCs. Furthermore, Vγ9/Vδ2 T cells that were activated with IPP and pulsed with the superantigen toxic shock syndrome toxin (TSST-1) were able to induce naive CD4 T cell proliferation as well as their polarisation into Th1 and Th2 functional subsets (Brandes, Willimann and Moser, 2005).

Although expression of MHC II by activated T cells can function as an amplifier of memory responses and is commonly seen in activated T cells (Barnaba et al., 1994),), to date, only Vγ9/Vδ2 T cells have shown the ability to prime naïve T cell responses. Thus, this study demonstrated for the first time that uniquely among T cells, Vγ9/Vδ2 T cells can function as pro-APCs. In another study, T_{EM} Vγ9/Vδ2 T cells isolated from PBMC and synovial fluid mononuclear cells (SMFCs) from Rheumatoid Arthritis (RA) patients were able to effectively present antigen Epstein Barr Virus gp110 (EBVgp110,) and Tetanus Toxin (TT) to patients' CD4 T cells. These SMFC-derived Vγ9/Vδ2 T cells also produce a high level of IL17, thus were suggested to have a role as professional APCs in the pathogenicity of RA (Hu *et al.*, 2012). A more recent study (Tyler *et al.*, 2017) demonstrated that microbially activated Vγ9/Vδ2 T cells could polarise both blood and colonic CD4+ T cells toward distinct effector fates in IL-6 independent mechanism that is different from the one induced by monocytes and DC. Microenvironmental factors in the intestine directly influence Vγ9/Vδ2 T cell's ability to prime and activate CD4+ T cells to promote local barrier defence via mucosal release of IL-22 and calprotectin (Tyler *et al.*, 2017).

Successful cross-presentation of a spectrum of viral and tumour derived antigens (Marlène Brandes *et al.*, 2009; Meuter, Eberl and Moser, 2010; Altvater *et al.*, 2011) confirmed that the APC machinery and surface expression of MHC class I molecules in Vγ9/Vδ2 T cells are functional. Transduction of EBV latent membrane protein-2 (LMP2) into Vγ9/Vδ2 demonstrated that they could present endogenous antigens to CD8 T cells and induce their cytotoxic function (Landmeier *et al.*, 2009). One of the most remarkable features of γδ T-APCs is their high proficiency in antigen MHC I cross-presentation and induction of CD8 T cell responses (Meuter *et al.*, 2010, Brandes *et al.*, 2009). Activated Vγ9/Vδ2 T cells showed a great ability to cross-present soluble exogenous antigens such as FluM1, PPD and TT to both naïve and memory CD8 T cells. After encountering antigen-loaded γδ T-APC, antigen-specific CD8 T cells displayed cytotoxic capability, perforin expression, IFNγ production, and migratory reprogramming, evidenced by the loss of CCR7 expression. Moreover, activated Vγ9/Vδ2 T cells are able to process and cross-present antigens derived from debris of cells infected by influenza viruses and whole virions (Meuter *et al.*, 2010). In addition, it was shown that larger beads coated with Flu M1 could be cross presented in a CD16 (FcγR)-dependent manner by Vγ9/Vδ2 T cells (Wu *et al.*, 2009). This robust professional APC function of Vγ9/Vδ2 T cells indicates their importance in the first line of defence and response to infections and cancer.

1.6 Cancer and the immune system

It has long been believed that the immune system primarily co-evolved with microbes to protect the host from infection with pathogens. With observations of the role of immune system function in clearance of mutated/transformed, dysfunctional and dead cells, as well as in wound healing, the vital importance of the immune system's role in surveillance and tissue homeostasis has become more and more recognised. The immune system has been implicated in pretty much every stage of cancer development, from initiation and growth to recurrence, invasion and metastasis (Hanahan and Weinberg, 2011).

Even though immune cells can directly kill cancer cells and help contain primary tumours, they can also produce factors that suppress anti-cancer immunity and benefit tumour growth and dissemination. Immunotherapies, based on the capacity of the immune system to antagonise tumour growth, have shown great promise but do not work as widely as hoped. Regardless of extensive research, there is a stark lack of information and understanding regarding why, how, when, and where different arms of the immune system influence tumour cells.

This section will describe the role of the immune system in the development/progression of cancer. As the main focus of my work is the regulation of immune responses mediated by γδ T-APCs, the emphasis of this part of my thesis will also be on tumour-associated immune cells, γδ T cells and cytotoxic T cells in particular. While other tumour-associated non-immune cells add important weight to cancer biology and treatment, they will not be discussed in detail.

1.6.1 Immune surveillance

The concept of immune surveillance describes that an ever-alert immune system continuously monitors cells and tissues and that such immune surveillance serves ` identifying and eradicating (most of) emerging cancer cells and thus budding tumours. Fitting in this concept, solid tumours that do appear have somehow been undetected by various arms of the immune system or have arisen due to the limited extent of immunological killing, thereby 'evading' eradication. Alternatively, when the immune system function is not in mint condition, thus unable to identify and/or destroy stressed/dysfunctional cells, a complex relationship between cancer and the immune system develops that shapes and sponsors an outgrowth of cancer cells and metastases. Collectively, the sequence and process of these events and circumstances leading from immune surveillance to immune escape have been then termed 'cancer immunoediting' and have been proposed to be grouped into three phases: elimination, equilibrium, and escape (Dunn *et al.*, 2002; Kim, Emi and Tanabe, 2007). Research using genetically modified mice (GMM), whereby specific components of the immune system were removed, has added great value to our understanding of the function of the immune system in cancer, demonstrating that several immune effector cells and pathways are important for either suppression or promotion of tumour development. An increasing body of evidence, both from GMM and clinical epidemiology, demonstrates that the immune system operates as a critical barrier to tumour formation or progression.

1.6.1.1 Cancer surveillance/immunoediting in humans

In addition to studies in mice, antitumoural immune responses have been increasingly recognised within clinical epidemiology. For example, immunosuppressed patients have a higher risk (3- to 100 fold) of developing certain types of solid tumours and cancers, most commonly lymphomas (Penn, 1988; Buell, Gross and Woodle, 2005). Generally, a better prognosis has been noted in patients with high levels of infiltrating effector CD8 T cells, NK cells and γδ T cells, and particularly in patients with colorectal, ovarian and breast cancer (Gentles *et al.*, 2015). A favourable prognosis is linked with the

presence of CD8 T cells in colorectal cancer cell nests (Naito *et al.*, 1998) and this association is further exhibited in tumours with high levels of microsatellite instability (MSI) MSI (MSI-H) (Guidoboni *et al.*, 2001; Buckowitz *et al.*, 2005). These MSI-H tumours show a high mutational rate which results in higher production of neoantigens that can be recognised by B cells (Ishikawa *et al.*, 2003), CD4 T cells (Saeterdal *et al.*, 2001) and CD8 T cells (Ishikawa *et al.*, 2003). It is not unusual for MSI-H tumours to contain lymphoid follicles and host a high amount of lymphocytes, including activated CD8 T cells (Buckowitz *et al.*, 2005) . (Nakata *et al.*, 2002) (Lu *et al.*, 2004).; which is representative of a powerful local immune response. Collectively, these findings indicate that the generation of antigenic peptides as a result of genomic instability might result in the priming of a protective adaptive immune response in patients with MSI-H cancers.

A large bioinformatics study of thousands of cancer patients of 39 cancer types found that among 22 different tumour-associated immune cell populations, the presence of γδ T cells is favourable correlate with overall better prognosis and survival (Gentles *et al.*, 2015).). In particular, in melanoma and breast adenocarcinoma lesions, γδ T cell infiltration has been negatively associated with the cancer stage and presence of metastases, supporting a role for these cells in immunosurveillance. Together with the evidence from *in vivo* studies in mice and *in vitro* human studies, these findings indicate that γδ T cells play an important role in controlling tumour growth. On the other hand, however, evidence suggests γδ T cell can also promote tumour growth and progression (see below). Hence, there is a need to define how human epithelia and tumour cells interact with tissue-resident γδ T cells.

In summary, many cell types, including αβ and γδ T cells, NKT cells, and NK cells, together with several cytotoxic molecules, such as perforin and TRAIL, and cytokines such as IFN-γ, type I IFNs, and IL-12 have been shown to contribute to successful immune surveillance and tumour elimination. It is clear that in cancers with such cellular composition, the immune system functions protectively, as noted for genetically unstable cells and/or carcinogen-challenged cells. However, it is important to acknowledge that the selection of effector cells and cytokines involved in elimination and immunoediting can be different among models and cancer types. Indeed, in some cancers, the immune system seems to have little influence on the rate of tumour onset or progression or even a counteractive function (see next section). In such cases, the recruitment or presence of certain cell types such as myeloid cells or Tregs and an inflammatory cytokine milieu sponsor the tumour growth and progression. The level of immune regulation and tolerance induced in such cancers might explain, at least in part, why in some instances, the removal of certain immune elements has an obvious effect on tumour progression.

1.6.2 Tumour microenvironment

For a long time, a tumour-centric view has dominated cancer research, focused on the interpretation of the inherent characteristics of individual cancer cells (such as sustained proliferative signalling, resisting cell death, inducing angiogenesis, enabling replicative immortality, evading growth suppressors, and activating invasion and metastasis) (Hanahan and Weinberg, 2011) with diminutive, if any, attention paid to their surrounding environment. However, over the past few decades, experimental evidence has accumulated and solidified the notion that the tumour microenvironment (TME) plays a crucial role in fostering or limiting tumour development - a view that has now become

widespread (Maman and Witz, 2018). Finally, the concept that cancers arise and develop due to many different but interacting circumstances has gained undeniable recognition over the past decade (Laconi, 2007; Li, Fan and Houghton, 2007; Hu and Polyak, 2008; Whiteside, 2008; Witz, 2008; Siemann, 2010; Hanahan and Weinberg, 2011; Finn, 2012; Chen and Mellman, 2013; Di Martino, Mondal and Bravo-Cordero, 2019).

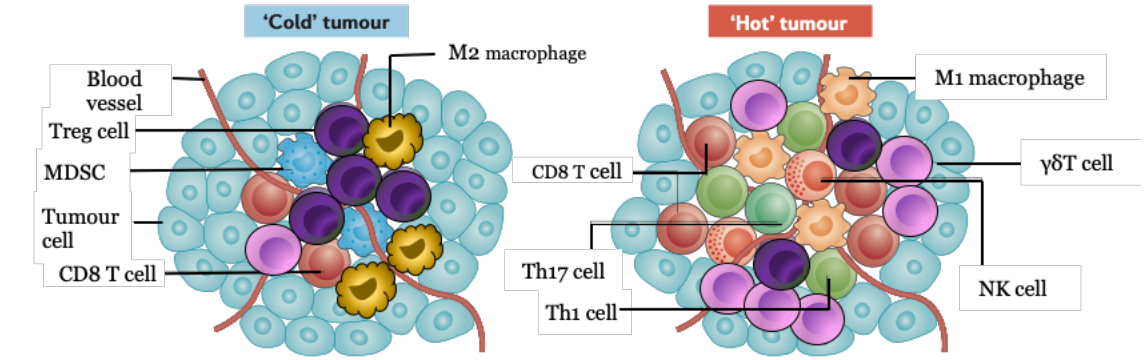
Pathologists have long acknowledged that some tumours are heavily infiltrated by innate and adaptive immune cells, thereby mirroring inflammatory conditions arising in non-neoplastic tissues (Dvorak, 1986). Since then, the evidence that the frequency of particular immune cells that functionally enhance cancer cell features began to accumulate; that the immune cell infiltration of neoplastic tissues acts counterintuitively, paving tumour progression. The concept of such studies was based on the observation of resemblance of chronic inflammation within tumour formation and the observation that tumours could be portrayed as wounds that never heal (Schäfer and Werner, 2008)(Dvorak, 1986). During the acute infection and normal wound healing, immune-inflammatory cells appear transiently and then disappear. This is in contrast to their persistent appearance in sites of chronic inflammation, where their presence has been associated with various tissue pathologies, including fibrosis, oedema and neoplasia (Karin, Lawrence and Nizet, 2006; Grivennikov, Greten and Karin, 2010). Thus, it has become increasingly clear that chronic inflammation also alters the tissue environment and creates a pro-tumourigenic environment through cellular mechanisms such as the production of cytokines, chemokines, pro-inflammatory factors, aberrant angiogenesis, reprogramming of energy metabolism, and epigenetic and tissue remodelling (Coussens and Werb, 2002). A large spectrum of different pressures (intrinsic and extrinsic) can induce-promote chronic inflammation and, in turn, tumourigenesis; these include exposure to certain pathogens, carcinogens, chemicals, radiation, dietary habits, immune dysfunction, and obesity, leading to epigenetic & genetic alterations, tissue heterogeneity, oncogene activation and/or loss of tumour suppressors (Swann and Smyth, 2007; Elinav *et al.*, 2013).

The tumour microenvironment is complex and consists of cells from both the innate and adaptive arms of the immune system, which interact with the tumour cells either directly or through the production of soluble factors. Moreover, over the past decades, it has become very clear that tumour associated non-immune cells also play a crucial role not only in orchestrating the behaviour of cancer cells but also in the recruitment of other cells and (un)tuning responses of local immune cells, thereby shaping tumour regression/progression and its response to therapy. The exact composition of the TME will be different from tissue to tissue within different tumour stages of the same tissue as well as between different patients. The immune cell compartment of TME is dynamic and can include any immune cell type imaginable in various proportions. In addition to T lymphocytes, natural killer (NK) cells and B cells, the myeloid cells in TME include monocytes, tumour-associated macrophages (TAMs), dendritic cells, and neutrophils.

Furthermore, studies have also reported the presence of eosinophils, basophils, mast cells, and platelets. All these tumour-associated immune cells (TAICs) can function either as pro-tumour or anti-tumour actors, while their mode of action can be managed by cancer and surrounding cells, and it can be interchangeable depending on the signals received from other cells. It is known that epithelium-

cancer initiating events cross-talk to inflammatory cells during cancer initiation and progression, and both the extrinsic and intrinsic mechanisms in the TME can stimulate an inflammatory response to pro-tumourigenic events (Comen, Bowman and Kleppe, 2018). The influence of specific immune cell compartments in controlling the neoplastic environment is an important and very interesting aspect of cancer research, and while the non-immune cells have a huge impact, this is out of the scope of this thesis and will not be discussed further here.

Accumulating evidence has illustrated that the chemokine milieu directly or indirectly influences the recruitment of immune cells into TME and/or their activation (Nagarsheth, Wicha and Zou, 2017) produced due to oncogenic (Spranger, Bao and Gajewski, 2015) and epigenetic pathways (Timp and Feinberg, 2013; Peng *et al.*, 2015; Nagarsheth *et al.*, 2016). For example, epigenetic silencing and β-catenin signalling are key tumourigenic mechanisms that correlate with cancer TME and a stem cell-like biological phenotype. The oncogenic genetic and epigenetic pathways simultaneously determine the biological and immunological phenotypes in the TME, affect tumour progression, and influence spontaneous and therapy-induced tumour-specific immunity. Thus, based on having a high or low density of a particular type of immune cells and their location, tumours may be immunologically classified into 'hot' (inflamed) or 'cold' (non-inflamed) phenotypes, respectively **(see Figure 1.12**). The manipulation of these tumour- intrinsic pathways may promote the infiltration of T cells into tumours, alter tumour immune phenotype and ultimately lead to tumour regression.



Biological characteristics	• Epigenetic silencing • Active β-catenin signalling • Mesenchymal-like cells • Stem cell-like cells • Less differentiated cells	• Epigenetic reprogramming • Suppressed β-catenin signalling • Epithelial cells • Highley differentiated cells • High PDL1 expression
Immunological characteristics	• Enriched Immunosuppressive cytokines • High numbers of Treg cells and MDSC • Few type 1 effector immune cells • Few functional APCs	• Enriched in Th1 type chemokines • High numbers of functional APCs • High numbers of effector immune cells

Figure 1.12. Characteristic of the 'hot' and 'cold' tumour microenvironment.
APC, antigen presenting cell; MDSCs, myeloid-derived suppressor cell; NK, natural killer cell; PDL1, programme cell death protein ligand 1; Th1, helper type 1 T cell; Treg, regulatory T cell.

1.6.3 Vγ9/Vδ2 T cells and cancer

In the most comprehensive study on tumour biopsy samples analysed at the transcriptomic level (>18,000 samples from 39 cancer types), Gentles and colleagues ranked γδ T cells as the number 1 (out

of 22) immune cell population associated with favourable prognosis (Gentles *et al.*, 2015). However, the bioinformatics analysis of these data has been challenged since the distinction of γδ T cell signature from CD4, CD8, or NK cell was not possible. The study by Tosolini improved the computational identification of circulating Vγ9/Vδ2 T cells and found considerable interindividual variation in abundance of these cells within each type and across the spectrum of cancers (~10,000 cancer biopsies from 50 types of haematological and solid malignancies). In contrast, the abundance of αβ TILs and Vγ9/Vδ2 TILs did not correlate across all cancers (Tosolini *et al.*, 2017). Accumulating evidence over the past decade has also proved that γδ T cells play a role in tumour progression. This section will describe the function of Vγ9/Vδ2 T cells when they act as anti-tumour or pro-tumorigenic effectors.

1.6.3.1 Antitumour effects of Vγ9/Vδ2 T cells

One of the common features of tumour cells is a switch in their metabolic pathway usage in response to a changing microenvironment. The extreme changes in energy supply requirements demand tumour cells change their usage of metabolic pathways such as glycolysis and mevalonate pathway. Vγ9/Vδ2 T cells can directly recognise a broad range of tumour cell types (Silva-Santos, Serre and Norell, 2015). As discussed before, high levels of IPP in tumour cells are critical determinants of Vγ9Vδ T cell activation through the association of IPP with BTN3A1 and BTN2A, which causes conformational changes in BTN proteins that are recognised by the Vγ9/Vδ2 TCR (Gu *et al.*, 2018; Rhodes *et al.*, 2018; Karunakaran *et al.*, 2020; Rigau *et al.*, 2020). On the other hand, Vγ9/Vδ2 T cells can also directly recognise stress-inducible ligands MICA and B and ULBPs via NKG2D (Raulet and Guerra, 2009),), but whether targeting of cancer cells is initiated by either TCR or NKG2D has raised many hot debates. Both, recognition of target cells via TCR or NKG2D, initiates killing that can involve the perforin/granzyme/granulysin pathway (Dieli *et al.*, 2001; Lawand, Déchanet-Merville and Dieu-Nosjean, 2017), TNF-related apoptosis-inducing ligand (TRAIL) (Vermijlen *et al.*, 2007; Dokouhaki *et al.*, 2013) pathways, and/or TNF family death receptor 6 (Fas)/Fas ligand (FasL) interaction (Li *et al.*, 2011; Xiang *et al.*, 2014). Furthermore, Vγ9/Vδ2 T cells also employ antibody-dependent cellular cytotoxicity (ADCC) to recognise targets via Fc receptors. However, the choice of the killing mechanism is mostly dictated by the nature of the target cell itself (Todaro *et al.*, 2013).

1.6.3.1.2 Indirect targeting of cancer cells

In addition, Vγ9/Vδ2 T cells can target cancer cells indirectly via the secretion of Th1 type cytokines that stimulate other immune cells. For example, Vγ9/Vδ2 T cells interact with DCs to induce their optimal maturation (Eberl *et al.*, 2009), and they can repolarise M2 macrophages to M1 phenotype, both of which would be favourable for anti-tumour responses (Devilder *et al.*, 2006). Moreover, Vγ9/Vδ2 T cells can also boost the cytotoxic potential of other immune cells in a contact-dependent manner. For example, activated Vγ9/Vδ2 T cells enhance NK cell cytotoxicity via co-stimulation of 4-1BB (Maniar *et al.*, 2010). However, conflicting data come from *in vitro* co-cultures of human Vγ9/Vδ2 T cells activated with ZOL, IL-2-primed NK cells and moDCs. Here, by killing moDCs that supply NK cell-activating cytokines, Vγ9/Vδ2 T cells show a negative control of IFNγ production by NK cells

(Nussbaumer *et al.*, 2011). These data suggest that the effects of γδ T cells on anti-tumour immunity are context-dependent and may be modulated by zoledronate.

1.6.3.2 Pro-tumourigenic effects of γδ T cells

In addition to their anti-cancer properties, γδ T cell subsets also have the power to contribute to tumour progression. Much of what we know about the pro-tumorigenic roles of γδ T cells originates from the observation that they can produce IL-17.

γδ T cell-derived IL-17 supports cancer progression via several mechanisms acting directly on cancer cells via IL17 receptor and indirectly through endothelial cells and other immune cell populations. For example, they act via endothelial cells to stimulate tumour growth via angiogenesis (Wakita *et al.*, 2010; Van Hede *et al.*, 2017) or to enhance the expression of adhesion molecules and endothelial cell permeability that supports metastases at secondary sites (Kulig *et al.*, 2015). In addition, secretion of GM-SCF by γδ T17 cells induces a positive feedback loop with neutrophils, expanding and polarising neutrophils towards an immunosuppressive phenotype (Ma *et al.*, 2014; Benevides *et al.*, 2015; Coffelt *et al.*, 2015), and neutrophils, in turn, stimulate IL17 production in γδ T cells (Ma *et al.*, 2014).

Other pro-tumorigenic effects of Vγ9/Vδ2 T cells have been suggested to drive cancer growth. In particular, the adoption of suppressive functions that impede DC maturation has been noted by several investigators (Peng *et al.*, 2007; Ma *et al.*, 2012; Ye *et al.*, 2013). A human pancreatic ductal adenocarcinoma study found that γδ T cells infiltrating express PDL1 (Daley *et al.*, 2016). It would be interesting to explore if γδ T cells express PDL1 exclusively in pancreatic TME or acquire such phenotype in other cancer types.

Together, these studies highlighted that Vγ9/Vδ2 T cell interaction with tumour cells generally involves cytolytic effector functions with a preference towards Th1 cytokine production; however, they also show great plasticity. Their function depends on a wide range of factors, from intrinsic properties like TCR avidity and phenotype to extrinsic factors like costimulatory or regulatory signals from neighbouring cells and cytokine milieu. A deeper understanding of all of these factors will be required in order to optimally design new therapies to harness Vγ9/Vδ2 T-cells for immunotherapy of cancer.

1.7 Cancer treatment options

Generally, the first three standard lines of cancer treatment include surgery, cytotoxic intervention and gamma radiation. Although these interventions aim to remove or destroy solid tumours directly and, in some cases, significantly restrict cancer progression, successful treatment is a major challenge.

Recent research advances and breakthroughs in cancer biology have substantially improved the standards of cancer care, not only in terms of overall response rates and progression-free survival but also the quality of life of cancer patients. In this context, immunotherapy has revolutionized the standard of care for cancer patients and their well-being in the long term. Although advances have also been made in standard lines of therapy, chemical reagents still target “public” cellular signalling

pathways. At the same time, radiation induces damage to healthy cells, and no reliable biomarkers exist so far that would predict the normal tissue sensitivity to a given treatment by individual patients. With the Cancer research UK £56m fund allocated for an acceleration of progress in radiation research in 2019, it is not surprising that immunology experts are attracted to conduct clinical studies combining radiotherapy and immunotherapy. However, since normal tissue toxicity induced by chemotherapy and radiotherapy may involve immunologic processes, combining immunotherapy with radio(chemo)therapy may increase the risk of normal tissue complications.

1.7.1 Cancer Immunotherapies

Immunotherapy aims to manipulate and exploit the inherent ability of the immune system to eliminate tumours and prevent metastasis. Research from the past couple of decades has led to a substantial success of immunotherapeutic drugs for cancer treatment, including molecules targeting various co-signalling receptors/ligands and therapies employing various cancer vaccines. Due to the breadth and pace of development of this field, a truly exhaustive review of all this literature is beyond the scope of this introduction. Here, I will introduce the main immunotherapy approaches relevant to my studies, including checkpoint inhibitors and T-cell and DC-based treatments. Some expansion of this background will be provided in the introduction to specific results chapters and general discussion.

1.7.1.1 Adoptive T Cell Transfer (ATCT)

Adoptive T cell transfer (ATCT) is a cell-based approach in which autologous T-cells are expanded, manipulated *ex vivo*, and then re-infused into the patient to exert an anti-tumour response.

1.7.1.1.1 Tumour Infiltrating Lymphocytes (αβT cells)

Tumour Infiltrating Lymphocytes (TILs) represent a heterogeneous population of T-cells within a tumour and can be associated with killing malignant cells. The rationale of TIL-based therapy is to extract cells with anti-tumour potential from an immunosuppressive tumour microenvironment and boost the natural anti-tumour immune response *in vitro* setting where these cells are also expanded before being re-infused back into the patient. The advantage of using TILs instead of T cells isolated from blood is that more TILs generally have TCR sequences appropriate for recognising tumour antigens. Depending on the TME, they can show reduced functional phenotype; however, the use of cytokines such as IL-15 and IL-21 during *ex vivo* expansion can enhance their cytolytic potential (Geukes Foppen *et al.*, 2015).

Re-infusion of TILs requires sufficient numbers to ensure trafficking to tumour sites and selective killing of tumour targets (and other stromal cell targets that support the tumour growth, such as vascular endothelial cells). In 1985 Rosenberg and colleagues published the first human study demonstrating how TILs could induce cancer regression when administered to patients with metastatic melanoma (Rosenberg *et al.*, 1985). Many efforts have been made and considerable advancements of these first promising results; in terms of modifying both the TIL generation and selection protocols and

the preparative regimens given prior to ATCT, which improved TILs (Tran *et al.*, 2008; Dudley *et al.*, 2010; Somerville *et al.*, 2012; Donia *et al.*, 2014). TIL manufacturing is a technically complex and resource-consuming process that requires cGMP compliant protocols. TIL-based approaches have shown great promise, but they are expensive. In addition, the success rate is variable and depends on tumour type, TME and the differentiation (exhaustion) state of TILs. For these reasons, many clinical trials employing TILs have joined therapeutic forces of ATCT together with checkpoint inhibitors (see below) such as ipilimumab in the hope of boosting efficacy (Rohaan *et al.*, 2018).

1.7.1.1.2 Genetically modified T cells

With the notion to boost anti-tumour T cell responses, approaches to adoptive cell transfer include modification of patient T cells prior to transfusion. Highly reactive anti-tumour T cells can also be generated *ex vivo* using genetic engineering approaches. Modified TAA-specific surface receptor genes and /or other costimulatory receptors are transduced or transfected into T cells and expanded prior to infusion. The reasoning for the infusion of genetically modified T-cells is that typically the endogenous repertoire of effective tumour-specific T cells has been compromised due to central tolerance and exhaustion. Evidence from studies performing comparative analyses has shown that tumour-specific TCRs have considerably lower antigen affinity than those directed against virus-derived antigens (Bridgeman *et al.*, 2011), which may also explain the poor clinical effectiveness of some approaches. Two principal antigen surface receptors are used in a genetic redirection of autologous T cells (reviewed by Park, Rosenberg and Morgan, 2011). The first uses the native a- and β -chains of a TAA- specific TCR; the second is a chimeric antigen receptor (CAR).

CAR T-cells are T cells transduced with CAR construct containing a tumour-specific antibody (Ab) variable region chain combined with intracellular domains of signalling molecules, such as CD3, αβTCRs, or CD28, among others. Thus, CAR-T cells detect surface antigens in an MHC-independent manner and do not need additional co-stimulation, which has been shown to increase their expansion and anti-tumour activities in some cases (Allegra *et al.*, 2016). So, when the Ab domain engages with its ligand, these CARs can induce specific intracellular signalling events. Ligation of these CAR molecules causes the same intracellular signalling cascades as ligation of conventional TCRs, leading to similar downstream effects, including proliferation, cytokine release and cytotoxicity. This provided a clear advantage of CAR application and presented an exceptional outcome in clinical trials of CD19 positive haematological malignancies, resulting in complete remission rates in 70–93% of patients. This has led to the Food and Drug Administration (FDA) approval of CAR-T therapy in 2017 (Chavez, Bachmeier and Kharfan-Dabaja, 2019). There are over 700 ongoing clinical trials with CAR T cells (US National Library of Medicine, clincalTrials.gov).

Despite this success, CAR-T cell therapy comes with some pitfalls. First, CARs based on single antibodies cannot distinguish tumour from healthy cells that express the target antigen, raising a high risk of off-tumour toxicity such as cytokine release syndrome (CRS) and neurotoxicity (Brudno and Kochenderfer, 2019). Second, unlike in TCR-dependent activation, CAR activation and functions are not subjected to regulatory mechanisms (Harris and Kranz, 2016). Leaky antigen-independent CAR activation can have deleterious effects on CAR-T cell phenotype and efficacy (Long *et al.*, 2015; Eyquem

et al., 2017; Quintarelli *et al.*, 2018). Third, a similar success rate is not achieved when treating solid tumours. Successful treatment of solid tumours depends on the ability of CAR-T cells to home to the tumour site, overcome the immunosuppressive tumour microenvironment, and persist long term.

1.7.1.2 Peptide vaccines

The field of tumour vaccination comprises a broad array of approaches that aim to generate and amplify cancer-specific immune responses. A critical step of all vaccines depends on the ability of APCs to induce T cell responses. Cancer vaccines include viruses, short peptides and full-length proteins or DNA pulsed dendritic cells or administered alone, or whole tumour cells and cell lysates (Blanchard, Srivastava and Duan, 2013).). In addition to low toxicities in preclinical studies, low production costs and ease of use makes peptide cancer vaccines attractive compared to other approaches. As a large collection of melanoma-associated antigens have been identified, several vaccines have been developed and applied to advanced melanoma patients. Even though such cancer vaccines can extend survival (by months, not years), broad reviews of clinical trials have shown poor cancer regression is achieved (less than 4% of patients) (Rosenberg, Yang and Restifo, 2004; Klebanoff *et al.*, 2010; Blanchard, Srivastava and Duan, 2013). These suboptimal clinical responses may correlate with the quality of the induced T-cell response. The insufficient immune response to eradicate cancer *in vivo* can also be caused by the poor immunogenicity of natural epitopes expressed by tumour cells.

In contrast to the immunodominant melanoma antigens Melan-A and gp100, which readily induce responses of their cognate T- cells *in vitro* (Rivoltini *et al.*, 1995) and *in vivo* (Salgaller *et al.*, 1996; Cormier *et al.*, 1997), T cells reactive to other TAA epitopes required repeated *in vitro* stimulation and showed limited immunogenicity when used as vaccines for melanoma patients (Marchand *et al.*, 1999; Weber *et al.*, 1999). Various strategies have been designed to boost peptide immunogenicity. One approach involves using modified peptides with increased affinity for cognate TCRs or MHC molecules (Berzofsky, Wood and Terabe, 2015; Tsang, Jochems and Schlom, 2015).

1.7.1.3 Dendritic cell vaccines

Targeted DC-based cancer vaccination can be put into two broad categories: (i) adoptive transfer of *ex vivo* generated and antigen-loaded DCs.; (ii) vaccines composed of various adjuvants promoting DC activation and mobilisation with/without antigens. The latter approaches utilise adjuvants containing various formulations of cytokines or PAMP/DAMP agonists, which can be injected directly or fused with antibodies targeting DCs *in vivo*, such as DEC205, CD40L, or DC-SIGN among others (Palucka and Banchereau, 2013) with/without antigens. Multiple trials have been conducted with such approaches but have not shown outstanding clinical success.

Most of clinical trials utilize moDCs generated *ex vivo* for practical reasons, as in contrast to PBMC-purified pDCs or cDCs, a larger number of mature antigen-loaded moDCs can be generated from blood-derived CD14+ monocytes or CD34+ HSPCs (Saxena *et al.*, 2018)(Mastelic-Gavillet *et al.*, 2019). These studies predominantly employed patients with melanoma, prostate cancer, glioblastoma or renal cell carcinoma due to the immunogenic nature of these cancers. Although clinical safety and induction of

anticancer NK cell, CD8 T cell and CD4+ T cell responses was demonstrated, the average overall response rate is only 8-15% (Melero *et al.*, 2014; Bol *et al.*, 2016; Garg *et al.*, 2017; Saxena and Bhardwaj, 2018). Even though DC vaccines were tested in more than 200 completed clinical trials to date, Sipuleucel- T is still the only method approved by the FDA for use in clinics for the treatment of metastatic prostate cancer (Kantoff *et al.*, 2010). In this approach, a cellular product composed of enriched blood APCs (with 90% CD14+ cells) is cultured with a fusion protein antigen composed of prostatic acid phosphatase (PAP) and GM-CSF (Burch *et al.*, 2000). Only a 4-month-prolonged median survival was achieved in prostate cancer patients treated with Sipuleucel-T (Kantoff *et al.*, 2010).

Ex vivo moDCs generated by stimulation of CD14+ cells with GM-CSF and interleukin 4 (IL-4) and various maturation reagents have been tested extensively in vaccination studies (Huber *et al.*, 2018; Saxena *et al.*, 2018; Mastelic-Gavillet *et al.*, 2019). Such moDCs demonstrated capacity to cross-prime T cells (Helft *et al.*, 2015; Briseño *et al.*, 2016; Boulet *et al.*, 2019) and produce anti- tumour cytokines, such as IL-12 (Carreno *et al.*, 2013). Furthermore, anti-tumour activity was noted in a subset of treated patients, which illustrated a potential for moDCs as a valuable vaccine (Carreno *et al.*, 2013) (Tanyi *et al.*, 2018). Nevertheless, the majority of patients exhibited only modest clinical responses..

The critical factor driving the success or failure of such a DC vaccine can be attributed to the type of DC maturation stimulus. DC maturation is key for immunogenic antigen presentation (Saxena and Bhardwaj, 2017). Various maturation cocktails have been used in the clinic to stimulate and mature moDCs (Palucka and Banchereau, 2013). Importantly, tolerance has been observed when vaccinating with improperly matured DC (Hawiger *et al.*, 2001; Bonifaz *et al.*, 2002). Improvement in the generation of mDCs with superior abilities to induce high avidity cytotoxic T cell responses has been achieved with strategies using electroporation of IL-12 and MART1 encoding DNA into mDCs (Bontkes *et al.*, 2007). More recently, induction of maturation has been achieved with mRNA transfection-based delivery of a cocktail of costimulatory molecules (CD40L, CD70, and constitutively active TLR-4) called TriMix. This process has shown an enhanced DC maturation and T cell activation potential. TriMix-DCs that were co-electroporated with mRNA encoding melanoma-associated antigens was reported to be feasible and safe, while it has resulted in antigen-specific immune responses in vaccinated subjects (NCT00074230, NCT01066390). New trials are also exploring combination therapies administering TriMix-DCs with other immune checkpoint inhibitor reagents such as an anti-CTLA-4 antibody (NCT01302496) (Wilgenhof *et al.*, 2016).

1.7.1.4. T cell checkpoint inhibitors

Immune checkpoints are part of the complex natural feedback mechanism of positive and negative signals that maintain and regulate immune homeostasis and self-tolerance. Upon activation, T cells start expressing inhibitory receptors aka 'checkpoints', such as programmed death 1 (PD-1), cytotoxic T-lymphocyte antigen 4 (CTLA-4), T-cell immunoglobulin 3 (TIM-3) and lymphocyte-activation gene 3 (LAG-3), which act as 'brakes' on T-cell activation (Pardoll, 2012). Ligands for these inhibitory receptors are often overexpressed on tumour cells of patients with advanced cancer, while their T cells also

express inhibitory receptors, which negatively regulate T-cell tissue effector functions. Thus, such a biological setting can contribute to tumour immune evasion, development of resistance to immune attacks, and prevent the induction of an optimal anti-tumour response. Therefore, this form of immunotherapy intervenes with this regulatory mechanism by blocking the membrane-bound T cell immune checkpoints or their ligands (Pardoll, 2012). Immune checkpoint blocking mAbs are considered attractive drug targets as instead of targeting tumours directly, they enhance endogenous anti-tumour activity. **Figure 1.13** schematically shows their mechanism of action. To date, seven mAbs have been approved by the US Food and Drug Administration (FDA), which target T cell checkpoints CTLA-4 (Ipilimumab), and PD-1 (Nivolumab, Pembrolizumab, Cemiplimab) and its ligand PDL-1 (Atezolizumab, Avelumab, Durvalumab). To date, over 100 clinical trials have aimed to define the advantage of these drugs in a wide range of cancers and various regimens and combinations..

Collectively, treatment with checkpoint inhibitors produces long-term responses, but in a small proportion of patients. The improved survival is often associated with autoimmune side effects (Ribas and Wolchok, 2018). In addition to their use in monotherapy, a few T cell checkpoint inhibitors are now being tested in combination with other treatments (such as ATCT-based therapies) in clinical trials, which showed promising response rates (NCT03287674).

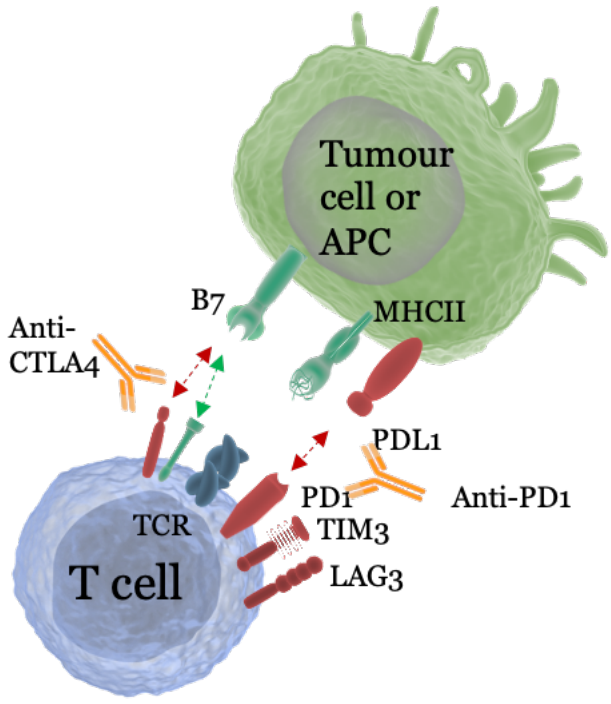


Figure 1.13. Immune checkpoint blockade.
This approach of immunotherapy employs antibodies directed against immune-suppressive receptor/ligands. For example, antibodies against CTLA4 prevent the binding of CTLA4 (on T cells) to B7 family members (on APCs). Additional intervention includes targeting interactions between PD1(on T cells) and PDL1 (on tumour or APCs). Other inhibitory receptors are TIM3 and LAG3. Adapted from Drake et al. 2013.

1.7.2 Vγ9/Vδ2 T cells in cancer immunotherapy

Numerous *in vitro* and *in vivo* studies have demonstrated that Vγ9/Vδ2 T cells efficiently recognise a wide spectrum of tumour cells and can induce strong anti-tumour effects. As noted in previous sections, these anti-tumour functions materialise via different mechanisms, such as the release of cytolytic and cytotoxic molecules and engagement of apoptosis-inducing receptors.

In addition to their favourable anti-cancer function, other reasons for the therapeutic employment of Vγ9/Vδ2 T-cells include their relatively high abundance in the peripheral blood (compared to other γδ T cell subtypes) and the ease of efficient expansion using amino bisphosphonates (NBP) or synthetic pAgs. Many early phase clinical trials have investigated the potential of Vγ9/Vδ2 T cells as cancer immunotherapy in a variety of haematological malignancies solid tumours, including head and neck cancer, hepatocellular carcinoma, renal carcinoma, mammary carcinoma, prostate cancer, neuroblastoma, and lung cancer, among others, reviewed by Fisher *et al.* (Fisher *et al.*, 2014).

Clinical trials demonstrated that γδ T cells have great potential in the immunotherapy of cancer since they can be easily expanded both *in vivo* and *in vitro*, they are safe, and able to induce anti-tumour immunity without issues related to off-targets or GVHD, as can be seen with CD8 T cell therapies. Nevertheless, generally, the results from completed early-stage clinical trials are rather disappointing (see below). **Figure 1.14** summarises the strategies using Vγ9/Vδ2 T cell application for cancer treatment.

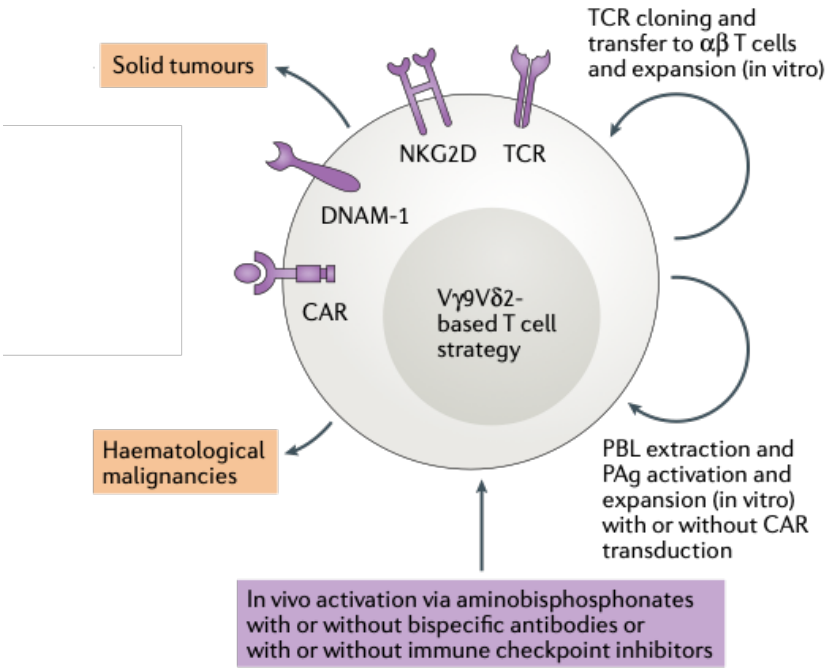


Figure 1.14. Summary of current strategies for therapeutic administration of human Vγ9/Vδ2 T cells in cancer treatment.
Vγ9/Vδ2 T cell based strategies involve *in vivo* activation, or peripheral blood extraction and *in vitro* activation with phosphoantigens (PAg). Another strategy relies on the generation of T cells engineered with

defined γδ TCRs, which consists of the cloning and transfer of Vγ9/Vδ2 TCRs into αβ T cells. CAR, chimeric antigen receptor; NKG2D, natural killer group 2D; PBL, peripheral blood lymphocyte. Adapted from Silva-Santos 2019.

1.7.2.1 Clinical studies involving Vγ9/Vδ2 T cells

Most of the clinical trials primarily employ ZOL and IL-2 for the Vγ9/Vδ2 T cell activation and expansion strategy, but they can be divided according to: and (i) ATCT strategies and (ii) *in vivo* stimulation (Hoeres *et al.*, 2018; Sebestyen *et al.*, 2020).

The latter approach involves 'in-patient' stimulation and expansion of Vγ9/Vδ2 T cells by systemic administration of pAg or NBPs. Administration of NBPs directly to patients serves a double purpose. Firstly, target cells are sensitised, rendering many primarily resistant tumour cells vulnerable to Vγ9/Vδ2 T cell-mediated attack (Gundermann *et al.*, 2014). Second, the degree of inhibition of farnesyl pyrophosphate synthase correlates well with anti-tumour functions of Vγ9/Vδ2 T-cells against a variety of tumour cell lines (Idrees *et al.*, 2013). As discussed in previous sections, NBPs stimulate Vγ9/Vδ2 T cells indirectly via accumulation of upstream metabolites like IPP in accessory cells or cancer cells due to inhibition of farnesyl pyrophosphate synthase. In addition, the increased production of toxic mevalonate pathway products and a decrease of downstream metabolites essential for normal cellular function induced by NBPs also result in direct anti-cancer effects on cancer cells (Jauhiainen *et al.*, 2009; Van Acker, Anguille, Willemen, Smits, *et al.*, 2016). This notwithstanding, multiple clinical trials utilising *in vivo* stimulation of Vγ9/Vδ2 T cells directly in patients were carried out, and although no severe toxicity was observed, no substantial antitumoural activity was reported either (Hoeres *et al.*, 2018; Sebestyen *et al.*, 2020) (Fisher *et al.*, 2014).

For ATCT, γδ T cells are extracted from blood, activated and expanded *ex vivo*. Various protocols for expanding human Vγ9/Vδ2 T cells exist, which vary regarding the culturing conditions, timing and dosage of NBPs or PAgs used, and added cytokines and co-stimulators. For example, high cell numbers of Vγ9/Vδ2 T cells can be obtained by simple selective stimulation and expansion (to up to 800-fold) using pAg or NBP plus the appropriate cytokines (Sakamoto *et al.*, 2011). In clinical studies, Vγ9/Vδ2 T cells are generally expanded *ex vivo* using ZOL, synthetic pAg BrHPP and 2M3B1-PP (Bennouna *et al.*, 2008) (Hoeres *et al.*, 2018). There are continuous improvements and further development of new approaches, which currently include the use of γδ TCR transduced T cells and CAR γδ T cells (reviewed in Hiasa *et al.*, 2009; Capsomidis *et al.*, 2018; Yazdanifar *et al.*, 2020).

Taken together, multiple clinical trials explored *ex vivo*-expanded polyclonal autologous or allogeneic Vγ9/Vδ2 T cells in ATCT alone or in combination with stimulating agents. These trials present γδ T cell immunotherapy as feasible, i.e. γδ T cells can be easily expanded both *in vivo* and *in vitro*, they are safe, and able to induce anti-tumour immunity without issues related to off-targets or GVHD as can be seen with CD8 T cell therapies. However, the activation or transfer of polyclonal γδ T cells outside the context of allogeneic stem cell transplantation to control tumours has been rather unsatisfactory. The results of large phase 3 clinical trials are, however, still pending. Disappointing outcomes of these strategies are

most likely a consequence of current underestimation of the impact of functional diversity and plasticity of different γδ T cell subtypes and/or their effector/memory states, along with the limited understanding of the γδ TCR repertoire diversity and the mechanisms of different receptor-ligand interactions. Accordingly, various justifications of therapeutic failure have been put forward, including functional instability, dysfunction or exhaustion of chronically activated Vγ9/Vδ2 T cells, and a highly variable capacity of the polyclonal Vγ9/Vδ2 TCR repertoire to recognise cancer cells.

1.7.3 Potential γδ T-APCs -based cancer vaccine

There is a need for the development of improved Vγ9/Vδ2 T cell strategies for cancer immunotherapy to overcome the impaired activation status and low persistence of Vγ9/Vδ2 T cells in patients with advanced cancer. Previously reported findings from our laboratory suggest that γδ T-APC represent a promising alternative to moDCs. By using γδ T-APC-based vaccines in a similar fashion as DC-based therapies, the bottlenecks associated with the difficulty of obtaining large numbers of homogenous functional moDCs could be overcome.

An *in vitro* method developed in our laboratory for the generation of expanded γδ T-APC may provide the basis for their large-scale manufacture under clinical-grade conditions. From the point of expansion yield and quality, optimal results are achieved when freshly isolated PBMC are stimulated with zoledronate and expanded in the presence of IL-2 and IL-15 (Khan *et al.*, 2014). It was demonstrated that large numbers of blood-derived Vγ9/Vδ2 T cells can be obtained in two weeks from healthy individuals and melanoma patients; yielding 10–50 million Vγ9/Vδ2 T cells per millilitre of whole blood. Furthermore, several investigations have shown that day 14 Vγ9/Vδ2 T cells retain their cytotoxic potential and functionality as assessed by cytokine secretion (IFNγ and TNFα) and proliferation in response to phosphoantigens without need for re-stimulation (Marlène Brandes *et al.*, 2009; Meuter, Eberl and Moser, 2010; Khan *et al.*, 2014). Importantly, immune inhibitory cytokines (TGFβ, IL-10) were not detected.

Furthermore, these expanded Vγ9/Vδ2 T cells also show potent tumour cell killing during *in vitro* culture. Importantly, and similar to short-term activated Vγ9/Vδ2 T cells, expanded Vγ9/Vδ2 T cells largely retain many cell surface receptors normally associated with APC, including MHC I and II, co-stimulatory molecules (CD80, CD86), and adhesion molecules (CD11a, CD54)(Khan *et al.*, 2014). In fact, day 14 Vγ9/Vδ2 T cells can process simple (influenza M1) and complex (PPD) antigens and to induce antigen-specific αβT cell responses in both cultured responder cell lines and primary responder cells present in unfractionated PBMC. Additionally, the beneficial effects could include tumour control through immediate recruitment of tumour-specific effector T cells or establishing tumour immunosurveillance involving the generation of long-lived, tumour-specific memory T cells (or a combination of both processes). Along these lines, a recent study demonstrated that a synergy of Vγ9/Vδ2 T cells and antigen-specific CD8 T cells successful eliminated breast cancer cells and cancer stem-like cells (Chen *et al.*, 2017).

Thus, the current data support the following protocol for the first-in man clinical study with γδ T-APCs **(Figure 1.15).** Briefly, following two weeks expansion as described above, tumour antigen-loaded γδ T-APC will be prepared by culturing expanded Vγ9/Vδ2 T cells in the presence of defined tumour antigen(s) or tumour cell extracts. Alternatively, a personalised immunotherapy protocol may involve the treatment of autologous γδ T-APCs with the patient's tumour cell preparations. Since γδ T-APCs retain functionality and responsiveness to stimulation after freezing, at this point, γδ T-APC may also be divided into individual samples and frozen in liquid nitrogen for shipment to corresponding cancer clinics. The frozen tumour antigen-loaded γδ T-APC samples can then be prepared locally for i.v. infusion into cancer patients according to treatment regimens that need to be defined during clinical trials. Ideally, a single round of expansion will provide enough tumour antigen-loaded γδ T-APC for an entire round of treatment, which may also involve a prime-boost protocol. Lastly, the effective cell doses need to be established before being able to apply a protocol for the generation of expanded γδ T-APCs in clinics (Khan, Eberl and Moser, 2014).

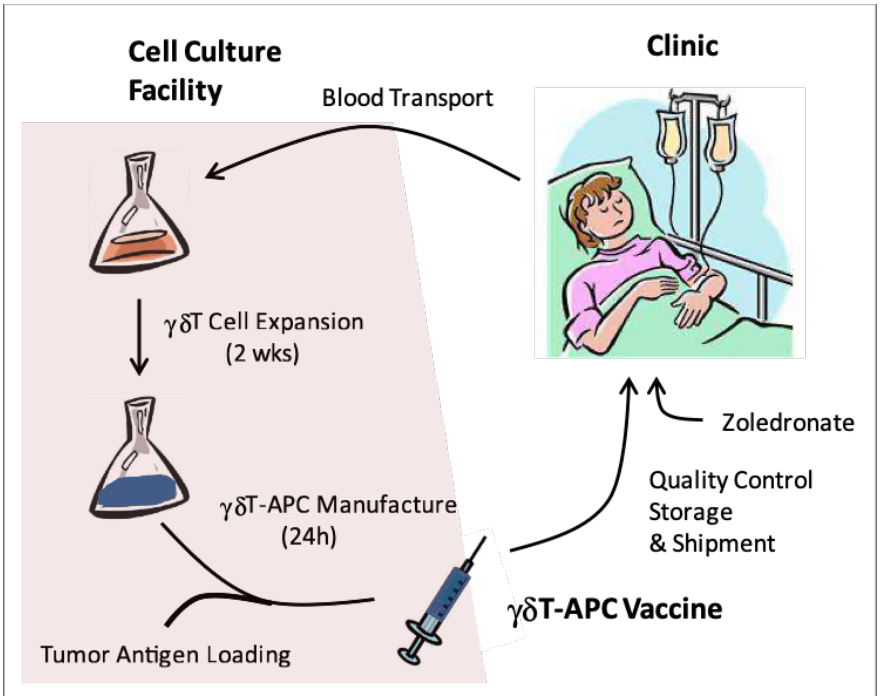


Figure 1.15. Production of tumour antigen-loaded γδ T-APCs for cancer immunotherapy. Cancer patient PBMC are prepared in a centralised γδ T-APC facility. Vγ9/Vδ2 T cells are expanded from PBMC in the presence of IL-2 and IL-15 following a single dose of zoledronate. Following two weeks of culture, γδ T-APCs are treated with soluble tumour antigens or whole-cell extracts for 24 h. As the γδ T cells pass quality control tests, they are frozen for transport to the clinic. γδ T-APC vaccine infusions may be followed by *in vivo* stimulation with zoledronate to ensure activated phenotype and responses. Adapted from Khan *et al.* 2014.

Finally, a few open questions remain. It is known that CD8 T cells specific for self-peptide usually possess low-affinity TCR and require strong signals for optimal activation. Firstly, will be day 14 Vγ9/Vδ2 T cell functionally appropriate and robust for optimal induction of anti-tumour responses in

tumour antigen-specific CD8 T cells? When injected into patients, will the generated γδ T-APCs retain their functional phenotype, and will they migrate to lymph nodes? Finally, it must be determined whether γδ T-APCs can overcome the potent immunosuppressive tumour microenvironment and whether repeated activation and (prolonged) expansion of Vγ9/Vδ2 T cells result in upregulation of inhibitory molecules such as immune checkpoint inhibitors, which would limit their APC as well as cytotoxic capacity

1.8 Hypothesis and aims

Hypothesis:

Based on the studies that showed activated Vγ9/Vδ2 T cells possess all phenotypical features of pro-APCs and have shown excellent capabilities of processing and presenting various antigens of microbial origin to CD8 and CD4 αβT cells, I propose that Vγ9/Vδ2 T cells are also able to process and present tumour antigens for induction of tumour-specific CD8 αβT cell responses. However, the expected difficulty lies in the fact that, unlike antigens of microbial origin, antigens from tumours are self-derived proteins and, thus, generally exhibit low affinity for αβTCRs.

Aims:

- To develop the molecular reagents for studying tumour antigen-cross presentation by γδ T-APCs, namely production and purification of recombinant tumour antigens from bacterial systems.
- To establish cellular tools to be used for studying antigen cross-presentation in γδ T-APCs. In particular, to establish functional tumour-antigen specific αβT cell lines and clones from healthy individuals and cancer patients.
- To determine conditions in which Vγ9/Vδ2 T cells acquire an optimal APC phenotype and relevant functional capabilities to induce responses in tumour antigen specific αβT cells.
- To optimise *in vitro* model system for studying presentation of soluble tumour antigens and antigens derived from cancer cells in coculture systems.

Chapter 2 Materials and Methods

2.1. Molecular Cloning and Construction of plasmid vectors for protein expression

Throughout this section for all bacterial plasmid extractions, LB broth supplemented with either 50 µg/mL carbenicillin (Sigma) alone or together with 34 µg/mL chloramphenicol (Sigma) was used. Carbenicillin is more stable than ampicillin and can be used as a selection with plasmid caring ampicillin resistance gene.

2.1.1 NY-ESO-1 expression plasmid

pGMT7-ESO1 expression vector was constructed by Mr. Andrew Thomas, Cardiff University. Briefly, plasmid pCDNA3 caring NY-ESO-1 coding sequence (CDS) (GenBank Accession No NM_001327.2) was obtained from Dr. Frederic Levy (University of Lausanne). The insert within the pCDNA3 vector was sequenced by the Sanger sequencing which confirmed the presence of NY ESO-1, and showed 100% sequence homology with the published CTAG1B mRNA sequence (data not shown). NY-ESO-1 CDS was amplified by PCR using primer NYESO-1-For, which added Bam HI restriction site at the N-terminus, and NYESO-1-Rev which added 6 histidine sequences (6 x His Tag) followed by Eco RI restriction site at the C-terminus. The generated NY-ESO-1 amplicon was cloned into pGMT7 expression plasmid by restriction cloning into Bam HI – Eco RI sites.

Prior to its use in protein expression studies, pGMT7-ESO1 construct was analysed by restriction digestion with Bam HI and Eco RI enzymes (Life Technologies), PCR using primers T7For and T7Rev (Table 2.1) and Sanger sequencing performed by Eurofins Genomics (Germany), and sequences were analysed using SnapGene software to confirm the CDS. A silent mutation TT**T**→TT**A** (Phe→Phe) was noted at position 510 of the coding sequence (CDS) (**Figure 2.1**).

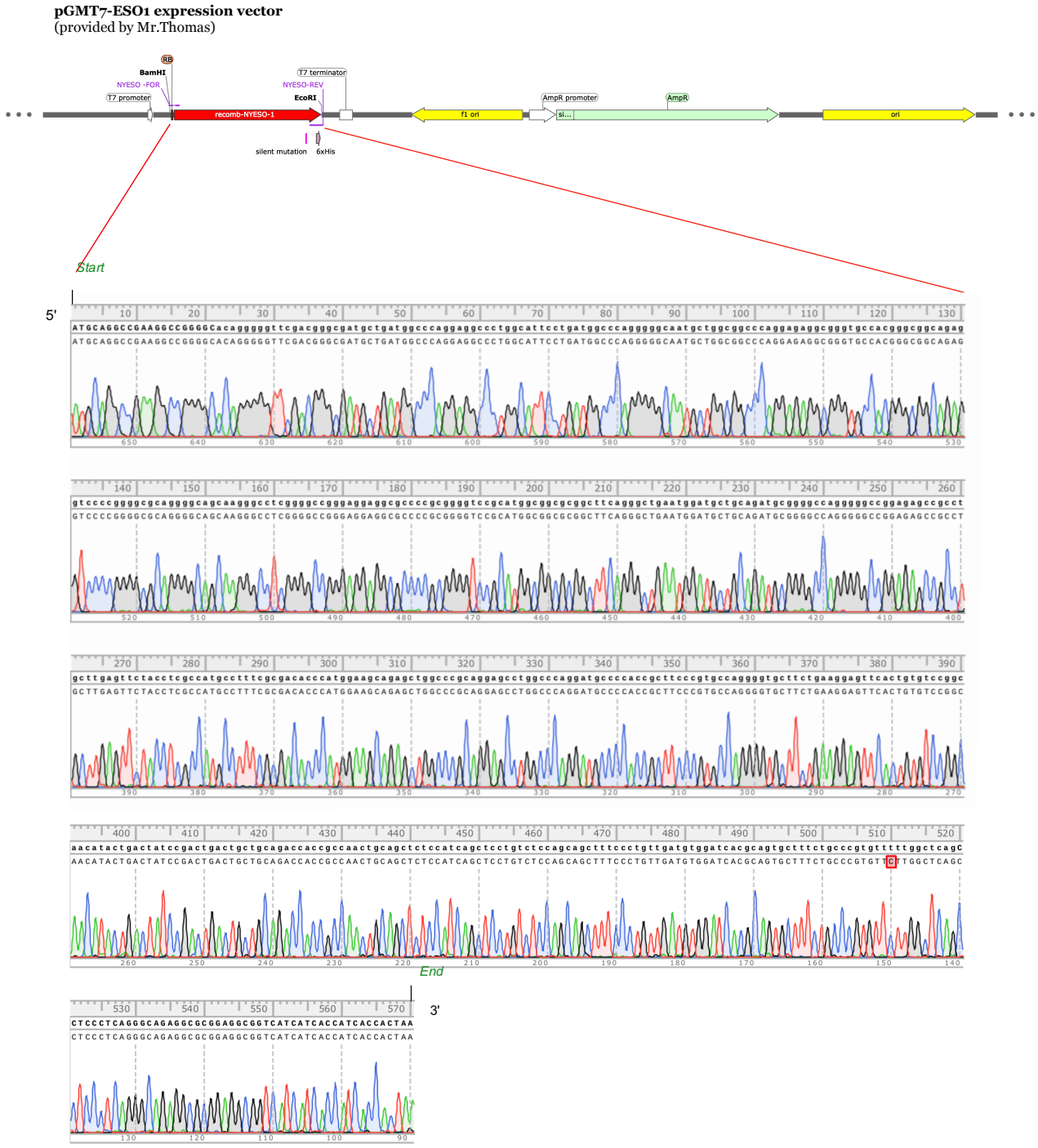


Figure 2.1. NY-ESO-1 CDS sequence within pGMT7-ESO1 vector.
Expression plasmid vector pGMT7-ESO-1 was constructed by cloning out the NY-ESO-1 CDS (GenBank Accession No NM_001327.2) from plasmid pCDNA3 (provided by Dr. Frederic Levy, University of Lausanne). NY-ESO-1 CDS was amplified by PCR using forward primer NYESO-1-For, adding Bam HI restriction site at the N-terminus, and reverse primer NYESO-1-Rev which added 6 x His Tag sequences followed by Eco RI restriction site at the C-terminus, and was cloned The into pGMT7 expression plasmid backbone by restriction cloning (constructed by Mr. Andrew Thomas, Cardiff University). Shown is DNA sequence analysis of the NY-ESO-1 amplicon flanking the whole CDS (using T7 forward and reverse primers). The silent mutation TTT→TTA (Phe→Phe) at position 510 highlighted in red.

2.1.2 Construction of 5T4 plasmids

2.1.2.1. RNA extraction and cDNA Synthesis

The 5T4 mRNA sequence was obtained from the GenBank database (Accession No NM_001166392). Fresh breast cancer cell line MCF7 was used as a source for TPBG gene. Total RNA was extracted from MCF7 cells using RNAeasy kit (Qiagen) according to manufacturer's instructions, and the quality and quantity of RNA was examined by NanoDrop ND1000 (Thermo Scientific). cDNA was subsequently synthesised from 1µg RNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems), according to manufacture's instructions; 10 µL of purified mRNA was incubated with 10 µL 2x transcription master mix under the following conditions : 10 min at 25 °C, followed by 120 min at 37 °C, and 5min at 85˚C.

2.1.2.2. PCR amplification of 5T4 gene from cDNA

Two PCR steps were used to amplify the 5T4 product from cDNA, using a single primer pair. The first step: 2.5 µL of cDNA sample with 10 µL 5 x high fidelity (HF) buffer, 0.5 µL 100 mM DMSO, 1 µL 20 mM dNTPs, 5 µL 10 X Universal Primer A (forward primer), 1 µL Primer 5T4-R1 (reverse primer) and 0.25 µL Q5® High-Fidelity DNA Polymerase (NEB) were mixed and made up to a final volume of 50 µL final volume with nuclease free water (Ambion). The mixture was incubated for the starting denaturation (98 °C, 5 min) followed by 30 cycles (1 cycle consists of 10 secs at 98 °C, 10 secs at 59 °C and 1 min at 72 °C) before a final extension (72 °C, 2 min).
The second step: 2.5 µL of sample from the step one PCR was mixed with 10 µL 5x high fidelity (HF) buffer, 0.5 µL 100 mM DMSO, 1 µL 20 mM dNTPs, 1 µL Primer 5T4-F1A (forward primer), 1 µL Primer 5T4 -R1 (reverse primer) and 0.25 µL Q5® High-Fidelity DNA Polymerase (NEB) and made up to a final volume of 50 µL final volume with nuclease free water (Ambion). Primer 5T4-F1A and primer 5T4 -R1 (Table 1) place Bam HI restriction site at the N-terminus, and a linker sequence (3 x glycine) with 6 consecutive histidine sequences followed by Eco RI restriction site at the C-terminus to generate 5T4-amplicon. PCR was initiated by denaturation (98˚C, 2min) and followed by 30 cycles of 10 secs at 98 °C, 10 secs at 59 °C and 1 min at 72 °C) before a final extension (72 °C, 2 min). Samples were analysed by electrophoresis on a 1% agarose gel (45 min, 90 V). Amplified samples of 1.3 kb were excised and purified using a Gel extraction kit (Nucleospin).

Table 2.1. Primers used for construction & sequencing of plasmid vectors.
Restriction sites are annotated in red and HIS-tag is annotated in green.

Primer name	*Sequence 5' —> 3'*	*comments*
5T4F1A	ATAGGATCCAGGAGGATGCCTGGGGGGTGCTC	Used to amplify genomic 5T4 cDNA and to insert Bam HI restriction site at the N-terminus
5T4-R1	TATGAATTCTTAGTGGTGATGGTGATGATGACCGCCTCCGACATCCGAGTTAGAACTGAGGTTC	Used to amplify genomic 5T4 cDNA to insert Eco RI restriction site and 6x His tag at the C-terminus of 5T4 CDS
5T4-F2	TTAACCGCAATCTGACCGAG	Sequencing of the CDS of 5T4
5T4-F3	TGAGCCTGACCTACGTGTCC	
M13For	CAGGAAACAGCTATGAC	To sequence amplicons within Bam HI – Eco RI sites of the pB-SSK(-) vector.
M13Rev	GTAAAACGACGGCCAGT	
T7For	TAATACGACTCACTATAGG	Used for sequencing amplicons within Bam HI – Eco RI sites of pGMT7 vector.
T7Rev	AACCCCTCAAGACCCG	
NYESO-1-For	ATAGGATCCAGGAGGATGCAGGCCGAAGGCCGGGGC	Used to amplify NY-ESO-1 CDS from pCDNA3 vector and to insert Bam HI restriction site at the N- terminus.
NYESO-1-Rev	ATGAATTCTTAGTGGTGATGGTGATGATGACCGCCTCCGCGC CTCTCGCCCTGAGGGAGG	Used to amplify NY-ESO-1 CDS from pCDNA3 vector and insert Eco RI restriction site and 6x His tag at the C-terminus.

2.1.2.3. Restriction cloning of recombinant 5T4 product into high copy plasmid

The resulting recombinant 5T4 PCR product (1.3 Kb) was inserted into high copy plasmid vector pBS-SK(-) by restriction cloning *via* Bam HI and Eco RI sites. First, the PCR product was cleaned using Clean & Concentrate kit (Zymo research). Both, vector backbone and the PCR insert were digested with Bam HI and Eco RI restriction enzymes (Life Technologies) according to manufacturer instructions to create sticky ends. Digestion reactions were prepared with buffer E in 50uL final volume and incubated overnight at 37˚C, and subsequently purified by gel extraction using the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel). Ligation of recombinant 5T4 product into a pBS-SK(-) vector was performed with T4 ligase (NEB) according to manufactures instructions, overnight at 16°C at vector: insert ratios of 1:1, 1:2, 1:3, 1:5 , to create pBS-SK-5T4 construct.

2.1.2.4 Analysis of pBS-SK-5T4 construct

Ligated products were transformed into 50 µL XL10-Gold Ultracompetent Cells (Agilent Technologies). 1 µL of ligation reaction was added to the bacterial aliquot and incubated for 10 min on ice. The cells were then heat-shocked at 42˚C for 2min and placed directly on ice for 5min recovery. After the recovery, 200 µl SOC medium (Invitrogen) were added and the mixture was cultured as described above. Bacteria colonies were selected by growth on carbenicillin plates and cultured overnight at 37 °C in 5 ml carbenicillin supplemented LB broth. 4 ml bacterial culture was used for extraction of plasmid

DNA using Plasmid Miniprep Kit (Qiagen) according to the manufacturer's instructions. The quality and concentration of purified plasmid DNA was examined by NanoDrop ND1000. To check if the recombinant inserts incorporated correctly into the vector, plasmid DNA was digested by FastDigest Eco RI and Bam HI (Life Technologies) in Buffer E for 30 minutes at 37°C and analysed by gel electrophoresis.

Alternatively, colonies containing the insert were selected and screened using ThermoPrime ReddyMix PCR Master Mix (Thermo Scientific) and primers flanking the insertion site (M13F, M13R). The final 1X reaction consisted of 0.625 µlThermoPrime Taq DNA Polymerase, 75 mM Tris-HCl (pH 8.8 at 25°C), 20 mM $(NH_4)2SO_4$, 1.5 mM $MgCl_2$, 0.01% (v/v) Tween ® 20, 0.2 mM each of dATP, dCTP, dGTP and dTTP, precipitant and red dye for electrophoresis. Using a pipette tip, isolated colonies were transferred to 50 µl of sterile water containing 10 mM NaOH, and boiled for 10 minutes. The reactions were spun at 16,000 x g for 5 minutes, and 5 µl of the supernatant was used in a 50 µl PCR. Pipette tips used for colony picking were dipped into Carbenicillin supplemented LB media for overnight culture.

Colony PCR reactions were prepared as follows:

Reagent	**Amount**
Primer For (10 µM)	1.25µl
Primer Rev(10 µM)	1.25µl
Colony DNA	5 µL
2X ReddyMix PCR Master Mix	12.5 µL
Water to Final Volume	25 µL

Colony PCR was initiated with denaturation at 95˚C for 2min, and run for 35 cycles as follows:

Denaturation:	**95C**	**25s**
Annealing	**54˚C**	**25s**
Extension	**72˚C**	**65s**
Final extension	72˚C	5min

5 µl of several individual products from the plate were run on an agarose gel.

Plasmid DNA from positive colonies were sent for sequencing to Eurofins Genomics (Germany,), using primers 5T4-F2 & 5T4-F3 and M13For & M13Rev (Table 2.1). The resulting sequences were analysed using SnapGene software. **Appendix 1** shows 5T4 CDS data and sequence alignment data of one representative colonies screened.

2.1.2.5 Sub-cloning of recombinant 5T4 gene into expression plasmids

250 µl bacterial culture of a successfully sequenced clone was expanded in 250 ml LB broth supplemented with carbenicillin at 37°C for overnight, shaking at 220 rpm. Plasmid DNA was extracted from bacterial cultures using the PureLink HiPure Plasmid Filter Maxiprep Kit (Invitrogen) according to the manufacturer's instructions, and examined by NanoDrop ND1000 for DNA concentration and quality, and stored at –20°C for later use.

The recombinant 5T4 amplicon was sub-cloned into low copy expression plasmids pGMT7 and pET32a by restriction cloning between Bam HI and Eco RI sites. The choice of pGMT7 vector was attributed to previous success for other recombinant protein expression, e.g. NY-ESO-1. The pET32a expression plasmid was a kind gift from Prof. Guilio Spagnoli (University of Basel), and it was designed for high-level expression of protein sequences fused with the 109aa Trx•Tag™ thioredoxin to circumvent inclusion body formation (Edward R LaVallie *et al.*, 1993). In both plasmids, the expression of target genes is driven by a strong bacteriophage T7 promoter. Generated expression plasmids were sequenced with three fold coverage, this time with T7For & T7Rev primers (Table 2.1) to cover each end of the 5T4 amplicon.

2.1.3 Bacteria strains and Transformation of competent cells

RosettaTM2(DE3): *E. coli* competent bacteria (generated in house) were used as a host strain for recombinant protein expression. This strain carries a chromosomal copy of the T7 RNA polymerase gene under control of the lacUV5 promoter inducible using IPTG. IPTG structurally mimics lactose and also binds to the lac repressor reducing its affinity for DNA, and hence driving gene transcription of the target gene under T7 promoter. Unlike lactose, IPTG is not part of any metabolic pathways and so will not be broken down or used by the cell. This ensures that the concentration of IPTG added remains constant, making it a more useful inducer of the lac operon than lactose itself. These bacteria also carry a pRARE plasmid, which encodes tRNAs rarely produced in *E. coli* and improves production of proteins of non-bacterial origin. pRARE carries a Chloramphenicol resistance gene.

Transformation of competent Rosetta cells was performed by thawing 30µL of competent cells on ice for 5 minutes. 50-100 ng of plasmid DNA was added to the bacterial aliquot and incubated for 5min on ice. The cells were then heat-shocked at 42°C for 2min and placed directly on ice for 5min recovery. After the recovery, 200 µl SOC medium (Invitrogen) were added and the mixture was cultured as described before. The bacterial cell culture was subsequently grown on carbenicillin and chloramphenicol supplemented LB plates. Resulting colonies were screened as described above.

For all plasmids cell banks were established from a single positive clone and maintained in -80˚C for subsequent uses. The banks were fully characterised by sequencing and performing diagnostic restriction digests of the plasmids.

2.2. Recombinant protein production and purification (RPPP)

2.2.1. Culture medium, buffers and reagents used in RPPP

All buffers were filtered using a 0.22 µm filter and vacuum pump system prior to use or application on AKTA FPLC. Chemicals were obtained from Sigma or Fisher Scientific unless otherwise stated.

Table 2.2 Media and buffers for bacterial culture

Media & buffers	*Composition*
LB medium	10 g/L tryptone (Fisher Scientific), 5 g/L yeast extract (Fisher Scientific), 5 g/L NaCl (Fisher Scientific)
LB agar plate medium	15 g/L agar bacteriological (Oxoid), 10 g/L tryptone (Fisher Scientific), 5 g/L yeast extract (Fisher Scientific), 5 g/L NaCl (Fisher Scientific) and
TYP medium	16 g/L tryptone, 16 g/L yeast extract, 5 g/L potassium phosphate dibasic (Acros Organics)
Terrific broth (TB)	Media base solution: 12 g/L Tryptone, 24 g/L Yeast extract, 4ml/L Glycerol Phosphate buffer solution: 2.31 g/100 mL KH_2PO_4 monobasic, 12.54 g/100 mL K_2HPO_4 dibasic pH 7.5
Bacteria Lysis buffer	10 mM Tris (Fisher Scientific), 10 mM MgCl2, 150 mM NaCl, 10% glycerol; pH 8.1
Triton wash buffer	0.5% Triton X, 50 mM Tris, 100 mM NaCl, 2 mM EDTA (Fisher Scientific); pH 8
Resuspension buffer	50 mM Tris, 100 mM NaCl, 2 mM EDTA; pH 8
Guanidine buffer	6 M guanidine, 50 mM TRIS, 2 mM EDTA, 100 mM NaCl
Urea buffer	8 M Urea, 20 mM TRIS, 5 mM EDTA, 20mM DTT (Sigma); pH 8
IMAC Binding Buffer 1	20 mM Na_2HPO_4-$2H_2O$, 0.5 M NaCl, 30 mM Imidazole, and 2 mM 2-ME; pH 7.5
IMAC Elution Buffer 1	0.5 mM NaCl, 0.5 M imidazole, 20 mM Na_2HPO_4-$2H_2O$, and 10 mM Tris; pH 7.5
IMAC Binding Buffer 2	8 M Urea, 20 mM Na_2HPO_4-$2H_2O$, 0.5 M NaCl, 30 mM Imidazole, and 5 mM 2-ME; pH 7.5
MAC Elution Buffer 2	8 M Urea, 0.5 mM NaCl, 0.5 M imidazole, 20 mM Na_2HPO_4-$2H_2O$, 5 mM 2-ME, and 10 mM Tris; pH 7.5
Storage buffer	4 M Urea, 20 mM TRIS, 5 mM EDTA, 20 mM DTT, 20% Glycine
Reducing sample buffer	125 mM Tris, 4% SDS, 20% glycerol, 20 µg/mL bromophenol blue, 10% DTT; pH 6.8

2.2.2 Protein sequences

Recombinant NY-ESO-1

MQAEGRGTGGSTGDADGPGGPGIPDGPGGNAGGPGEAGATGGRGPRGAGAARASGPGGGAPRGPHG
GAASGLNGCCRCGARGPESRLLEFYLAMPFATPMEAELARRSLAQDAPPLPVPGVLLKEFTVSGNILTI
RLTAADHRQLQLSISSCLQQLSLLMWITQCFLPVFLAQPPSGQRRGGGHHHHHH

Recombinant 5T4

MVVAALLAGRALQGLRRLELASNHFLYLPRDVLAQLPSLRHLDLSNNSLVSLTYVSFRNLTHLESLHL
EDNALKVLHNGTLAELQGLPHIRVFLDNNPWVCDCHMADMVTWLKETEVVQGKDRLTCAYPEKMR
NRVLLELNSADLDCDPILPPSLQTSYVFLGIVLALIGAIFLLVLYLNRKGIKKWMHNIRDACRDHMEGY
HYRYEINADPRLTNLSSNSDVGGGHHHHHH

Recombinant TrxA-5T4

MSDKIIHLTDDSFDTDVLKADGAILVDFWAEWCGPCKMIAPILDEIADEYQGKLTVAKLNIDQNPGTAP
KYGIRGIPTLLLFKNGEVAATKVGALSKGQLKEFLDANLAGSGSGHMHHHHHHSSGLVPRGSGMKET
AAAKFERQHMDSPDLGTDDDDKAMADIGSRRMPGGCSRGPAAGDGRLRLARLALVLLGWVSSSSPTS
SASSFSSSAPFLASAVSAQPPLPDQCPALCECSEAARTVKCVNRNLTEVPTDLPAYVRNLFLTGNQLAV
LPAGAFARRPPLAELAALNLSGSRLDEVRAGAFEHLPSLRQLDLSHNPLADLSPFAFSGSNASVSAPSPL
VELILNHIVPPEDERQNRSFEGMVVAALLAGRALQGLRRLELASNHFLYLPRDVLAQLPSLRHLDLSNN
SLVSLTYVSFRNLTHLESLHLEDNALKVLHNGTLAELQGLPHIRVFLDNNPWVCDCHMADMVTWLKE
TEVVQGKDRLTCAYPEKMRNRVLLELNSADLDCDPILPPSLQTSYVFLGIVLALIGAIFLLVLYLNRKGI
KKWMHNIRDACRDHMEGYHYRYEINADPRLTNLSSNSDVGGGHHHHHH

2.2.3 Small scale protein expression tests

From a Single colony a starter culture was set up in LB media containing carbenicillin and incubated overnight at 220 rpm and 37 °C. 10 mL of starter culture was used for plasmid DNA analysis; DNA was extracted using Plasmid DNA mini prep kit (Qiagen) according to manufacturer's instructions. 250 µl of starter was used to inoculate 5 ml of TYP or TB and cells were incubated at 220 rpm and 37 °C. Growth was monitored by OD at 600 nm using spectrophotometer.

For cultures in TYP, expression was induced at an OD_{600} of between 0.6 -0.8 at indicated temperature and IPTG concentration. For cultures in TB, expression was induced with indicted IPTG concentration at an OD600 of between 2 and 3.

In order to compare different expression temperatures, the induced cultures were either incubated for 4-6 h or overnight with shaking at 220 rpm and varying temperatures. Cells were replenished with antibiotics before overnight expression. A 0.5 mL sample was taken before and after IPTG induction to record the OD600 and to verify the protein expression by SDS-PAGE and western blotting. Cells were harvested by centrifugation for 10 min at 20,000 x g and 4 °C. Pellets were lysed in 500 µl of bacteria lysis buffer and water bath sonication for 10min. Total lysates were collected before separation of

soluble and insoluble material by centrifugation at 20,000 x g for 10 min and 4 °C. The supernatants were saved and pellets were resuspended in equal volumes of urea buffer. Protein expression was analysed by SDS-PAGE and western blotting (Section 2.2.8.1 and 2.2.8.2).

2.2.4 Large scale protein expression

A glycerol cell bank was used to inoculate 40 ml of LB. Cultures were incubated overnight shaking at 220 rpm and 37 °C, gently centrifuged at 300 × g for 10 min at room temperature and resuspended in fresh medium. 10 ml of starter culture was seeded into 90 mL fresh TB medium with antibiotics in 500 mL shake flasks. The initial OD_{600} of production cultures was between 0.0 and 0.1, and the cultures were incubated at 37˚C, shaking at 220 rpm. Expression was then carried out as for the small scale protocol.

2.2.5 Harvesting and cell lysis

Cells were harvested by centrifugation at 3500 x g for 30min and frozen at -20 overnight. Thawed pellets was resuspended in 40 mL of lysis buffer by vortexing and pipette mixing. Protease inhibitor cocktail III (Merk Millipore) was added to samples containing 5T4 protein. The lysates was then sonicated on ice at 70% power for 20 min and 2 sec intervals using a Sonopulse HD 2070 MS73 probe (Bandelin). If large volumes of cells were obtained then sonication could be repeated, as well as the addition of extra freeze thaw steps in between sonication. Complete lysis of all cells was essential for purity at later steps. Lysed cells were incubated with 0.2 mg/mL DNAase (Sigma) for 1hr at 37˚C, shaking, before high speed centrifugation at 10 000 x g for 20 min.

2.2.6 Inclusion body preparation

The inclusion bodies were resuspended in triton wash buffer, homogenised and centrifuged for 20 min at 10000 x g in order to remove irrelevant material . The triton wash was repeated until clear white pellet was obtained after centrifugation. Clean pellets were then resuspended in 40 mL resuspension buffer (to remove triton) and centrifuged for 20 min at 10000 x g. Finally, the inclusion bodies were dissolved in urea buffer and stored at -20°C. The concentration of inclusion bodies was determined using 1-3 µL of each sample and urea buffer as a blank (Section 2.1.6). The suspension was finally centrifuged at 20,000 × g for 30 min to remove insoluble debris, and dialysed into IMAC binding buffer prior to purification.

2.2.7 Purification of recombinant protein by immobilised metal affinity chromatography (IMAC)

Solubilised protein was filtered using a 0.45 µm filter and purified by by immobilised nickle column chromatography using 5-ml HisTrap FF (GE Healthcare) and AKTApure FPLC system (GE healthcare). The His-Trap column was equilibrated with 3 column volumes (CV) of IMAC binding buffer, and protein was loaded onto the column at 1mL/min. The column was washed with 5 CV of IMAC binding buffer. The protein was eluted in 1mL fractions at 1 ml/min with 3 CV of 100% IMAC Elution Buffer. The fractions that contained protein as indicated by UV absorbance (A280) on the AKTApure, were analysed by SDS-PAGE. Fractions with minimal impurities were pooled, the pool concentration determined as described in section 2.2.8.3. The purified protein samples were sent for MALDI/ TOF mass spectrometry analysis.

2.2.8 Protein analysis

2.2.8.1 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Protein samples were prepared by 1:4 dilution in 5 x reducing sample buffer and boiled at 95˚C for 5min. The samples and molecular wight marker were loaded into separate lanes. Proteins were separated by SDS-PAGE using Bolt Mini Gel Tank (all ThermoFisher Scientific), in 1x NuPAGE MES SDS running buffer (Life Technologies) at a constant voltage 120 V for 45min. Gels were subsequently washed in distilled water and stained with Commassie Blue (40% methanol (BDH), 7% acetic acid (BDH), 0.025% Brilliant Blue G) for 30min, followed by at least two incubations with fresh de-staining solution for 30min.

2.2.8.2 Western blotting

For Western blot analyses, Pierce Fast Semi-Dry Transfer (ThermoScientific) system was used to transfer onto a nitrocellulose membrane and subsequently rinsed with distilled water. Following overnight incubation in 5% milk PBS-T solution containing a primary antibody at 4˚C, membrane was washed three times with PBS-T and incubated with the secondary antibody in 5% milk PBS-T solution for 1hr at RT. NY-ESO-1 was detected with 0.5 µg/mL murine anti-human NY-ESO-1 monoclonal antibody (clone E978, Millipore) and 0.16 µg/mL HRP-conjugated goat anti-mouse antibody (Jackson Laboratory) as secondary antibody. 5T4 was detected with mouse anti-HIStag-HPR antibody. Pierce ECL Western Blotting Substrate (Thermo Scientific) was used for detection. All assays were performed according to manufacturer's instructions.

2.2.8.3 Estimating protein concentration by spectrophotometry

To determine the concentration of inclusion bodies and purified protein, samples were diluted 1/10 in urea buffer. Using a a NanoDrop ND100 (ThermoScientific) the machine was blank referenced using the buffers that the protein had been made in, i.e. resuspension buffer, urea buffer, PBS etc. For accurate results, the protein's unique extinction coefficient (ε) were entered. The theoretical extinction coefficients for recombinant proteins 5T4 (0.759), 5T4-TRX (0.753) and NY-ESO1 (0.368) were determined using Expassy ProtParam tool (https://web.expasy.org/protparam/). The absorbance measurements were taken at 280nm, and the machine then calculated the protein concentration [C] using the Beer-Lambert equation A280 = c * ε * b (where ε is the wavelength-dependent protein extinction coefficient, b is the path length). The given concentrations were adjusted for the dilution factor.

For protein mixtures (e.g. Total Cell lysate), a rough estimate of protein concentration was determined, assuming $\varepsilon_{percent}$ = 10. Most protein extinction coefficients ($\varepsilon_{percent}$) fall in the range 4.0-24.0.3 Therefore, although any given protein can vary significantly from $\varepsilon_{percent}$ = 10, the average for a mixture of many different proteins will likely be close to 10.

2.3 Human Cell culture, buffers and reagents

2.3.1 Cell culture Media and buffers

All reagents and buffers are listed below, with the exception of those supplied as part of commercial kits.

Media and reagents	Composition
DMEM	DMEM medium (Invitrogen), 10% Heat-Inactivated Fetal Bovine Serum (HI-FBS) (Life Technologies), 100 U/mL Penicillin (Life Technologies), 100 µg/mL Streptomycin (Life Technologies), 2 mM L-Glutamine (Life Technologies), 1% sodium pyruvate,100 µM NEAA
R0	RPMI-1640 (Life Technologies) 100 U/mL Penicillin (Life Technologies), 100 µg/mL Streptomycin (Life Technologies) 2 mM L-Glutamine (Life Technologies),
R5	R0 with 5% HI-FBS (Life Technologies), 1 mM Sodium Piruvate, 1X MEM Non-essential amino acids (NEAA) solution
R10	R0 with 10% FBS, 1 mM Sodium Piruvate, 1X MEM Non-essential amino acids (NEAA) solution
hR10	R0 with 10% human serum (HS), 1 mM Sodium Piruvate, 1X MEM Non-essential amino acids (NEAA) solution
T cell priming medium	hR10 10 mM HEPES 1 mM Sodium Piruvate 1x MEM Non-essential amino acids (NEAA) solution 20-50 IU/mL IL-2, 20 ng/mL IL-15 (PeproTech)
T cell expansion medium	R10 10 mM HEPES (Life Technologies) 1 mM Sodium Piruvate (Life Technologies) 1x MEM Non-essential amino acids (NEAA) solution (Life Technologies) 200 IU/mL IL-2, 25 ng/mL IL-15 (PeproTech)
REP medium	R10 2 mM HEPES 1 mM Sodium Piruvate (Life Technologies) 1X MEM Non-essential amino acids (NEAA) solution (Life Technologies) 800 IU/mL IL-2, 50 ng/mL IL-15 (PeproTech)
FACS buffer	1x PBS, 2% FCS
MACS buffer	1x PBS, 2% FCS, 2 mM EDTA

All media and buffers used for tissue culture were filtered using 0.2 µm syringe (Stericup®, Merck Millipore). T25, T75 and T175 flasks (Greiner Bio-One) and 24-, 48-, 96- multi well plates (Greiner Bio-One) were used for tissue culture. Cell lines were regularly screened for Mycoplasma infection (MycoAlertTM kit, Lonza) following manufacturer's instructions. Cells line were washed in PBS and adherent cell cultures were detached with 0.05% Trypsin-EDTA (Gibco). R5 medium was used to rest T cells overnight and to perform most of the *in vitro* assays (ELISPOT).

2.3.2 Cell culture

Cancer cell lines were grown at 37 °C with 5% CO_2 in DMEM R10 medium. Every 2 to 3 days, cells were split and a portion thereof seeded into fresh media. Care was taken to ensure the cells did not exceed 80-90% confluence and/or turned the media yellow, indicating a decrease in pH level due to the accumulation of waste products. For adherent cell lines, the whole content of the tissue culture flask was transferred into a 50 mL centrifuge tube and the flask washed with PBS to remove all remains of the media. 0.05% Trypsin-EDTA (Gibco) was added to detach the cells from the inner surface of the flask. The flask was then incubated at 37 °C for 2 min and then rinsed with an equal volume of R10 media. The bulk cell suspension was transferred to the 50 mL centrifuge tube, centrifuged at 400 x g for 5 min and resuspended in R10 medium for counting. Cells were plated into a new flask at the recommended seeding density. For suspension cultures, cells were resuspended thoroughly and counted if required. Cells were then split into fresh R10 medium in a new flask.

2.3.2.1 Cell count

Cells were thoroughly resuspended, 20 µL of cell suspension was mixed 1:1 with trypan blue 0.4% solution (Sigma-Aldrich) in a single well of a 96-well round bottomed plate. 10 µL were loaded onto a counting chamber and live cells were counted based on trypan blue exclusion according to the following formula: (number of cells in one section) x (trypan blue dilution factor) x 10^4 = number of cells/mL. Or alternatively cell were counted in automated cell counter (Countess, Thermofisher scientific)

2.3.2.2 Cytoperservation and thawing of cells

Cells were centrifuged at 400 x g for 5 min to remove culture media, and resuspended in freezing buffer. Viable cell numbers were enumerated by trypan blue exclusion and 1 mL aliquots (typically from 1 to 1 x 10^7 cells) were frozen in internal thread cryovials (Nunc) at -80 °C using a controlled-rate freezing device (Mr. Frosty® freezing pot) following manufacturer's instructions. Once frozen, cells were stored short- or long-term in liquid nitrogen. Vials of cryopreserved cells were removed from liquid nitrogen storage and thawed at 37 °C in a water bath. Immediately upon thawing, cells were transferred into a 15 mL centrifuge tube containing 10 mL pre- warmed R10 medium. Cells were centrifuged at 400 x g for 5 min and the supernatant discarded. The cell pellet was resuspended in 1 mL R10 media, counted and transferred to flasks or plates.

2.3.3 Immune cell isolation

2.3.3.1 Isolation of peripheral blood mononuclear cells (PBMCs)

Healthy PBMCs were isolated from venous blood collected locally from healthy volunteers. Venous blood was heparinised with anti-coagulant buffer, consisting of 20 U/ml heparin, to prevent coagulation. Blood was separated using Lymphoprep density gradient separation media (Axis-Shield) by layering blood on top of Lymphoprep, then centrifuging at 2000 rpm, at 21 °C for 25 minutes, without brake. Mononuclear cells within the buffy coat layer were collected and washed three times (400x g, 300 x g, 200 x g) with sterile PBS to remove platelets. PBMC were finally resuspended in R10 medium, counted and further processed or cultured in the incubator at 37˚C, 5% CO_2.

Alternatively, fresh blood samples were obtained from blood bags *via* the Welsh Blood Service (WBS, Cardiff) in accordance with the appropriate ethical approval. Samples were confirmed seronegative for HIV-1, HBV and HCV. Blood bags were diluted at 1:2 ratio with sterile PBS, and separated similarly using Lymphoprep.

2.3.3.2 HLA phenotyping

HLA phenotype of each donor was determined by flow cytomerty of freshly isolated PBMC stained with monoclonal antibody directed against HLA-A2 molecules (Table 2.3).

2.3.3.3 Purification of CD14 Monocytes from PBMC

CD14 positive monocytes were purified from PBMC by incubating cells with anti-CD14 Microbeads (Miltenyi Biotec) for 20 minutes at 4°C (positive selection). Labelled cells were separated over two LS column, and purity was assessed prior to use in assays (>95 % purity) .

.

2.3.3.4 Purification of CD8 T cells from PBMC

CD8 T cells were purified from bulk PBMC *via* negative selection, using the EasySep CD8 T cell enrichment Kit (Stem Cell Technologies), as per the manufacturer's instructions. Non-CD8 T cells labelled with antibody-magnetic particles were separated using an EasySep™ magnet (Stem Cell Technologies) according to manufacturer's instructions, without the use of columns, leaving CD8 T cells untouched.

2.3.3.5 Isolation of viable tumour-antigen specific T cells

Purified viable CD8 T Cells (described in *section 2.3.3.4*) were washed with FACS buffer, and labelled for 30 min at 4°C with pre-titred volumes of peptide-MHC tetramers conjugated to PE as described in section *pMHC tetramer staining 2.3.7.2*. Following incubation with LIVE/DEAD® Fixable Aqua Dead

Cell Stain (Thermofisher), cells were further labelled with surface markers CD3-BV421 and CD8- APC. Cells were then sorted on BD FACSAria™ III cell sorter, and directly collected into microfuge tubes containing 2 mL FCS.

Alternatively, pMHC-PE labeled CD8 T cells were subsequently washed with MACS buffer and labelled with anti-PE microbeads (Miltenyi Biotec) for 20 minutes at 4 °C. Antigen specific pMHC-anti-PE labelled cells were then separated from the bulk of CD8 T cell using a midi-MACS system and two LS columns.

2.3.4. Generation and expansion of T cells

2.3.4.1 Expansion of of Vγ9/Vδ2T cells

Vγ9/Vδ2 T cells were expanded from PBMCs of healthy donors with zoledronate (Zometa; Novartis) for around 14 days. Zoledronate in R10 medium was added to total PBMC cultures at day 0. Cells were supplemented with fresh T cell expansion medium every 2-3 days starting on day 5 until the end of expansion. After 14 days, Vγ9/Vδ2 T cells with purities <90% were enriched further by negative selection using a customised γδ T cell purification kit (Stem Cell Technologies) removing αβ T cells, B cells, NK cells, dendritic cells, stem cells, granulocytes, monocytes without depletion of CD16 positive cells, to a final purity > 95%. Expanded cells were phenotyped, The enriched cells were cryopreserved in liquid nitrogen until use in functional assays.

2.3.4.2 Generation of γδ T-APCs

PBMC or freshly isolated CD14 monocytes of a HLA-A2 negative donor were irradiated at 50 Gy, and plated into 96-well round-bottom plates as assessory cells. Expanded Vγ9/Vδ2 T cells (fresh or frozen) were plated into wells containing irradiated monocytes cells at a 1:10 monocyte : γδ T cell ratio, or 1:5 PBMC : γδ T cell ratio. Cultures were treated with HMB-PP (a kind gift from H. Jomaa, Giessen, Germany) with IL-2 (Proleukin, Chiron) and IL-15 (Miltenyi Biotec) at indicated concentrations. Cells were cultured up to 5 days in cR10 medium.

2.3.4.3 Expansion of FluM1- specific CD8 T cells

HLA-A2-restricted CD8 T cell lines with specificity to FluM1p58-66 peptide were established from PBMCs of healthy donors. FluM1-specific CD8 T cells were expanded in T cell priming medium and 0.1 µM M1p58- 66 peptide. Cells attained positive for M1p58-66 MHC tetramer were sorted and further expanded with 1 µg/ml PHA, in T cell expansion medium in the presence of irradiated PBMCs (Khan *et al.*, 2014).

2.3.4.4 Generation of tumour antigen specific CD8 T cells

FACS sorted CD8/TET double positive cells (described in section 2.3.3.5) were first established as cell lines in U-bottom 96 well plates. Up to 2000 CD8 T cells were seeded onto 96 well plate in T-cell priming medium together with feeder cells consisting of 10^5 irradiated (35 Gy) allogenic PBMC from at least 3 donors and 1 x 10^4 irradiated (80 Gy) EBV-transformed LCL per well, and 1 µg/mL phytohemagglutinin (PHA). On day 4, half the volume of medium in plates (100 µl/well) was replaced with fresh T-cell priming medium, and thereafter every 2-3 days the media was replenished with fresh T cell expansion medium supplemented with human serum (HS). Proliferating clones could be visualised by microscopy after 10 days, and on day 14-17 three random wells from each library plate were counted to establish an average T-cell number per well. Enough cells were removed from each library well to provide approximately 5 × 10^4 cells for screening by pMHC tetramer staining and flow cytometry. CD8/TET double positive wells were pooled and cells sorted as described before. Cells were then cloned by limiting dilution in U-bottom 96-well plates at 0.5 – 3 cells per well, or established as an enriched cell line in 24 well plate, in the presence of irradiated feeder cells and 1 µg/ml PHA stimulation. T-cell clones and lines were maintained at 37 °C in T cell expansion medium supplemented with HS. After 14-17 days CD8 /TET positive clones were identified by flow cytomentry and re-expanded until sufficient number of cells were obtained for functional analyses. Selected clones were cytopreserved.

2.3.4.5 Expansion of CD8 T cell lines and clones

An antigen-independent protocol was used to expand large numbers of CD8 lines and clones. Up to 1 x 10^6 T cells were seeded into a T25 tissue culture flask with 15 ml of T-cell priming medium and 15 x 10^6 irradiated (35 Gy) allogenic PBMC from at least 3 donors and 1 µg/mL PHA. The flask was placed at 37˚C, 5% CO_2 tilted at approx. 45˚ to enhance cell-to-cell contact. On day 3-4 of the expansion, half the medium was replaced with the same volume of T cell expansion medium. Cells were resuspended and incubated for further 2 days in upright position. On day 6 medium was replaced with T-cell expansion medium. Every 2-3 days, or when media turned yellow, half the media was replaced with fresh medium. Cell were split accordingly to maintain density at 2 x 10^6/mL. T cells were re-expanded with every 14 days, until sufficient numbers were obtained and used for experiments, or were cryopreserved until further use.

2.3.5. Generation of DCs

CD14 monocytes were isolated from fresh PBMCs by positive selection as described in *section 2.3.3.1* and seeded at 0.5 - 1.5 mio/well on 24-well plate, in complete RPMI medium supplemented with 8% HS, and 100 ng/mL GM-CSF and 100 ng/mL IL-4 (both cytokines from Miltenyi), and incubated at 37°C and 5% CO2. Medium supplemented with IL-4 and GM-CSF was replaced on day 3. For maturation, iDCs were harvested on day 6 and re-plated in medium supplemented with 1 µg/mL LSP (TLR grade from ENZO). Cells were assessed for their phenotype by flow cytometry throughout the process of monocyte differentiation into mDCs. DCs were defined by flowcytometry, as CD14 negative,

DC-SIGN positive and CD83 positive population, using commercially available antibodies (Table 2.3). Further phenotype characterisation was performed based on antibodies against HLA-DR, CD86, CD40, CCR7, and HLA-ABC (Table 2.3).

2.3.6 Functional T-cell assays

2.3.6.1 IFN-γ Enzyme-Linked ImmunoSpot (ELISpot) assay

Mouse anti-human IFN-γ antibody 1-DIK (Mabtech) was diluted to 10 µg/mL with PBS, and 50 µL were added to each well of an ELISpot plate (PVDF-backed plate, Millipore).Coated plates were incubated for 4 h at 37˚C wrapped in foil, washed thoroughly 5 times with 200 µL PBS/well, and blocked with 100µL of R10 medium for 1 h at RT. Per ELISpot well 50 x 10^3 T2-HLAA2$^+$ cells or T2-HLADR1$^+$ cells were used as antigen presenting cells. This suspension cell line was originally established by PEG-mediated fusion of the B-lymphoblastoid cell line (B-LCL) with a variant of the T-LCL CEM (Salter *et al.*, Immunogenetics, 1985), and is known for expressing small amounts of HLADR1 or HLA.A2 and high expression of co-stimulatory molecules such as CD40, CD86, CD80, CD83 on the cell surface (**Appendix 7**) and therefore is a standard line used as an APC cell target in intracellular IFN-γ secretion and ELISpot assay. T2 cells (174 x CEM.T2) were kindly provided by Dr Garry Dolton, Cardiff University. Rested APCs and T cells (overnight at 37˚C in R5) were each added to the wells (50 x 10^3 cells) in 50 µL of R5 medium. APCs were pulsed with peptides for 2h at chosen peptide concentrations in assay media. Cells were then washed in R0 three times in order to remove unbound peptide from the media, thus limiting T-cell to T-cell presentation. The final reaction volume was 100 µL/well made up in R5 medium. A well with PHA stimulated cells was included as a positive control. T cells cultured with/without APCs in the absence of peptide or PHA stimulation was included as a negative control. All tests were run in duplicate. The plate was then wrapped in silver foil and incubated at 37˚C for 16-18 hours.

Plates were washed 3 times with 150 µL PBS and incubated with 100µL sterile water for 10 min at room temperature (therefore lysing the remaining bound cells). The plate was further washed twice with 150 µL/well PBS. Secondary biotinylated antibody 7-B6-1-Biotin (Mabtech) (1:1000 in PBS) was added at 50 µL/well and incubated in the dark at room temperature for 2 hours. The plate was washed 5 times with PBS, and 50 µL of Streptavadin- Alkaline phosphatase (1:1000 in PBS) was added to each well. The plates were incubated for 2 h in the dark at RT, followed by 5 washes with 150 µL PBS. Developing solution was made fresh using 25X AP colour development buffer (BioRad), AP-conjugate substrate A and B solutions (BioRad), and sterile water. The solution was added at 50 uL/well and left to develop in the dark between 10 to 20 min, until spots were clearly visible. Developing reaction was stopped by washing plates with tap water; plates were then air dried in the dark before spots were imaged and counted using an automated CTL Immunospot analyser. CTL Single Colour software was used for spot counting and QC. Settings were kept constant for each reading. Assays were normalised for cumulative analysis by division of individual well spots by total number of spots across all wells (minus

background). This accounted for inter-assay variation derived from plate sensitivity differences, low or high T cell numbers or experimental error resulting in an inter-assay difference.

2.3.6.2 Cell Proliferation Assays

For the measurement of cell proliferation, relevant cells (peptide specific CD8 T cells) were stained with 1 μM CFSE (Life Technologies) for 5 minutes at room temperature, in complete RPMI. Cells were subsequently washed and used in assays. APCs (γδ T-APCs or T2-A2 cells) were pulsed with protein of interest for 24 h or with peptide for 1hr, and subsequently irradiated at 50 Gy prior to assays. APCs were plated out in 96 well round-bottom plates and responder CD8 T cells were added in 1:1 or 1:2 ration to γδ T-APC cultures. Co-cultures were incubated for 5 days in the presence of IL-2 (100 IU/mL) and IL-15 (20 ng/mL). Cultures were then stained for surface markers with fluorochrome conjugated monoclonal antibodies and analysed by flow cytometry determining CFSE dilution among CD8 T cells.

2.3.6.3 Antigen cross-presentation assay (ACPA)

γδ T-APCs were generated as previously stated. At 48 h into γδ T-APC generation, cells were pulsed with protein of interest for the final 24 h, to allow for antigen uptake and processing. Following culture with the protein, APCs were washed three times with R0. Subsequently, peptide specific CD8 responder T cells were added to APC cultures at a 1:1 ratio APC:responder cells in the presence of 5 μg/ml BRE A for 4-18 h.

For ACP experiments using cancer cells as an tumour antigen source, PC3, HT1080 and MCF7 cell lines were treated with 10μM zoledronate (ZOL) or 30 Gγ irradiation, and were were set up in 96-well U-bottomed plates at 5 x 10^3 cells per well and incubated for 48-72 h. Vγ9/Vδ2 T cells were then added to wells at 1:1 γδ : cancer cell ratio. After 48 h 5T4-specific CD8 T cells were added at a 1:1:1 ratio. 5 μg/mL BRE A (Sigma) and Monensin (0.7 μL/mL; Sigma) were added to the wells 1 hour later and the cultures were incubated overnight.

Intracellular cytokine (IFN-γ) was analysed in CD8 T cells by flow cytometry. Technical replicates were conducted in duplicate.

2.3.7 Flow cytometry

Unless otherwise stated, at least 50,000 events were collected using a BD FACS Canto II for all experiments. All Samples were acquired on an 8-colour BD FACSCanto II flow cytometer (BD Biosciences) using the FACSDiva software (BD Biosciences). All analyses of data were performed using FlowJo (version 9.3.2; TreeStar Inc.), by gating on intact cells (FSC-A/SSC-A), single cells (FSC-A/FSC-H), live cells (Aqua- negative), and expression of markers of interest according to appropriate isotype controls. Percentages of cells were transferred to GraphPad Prism 6 software (GraphPad Software, Inc., CA, USA) for further analysis.

Anti- mouse or anti-rat Igκ antibody capture beads (BD Biosciences) were used to prepare individual compensation tubes for each Ab used in the experiment. For all flowcytometric analyses cells were

treated with intravenous immunoglobulin (IvIg) (Kiovig; Baxter) at 1:100 dilution in order to block Fc receptors. Fc receptors specific for IgG, including FcγR1 (CD64), FcγRII (CD32) and FcγRIII (CD16) are present on many cell types, with particularly high expression on myeloid, granulocyte and B cell lineages. All antibody cocktails diluted to working concentrations contained 10 μg/ml of human Fc Block. This step prevents binding of conjugated Antibodies to Fc receptors, thus prevents non-specific background signal.

A complete list of all antibodies and appropriate dilutions can be found in Table 2.3. In all cases, cells were washed with FACS buffer between each stage of staining.

Table 2.3 List of antibodies

Antigen	Clone	Conjugate	Dilution	Company
CD3	UCHT1	BV421	1/100	BioLegend
CD4	RPA-T4	APC-H7	1/100	BD
CD4	RPA-T4	BV421	1/50	BD
CD8	RPA-T8	APC	1/20	BD
CD8	RPA-T8	PE	1/20	BD
CD14	M5E2	BV421	1/100	BioLegend
CD14	MOP9	PerCP	1/20	BD
CD19	HIB19	PE-Cy5.5	1/30	Biolegend
CD19-V500	HIB19	V500	1/40	BD
CD25	BC96	APC	1/20	eBioScience
CD27	O323	PE-Cy5.5	1/20	Biolegend
CD40	mAB89	PE	1/20	Beckman Coulter
CD45RA	HI100	FITC	1/20	BD Pharmingen
CD70	113-16	FITC	1/20	BioLegend
CD80	2D10.4	FITC	1/10	eBioScience
CD83	HB15c	FITC	1/100	BD
CD86	IT2.2	APC	1/20	BioLegend

Antigen	Clone	Conjugate	Dilution	Company
CD209 (DC sign)	DCN46	FITC	1/25	BD Pharmingen
CD209 (DC sign)	DCN46	APC	1/30	BD Pharmingen
CCR7	3D12	PECy7	1/20	BD Pharmingen
IFNγ	B27	FITC	1/100	BD Pharmingen
HLA-ABC	w6/32	PE	1/10	eBioscience
HLA-A2	BB7.2	AF88	1/10	Serotec
HLA-DR	L243	APC-H7	1/40	BD Pharmingen
TCRVγ9	Immu360	PE-Cy5.5	1/400	Beckman Coulter

2.3.7.1 Staining of cell surface markers

Cells were counted by trypan blue exclusion and transferred to either a 96 well plate or 5 mL FACS tube, washed twice in PBS (500 x g for 3 min) and resuspended in residual PBS with 3 µL of diluted (1:10; in PBS) Fixable Aqua LIVE/DEAD® Cell Stain (Life Technologies), or with 3 µL of diluted (1:70 in PBS) Fixable Zombi LIVE/DEAD® Cell Stain (Life Techologies), and incubated for 10 min at room temperature in the dark. The Aqua dye binds to free amines both in the interior and on the surface of the cells with compromised membranes, yielding intense fluorescent staining. Contrary, in viable cells the Aqua dye reacts only with the cell-surface amines, resulting in around a 50-fold less intense fluorescence (Makedonas *et al.* 2009).

After incubation with Aqua live/dead staining, 50 µl of freshly prepared antibody cocktail against cell-surface markers was added to cells directly, without washing, and incubated on ice for 20 min in the dark.

Cells were washed in FACS buffer and finally resuspended in 100-200 µL of PBS. Cells were kept on ice in the dark (or fixed in 2% PFA) until flow cytometric analysis.

For intracellular staining of antigens, surface-stained cells were treated with FixPerm Buffer (CytoFix kit, BD Biosciences) for 20 minutes at room temperature. Cells were subsequently washed twice in Perm wash buffer (CytoFix kit, BD BISciences) and incubated with fluorochrome-conjugated monoclonal antibodies diluted in PermWash buffer, for 30 minutes at room temperature according to the manufacturer's instructions.

2.3.7.2 pMHC tetramer staining

Soluble biotinylated monomeric pMHC-I were produced by NIH. Peptide-MHC-I tetramers were assembled by adding PE or BV421 conjugates (Life Technologies) in five separate 20 min steps at a molar ratio of 1:4. The desired number of cells, typically 0.5–2 x 10^5 of a mixed lymphocyte preparations or 1–3 x 10^6 of Cd8 T-cells, was transferred to flow cytometry tubes and cells washed with FACS buffer (700 x g 3 min). Cells were treated with the PKI (protein kinase inhibitor) (Dasatinib, Axon Medchem, Reston) at a final concentration of 50 nM for 30 min at 37 °C and then stained with tetramer (0.48 mg of tetramers per 1 x10^6 cells in 50 µL FACS buffer) without washing. Treatment with PKI prevents TCR triggering and internalization of the TCR and any pMHC tetramer bound to it. PKI is unstable when stored at 4 °C, so 1 mM DMSO aliquots of PKI were stored at -80 °C and working aliquots of 100 nM were prepared in PBS for each experiment. Following tetramer addition, cells were placed on ice and in the dark for 30 min. All subsequent Ab staining of the cells was performed for 20 min on ice and in the dark. To enhance tetramer staining intensity the cells were washed in FACS buffer (700 x g 3min) and labelled with anti-fluorochrome unconjugated primary Ab for 20 min on ice in the dark. Primary (1°) unconjugated mAbs were used at a concentration of 10 mg/mL (0.5 mg/test). Cells were subsequently washed first in PBS and samples were subsequently stained with Aqua live/dead stain and mAbs against cell-surface markers as described in sections above. Samples were prepared for flow cytometry by washing in FACS buffer or resuspended in 2% PFA.

2.3.8 Statistical analysis

Statistical analysis was performed with the use of GraphPad Prism 6 software (GraphPad Software, Inc.). Column statistics were carried out in the first instance to assess distribution of data sets and identify whether datasets were parametric or non-parametric. For the comparison of two variables, either Student's *t* test (parametric data sets), Mann-Whitney U test (unpaired, non-parametric data sets), or Wilcoxon matched-pairs signed rank test (paired, non-parametric data sets) were utilised. For comparison of multiple variables, one-way or two-way ANOVA was used. Following analysis, the Dunnent's or Fisher's LSD multiple comparison test was used for comparison of each condition within experiments. Descriptive statistics are displayed as mean ± standard deviation of the mean (SD) in all figures presented. Significance was defined as p values of <0.05, and resulting statistical significances of difference are indicated in figures as *=$p<0.05$, **=$p<0.01$, ***=$p<0.001$, ****=$p<0.0001$, ns=non-significant.

2.3.9 Ethics, healthy donors and patients

Blood bags were supplied by the Welsh Blood Service. Healthy volunteers were recruited locally for donations of venous blood. This study was conducted according to the principles expressed in the Declaration of Helsinki and under local ethical guidelines (Bro Taf Health Authority, Wales). All healthy blood donors provided written informed consent for the collection of samples and subsequent analysis. PBMC from melanoma patients, were acquired from the Human Biomaterials Resource Centre (HBRC),

University of Birmingham. The HBRC is a biorepository licensed by the Human Tissue Authority, UK and samples were taken with prospective written generic consent for research use.

Chapter 3
Generation of recombinant tumour proteins from *E. coli*

3.1 Background

Recent cancer immunotherapy trials involving *in vitro* expanded human γδ T cells rely on one of their great characteristics - as effector cells, they are able to kill various types of cancer cells – while they are easily expanded to a large number *in vitro*. Nevertheless, these types of immune cell-based therapies include problems associated with T-homing to tumour tissues. Our laboratory discovered that activated human blood-derived Vγ9/Vδ2T cells behave like professional antigen-presenting cells (APCs), prompting us to translate this finding into the clinics. When loaded with antigens of microbial or viral origin, human blood Vγ9/Vδ2 T cells can differentiate into professional antigen-presenting cells (γδ T-APCs) and induce potent CD4 and CD8 T cell responses. Based on these characteristics, it can be postulated that γδ T-APCs would be advantageous as a novel anti-cancer vaccine. Thus, investigation of the nature and mechanisms of tumour antigen processing and presentation in γδ T-cells is a fundamental step towards developing better, more effective anti-cancer treatments, which is presented in this thesis. However, strategies for studying antigen presentation essentially depend on sufficient quantities of purified (soluble) tumour antigens for functional experiments.

In-house recombinant protein production offers cost advantages over commercial products since milligrams of the target protein can be isolated per small volumes of the main culture. Generally, recombinant proteins can be expressed in various eukaryotic and prokaryotic host systems that can be divided into five categories: bacterial, yeast, plant, insect, and mammalian cell systems. What they have in common is that the gene encoding the protein of interest has to be cloned into an expression vector and transduced or transfected into the host cell prior to protein expression. Each host offers different advantages, and usually, the best system is determined on protein function, yield, stability, overall cost, and scalability. Bacterial systems are the most widespread but often lack post-translational modification mechanisms (such as glycosylation) provided by eukaryotic hosts.

On the other hand, bacterial hosts are typically simple to cultivate, less expensive and time-consuming. Furthermore, bacterial expression vectors are relatively easy to assemble, and thanks to a wide range of commercially available bacteria strains, many proteins can be over-expressed. Additionally, bacterial systems are easy to scale-up, and the genetic expression mechanisms are well understood (Consortium *et al.*, 2008)(Sørensen *et al.*, 2005; Peti and Page, 2007; Sivashanmugam *et al.*, 2009; Young, Britton and Robinson, 2012; Spriestersbach *et al.*, 2015). Therefore, *E. coli* was the favoured host for rapid and high-level production of recombinant tumour antigens in inexpensive growth conditions. Importantly, the antigenicity of the tumour antigens that I have chosen (see below) does not seem to depend on post-translational modifications. NY-ESO-1 and 5T4 antigens were chosen as model antigens because 1) they are expressed by many solid and malignant cancers whereas their expression is limited to particular

germ and embryonic stem cells, with no or little expression in other adult healthy tissues; and 2) they have already been extensively studied.

About NY-ESO-1

NY-ESO-1 protein, also known as CT6.1 and LAGE-2, is a widely studied target for tumour peptide-based vaccines. It was originally identified by screening a tumour-derived cDNA expression library with an autologous serum of an oesophageal cancer patient (Chen *et al.*, 1997). The CTAG1B gene encoding NY-ESO-1 is located in the q28 region of the X chromosome and is a member of the multi-gene CT-X antigen family (Simpson *et al.*, 2005). It transcribes a 180 amino acid long cancer/testis antigen (CTA), a cytosolic membrane protein of highly hydrophobic nature (Schultz-Thater *et al.*, 2000; Zendman, Ruiter and Van Muijen, 2003; Caballero and Chen, 2009). NY-ESO-1 has a differential expression among several solid tumour malignancies, including melanoma, sarcoma, lung cancer, prostate, ovarian, breast, and bladder cancers (Jungbluth *et al.*, 2001; Caballero and Chen, 2009). On the other hand, its expression in normal tissues is limited to testis germ cells. Although its function is still poorly understood, expression in sperm cells suggests a role in spermatogonia development. It has been proposed that its aberrant expression in tumours may give cells spermatogenic features supporting tumour progression (Zendman, Ruiter and Van Muijen, 2003) (Simpson *et al.*, 2005). NY-ESO-1 is one of the most immunogenic CTAs, eliciting humoral and adaptive immune responses spontaneously and after vaccine administration (Szmania, Tricot and van Rhee, 2006; Caballero and Chen, 2009). Clinical responses were also detected in chemo-resistant cancers. NY-ESO-1 encodes many peptides capable of binding to common HLA-class I and II alleles (https://caped.icp.ucl.ac.be), with the most immunogenic peptide being the HLA-A2 peptide 157–165 or analogue 165V. Numerous studies showed that not only can they be recognised by tumour-infiltrating and circulating T-cells, but NY-ESO-1 epitopes can also stabilise or elicit tumour regression in metastatic cancer patients (Jäger *et al.*, 1998, 2000a; Valmori *et al.*, 2000; Gnjatic *et al.*, 2002; Szmania, Tricot and van Rhee, 2006; Chen *et al.*, 2014). Importantly, since testis germ cells are MHC class I negative (Guillaudeux *et al.*, 1996) and will not trigger a cytotoxic response, NY-ESO-1 makes an excellent tumour vaccine candidate. Numerous studies have investigated the use of NY-ESO-1 peptide-pulsed DCs as cellular vaccines. Currently, the safety and efficacy of NY-ESO-1-pulsed DCs are being examined in various clinical trials, either alone (NCT02692976, NCT01883518, NCT02334735, NCT02224599) or in combination with a NY-ESO-1 protein vaccine and TLR4 agonist (NCT02387125). Moreover, studies with an infusion of DCs and tumour-infiltrating lymphocytes (TILs) are also ongoing (NCT01946373).

About 5T4

The oncofoetal antigen 5T4 (also known as trophoblast glycoprotein, TPBG; and Wnt-activated inhibitory factor 1, WAIF1) is a vertebrate-specific, cell surface single-pass transmembrane protein with a short cytoplasmic region and N-glycosylated extracellular domain containing multiple leucine-rich repeat regions (Myers *et al.*, 1994; Zhao *et al.*, 2014). It was first identified in human trophoblasts of placental tissues by indirect immunoperoxidase staining of frozen sections (Hole and Stern, 1988). The 5T4 gene maps on the long arm of chromosome 6 at q14-15 and transcribes a 420 amino acid long protein. It is an early marker of differentiation of mouse and human embryonic stem (ES) cells (Boyle

et al., 1990; Hole and Stern, 1990; Myers *et al.*, 1994). A crystal structure of the extracellular domain predicts a highly glycosylated rigid core with leucine-rich repeat (LRR) regions (Zhao *et al.*, 2014), which makes it a member of the LRR family of proteins. LRR regions mediate protein-protein interactions, such as those associated with cellular adhesion and attachment to the extracellular matrix. With this in mind, 5T4 expression correlates with the directional movement of cells through epithelial-mesenchymal transition and influences the cytoskeletal organisation and cell motility (Carsberg, Myers and Stern, 1996) *via* canonical Wnt/beta-catenin signalling and possibly in partnership with CXCL12/CXCR4 mediated chemotaxis (Stern *et al.*, 2014) (Kagermeier-Schenk *et al.*, 2011). It was shown that 5T4 modulates canonical Wnt/beta-catenin signalling in a unique inhibitory fashion (Zhao *et al.*, 2014), while its natural ligand(s) have not yet been identified . The expression of 5T4 by tumour cells is associated with metastatic potential (Carsberg, Myers and Stern, 1996), while increasing levels of 5T4 expression have been associated with increasing tumour stage of some cancers. 5T4 is highly expressed on many common epithelial tumours and in patients with more advanced disease, including breast, colorectal (CRC), cervical, ovarian, gastric, renal cell carcinoma (RCC) and non-small cell lung cancers (Hole and Stern, 1988; Starzynska, Rahi and Stern, 1992; Griffiths *et al.*, 2005; Stern *et al.*, 2014). While 5T4 is highly expressed on placental trophoblast cells, it is not detected on most other healthy tissues, except for expression on some epithelial cells (Hole and Stern, 1988; Southall *et al.*, 1990). These properties have sparked interest in development of 5T4 related anti-cancer treatments. Two candidate CTL epitopes from the 5T4 antigen presented by HLA-A2 (residues 17–25 and 97–105) have been identified (Harrop *et al.*, 2008; Shingler *et al.*, 2008). In one recent report, CTL clones specific for peptides $5T4_{17–25}$ or $5T4_{97–105}$ were established from healthy donors and cancer patients (Tykodi *et al.*, 2012), and showed lytic activity towards tumour cells expressing 5T4 in *in vitro* assays while they also prevented xenoengraftment in immune-deficient mice. In another study, a recombinant modified vaccinia Ankara (MVA) virus encoding 5T4 (MVA-5T4; TroVax®) induced an antitumour immune response in mouse cancer models (Woods *et al.*, 2002; Mulryan *et al.*, 2002). It has also been evaluated as a therapeutic cancer vaccine in RCC, CRC, or prostate cancer patients (Kim *et al.*, 2010; Rowe and Cen, 2014). Even though immune monitoring in the early phase of the study confirmed that 5T4-specific antibodies, proliferative, and/or CTL responses were elicited in subsets of vaccinated patients, the outcome of this study showed no difference between control and patient groups (Kim *et al.*, 2010). Nevertheless, an analysis of a phase 3 clinical study suggested that subgroups of patients could benefit (Amato *et al.*, 2010). However, the latest randomised clinical trial with MVA-5T4 in combination with low-dose cyclophosphamide, demonstrated improvement in antitumour immunogenic responses, and a prolonged survival in patients with metastatic CRC (Scurr *et al.*, 2017). So far, no studies have investigated 5T4 peptide/protein-pulsed APCs alone or in combination with other agents as an anti-cancer treatment.

Collectively, the above findings support our interest in using NY-ESO-1 and 5T4 antigens as model antigens in the development of tumour-antigen loaded γδ T-APCs for use in cancer immunotherapy. The costs of mg quantities of 5T4 and NY-ESO-1 would have been enormous if obtained from commercial sources.

Aims

The aim of this chapter was to establish molecular tools for studying tumour antigen presentation in γδT-APCs, namely, the generation and purification of recombinant NY-ESO-1 and 5T4 antigens from bacterial expression systems.

In particular, the aims were to:

- Optimise bacteria cultivation for high-yield production of NY-ESO-1 antigen
- Optimise purification of NY-ESO-1 by one-step nickel affinity chromatography
- To generate bacterial expression plasmid constructs encoding 5T4 protein with/without fusion partner and optimise protein expression conditions to generate high yield 5T4 protein
- Optimise chromatography purification conditions for recombinant 5T4 production

3.2 Production and purification of recombinant NY-ESO-1 protein

3.2.1 Verification of pGMT7-ESO plasmid and rNY-ESO-1 expression

The pGMT7-ESO expression plasmid (**Figure 3.1**) carrying full NY-ESO-1 coding sequence (CDS) (GenBank Accession No NM_001327.2) was generated previously in our laboratory (unpublished). In this construct, the CDS of NY-ESO-1 was fused with six consecutive histidine sequences (6xHis-tag) to facilitate recombinant protein purification by nickel column immobilised affinity chromatography (Ni-IMAC). The advantage of using the pGMT7 expression vector is attributed to the deletion of the *lacI* gene and its repressor-binding site to achieve higher protein expression levels. Prior to its use for the production of the NY-ESO-1 protein, the open reading frame (ORF) in the pGMT7-ESO construct was verified by PCR and sequenced to confirm the correct sequence and the presence of the six histidine tag (6xHis-tag). A silent mutation TT**T**→TT**A** (Phe→Phe) was noted at position 510 of the coding sequence (CDS) (**Appendix 1**). The pGMT7-ESO expression vector was transformed into Rosetta BL21 (DE3) *E. coli* (generated in-house) to express NY-ESO-1 protein. This strain was designed to enhance the expression of eukaryotic proteins by supplying six codon tRNAs rarely used in *E. coli*. These tRNAs are encoded in an additional chloramphenicol-resistant pRARE plasmid, which supplies the codons AUA, AGG, AGA, CUA, CCC, and GGA and improves the production of proteins of non-bacterial origin.

Furthermore, this bacteria strain is deficient in both lon and ompT proteases, which are known to be involved in heath shock-like responses to the production of recombinant proteins and in the degradation of inclusion body (IB) proteins (Jürgen *et al.*, 2010), thus minimising potential proteolytic degradation of the recombinant protein. To drive the expression of the gene of interest, the Rosetta strain carries a chromosomal copy of the T7 RNA polymerase gene under the control of the lacUV5 promoter inducible using IPTG (isopropyl β-D-1-thiogalactopyranoside). IPTG structurally mimics lactose and binds to the lac repressor reducing its affinity for DNA and hence driving gene transcription of the target gene under the T7 promoter. However, unlike lactose, IPTG is not part of any metabolic pathways and will not be broken down or used by the cell. This ensures that the concentration of IPTG added remains constant, making it a more useful inducer of the lac operon than lactose itself.

The sonicated bacteria lysates were fractioned into soluble and insoluble partitions to identify if the NY-ESO-1 expresses in the soluble cytosolic fraction or inclusion bodies. The controls were uninduced bacteria and induced bacteria carrying an unrelated plasmid (**Figure 3.2**). As expected, almost all of the recombinant protein expressed in the inclusion bodies, which was confirmed by a ~20 kDa band on SDS-PAGE and Western blot using an anti-NY-ESO-1 antibody. Two additional bands of ~60kDa and ~40kD detected by Western blot suggested the formation of dimers and multimers. Since the pGMT7 vector does not contain the*lacI* gene nor the *lac* repressor-binding site, I expected to see a minor basal expression of NY-ESO-1 in the uninduced bacteria (**Figure 3.2**.). While expression of membrane proteins is generally toxic to most bacteria strains, including BL21(DE) strain (Hattab *et al.*, no

date)(Miroux and Walker, 1996), surprisingly rNY-ESO-1 did not result in death of the Rosetta *E. coli* cultures.

In conclusion, since the NY-ESO-1 is produced in inclusion bodies (IBs), the recovery/purification of the soluble protein can be difficult and a time-consuming process. However, a prominent aspect about IBs is that the embodied protein can be relatively pure, constituting from 50% to 95% of total proteinaceous material (Villaverde and Carrió, 2003), while most of contaminant host proteins can be more easily removed during processing (Villaverde and Carrió, 2003). Thus, a protocol for purification of NY-ESO-1 from IBs was adopted and optimised.

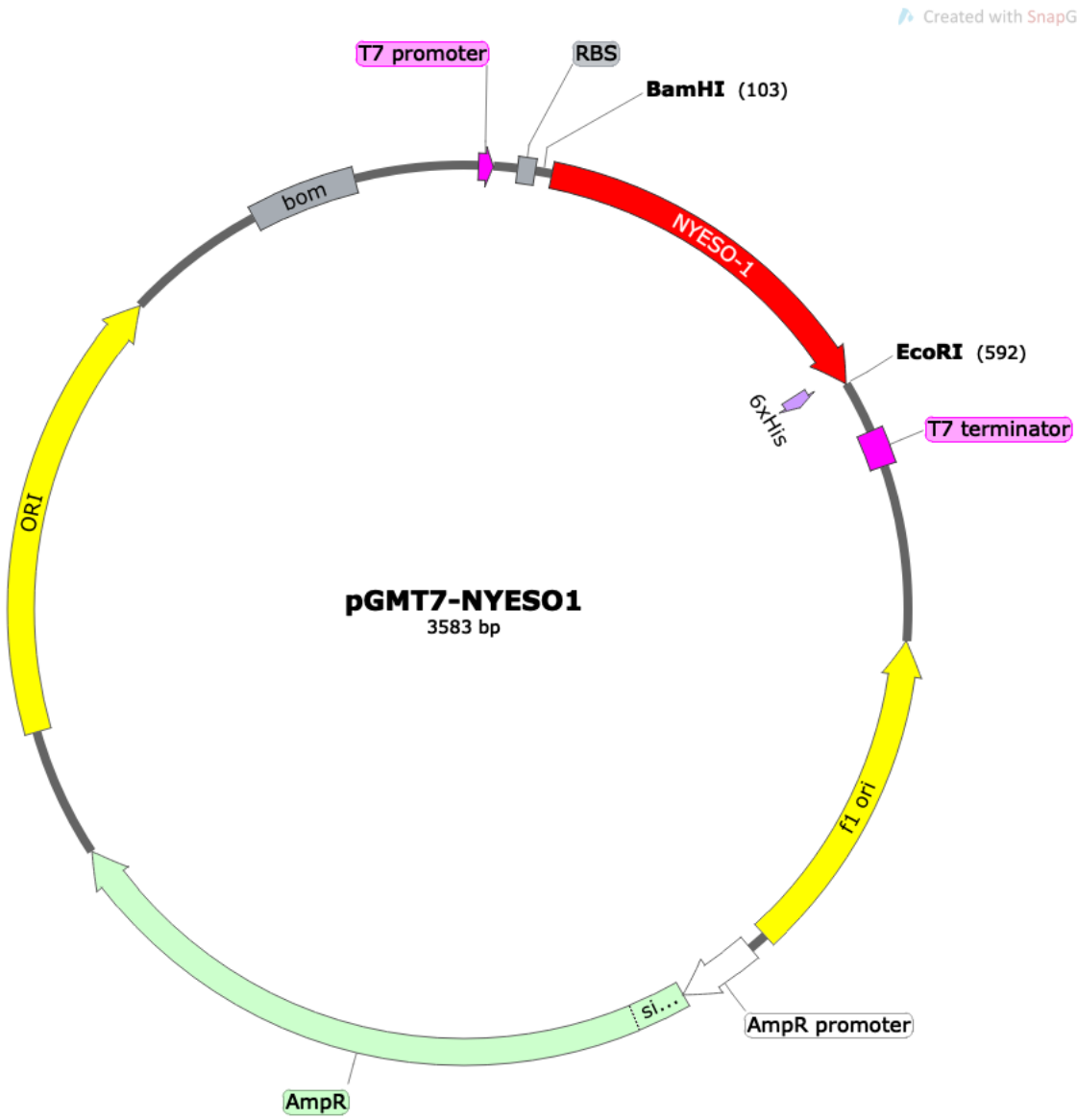


Figure 3.1 pGMT7-ESO1 expression vector.
The final pGMT7-ESO-1 expression plasmid construct was a kind gift from Mr Andrew. Thomas., Cardiff University The vector contains an AmpR selection cassette (Ampicillin resistance gene with a signal sequence and ampicillin promoter). The NY-ESO-1 CDS with 6x His tag at the C-terminus is contained within the expression cassette, including a promoter and terminator for bacteriophage T7 RNA polymerase and an efficient ribosome binding site (RBS) from bacteriophage T7 gene 10 (Olins and Rangwala, 1989). The NY-ESO-1 CDS was verified by Sanger sequencing (see **Appendix 1**).

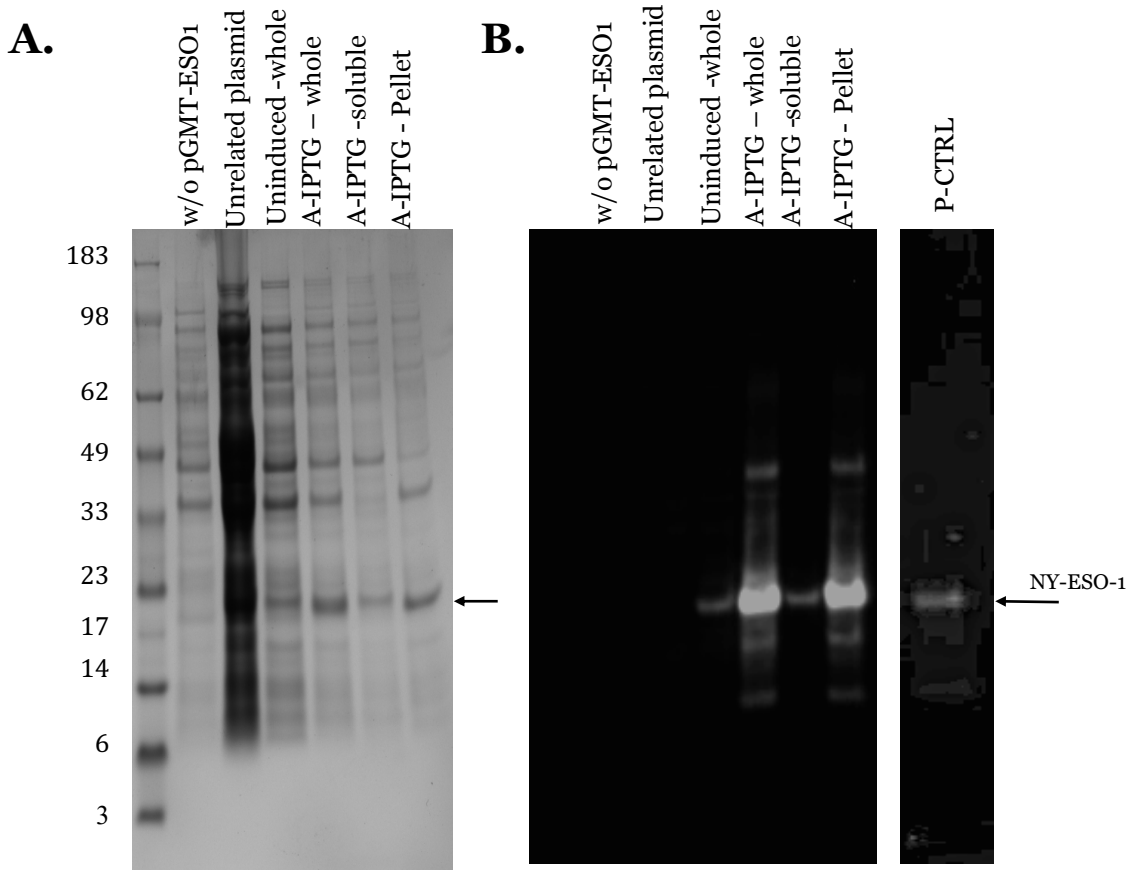


Figure 3.2. NYESO-1 protein expression analysis.

(A) SDS-PAGE protein analysis in plasmid transformed Rosetta cells. Samples were lysed by one freeze/thaw cycle and water bath sonication. The black arrow indicates NYESO-1 protein. *Lane "M"*- protein ladder (kDa); *Lane 1,* Rosetta BL21(DE) without pGMT7-ESO1 plasmid. *Lane 2,* bacteria carrying unrelated expression plasmid. *Lane 3* – uninduced bacteria carrying pGMT7-ESO1; *Lane 4,* bacteria carrying pGMT7-ESO1 after IPTG induction: total cell lysate; *Lane 5*- bacteria carrying pGMT7-ESO1 After IPTG: soluble protein fraction of cell lysate; *Lane 6*- bacteria carrying pGMT7-ESO1 After IPTG After induction: insoluble fraction of cell lysate. (B) Corresponding Western blot, using anti-human NYESO-1 monoclonal antibody. P-CTRL represents NYESO-1 protein standard.

3.2.2 NYESO-1 protein purification

3.2.2.1 Processing of rNY-ESO-1 inclusion bodies

The general strategy for protein recovery from inclusion bodies (IBs) includes three consecutive steps: 1) isolation and washing of IBs; 2) solubilisation of aggregated protein; and 3) refolding of the solubilised protein. Since downstream antigen presentation studies should not require a properly folded protein, an attempt of re-folding the NY-ESO-1 was omitted.

In a first attempt to purify NY-ESO-1 from IBs, an in-house protocol for producing and processing IBs of MHC molecules was adopted. In brief, the NY-ESO-1 production culture was set up in a 2TY medium under carbenicillin selection. Protein expression was induced with 0.5 mM IPG in the early log phase when OD600 reached ~0.4, and bacteria were maintained for another 3 h before harvesting (**Figure 3.3**). Fractionation of whole lysates confirmed a band of ~20 kDa corresponding to NY-ESO-1, mostly expressed in inclusion bodies (**Figure 3.3**). In preparing the inclusion bodies, agents that improve solubilisation of unwanted proteins include low concentrations of chaotropic agents and detergents. After cell lysis by sonication, the inclusion bodies were subject to three Triton X-100 washes, which cleared a significant amount of host protein contaminants (**Figure 3.3**). When guanidine solubilised IBs were dialysed into Ni-IMAC binding buffer A, the dialysate was not completely translucent in appearance, suggesting aggregate formation. NY-ESO-1 was then purified from solubilised inclusion bodies by Ni-IMAC.

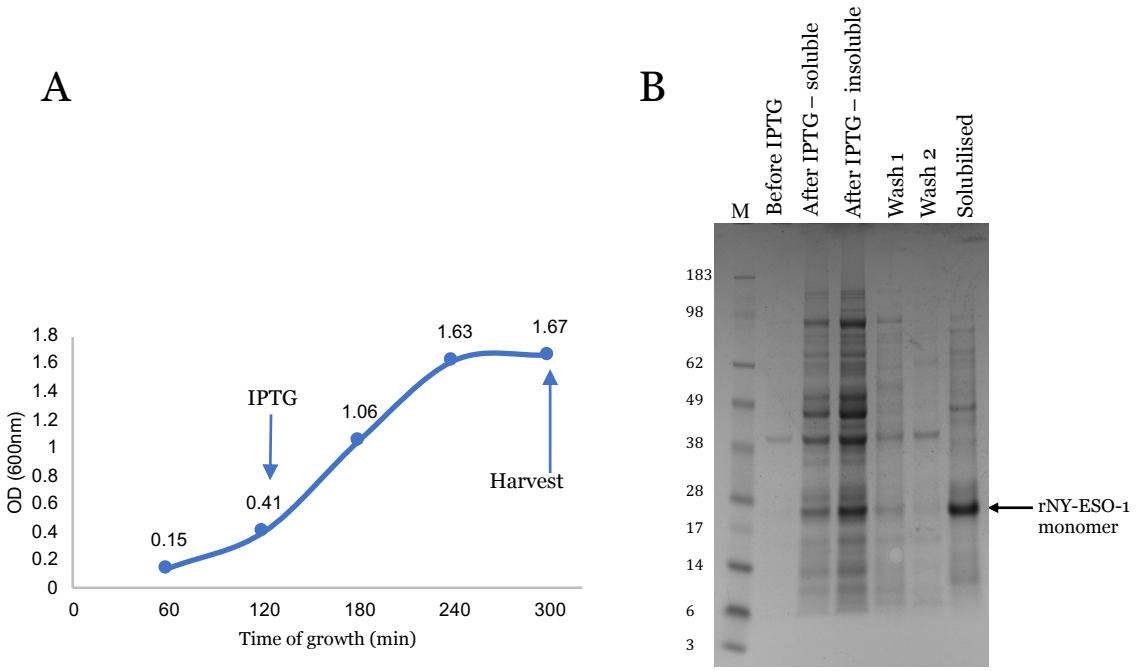


Figure 3.3. Initial cultivation of bacteria, expression and processing of NY-ESO-1 in inclusion bodies (next page):
(A) An overnight starter culture grown in LB was diluted in 1L fresh 2TY medium. Bacteria growth was monitored; when the OD600 reached 0.4, bacteria were induced with 0.5 mM IPTG and harvested 3 h after induction. (B) Coomassie-stained SDS-PAGE showing a comparison of a crude lysate of bacteria before

IPTG induction (*Lane 1*) and soluble (Lane 2) and insoluble (*Lane 3*) lysate fractions after IPTG at harvesting. Bacteria were sonicated (*Lane 4*), and inclusion bodies were processed by 2 Triton washes (Lane 5; combined washes), and finally, inclusion bodies were solubilised in guanidine buffer (*Lane 6*). Lane "M": molecular weight marker (kDa). Samples were lysed in bacteria lysis buffer and diluted(5x) before loading onto the gel before dialysis. A band corresponding to the NY-ESO-1 monomer can be seen as ~20 kDa and runs a bit higher on the gel.

3.2.2.2 Ni-IMAC purification

After the unbound protein passed through the nickel column, the column was first washed with 10% elution buffer A (50 mM imidazole) to remove unspecific host cell proteins lightly bound to the column, followed by elution of NY-ESO-1 with 100% elution buffer A. On the chromatogram (**Figure 3.4**), one peak is distinguishable; all three fractions of the peak (B4-B6) were analysed by Coomassie-stained SDS-PAGE (**Figure 3.4 B**). Elution fractions B5 and B6 showed a protein at ~20 kDa corresponding to NY-ESO-1, as well as dimers (~40 kDa) and multimers. In addition, a smaller protein (~6 kDa) visible in the B5 fraction could correspond to a degraded fragment of NY-ESO-1. Larger proteins (>60 kDa) were also detected and may represent host protein impurities that did not wash off the column or may have bound to the inclusion bodies. As the column was not loaded with an excess of protein, non-specific binding may occur at unoccupied nickel resin binding sites, even though 20mM imidazole was included in the binding buffer to reduce the non-specific binding of host protein to the column. SDS-PAGE analysis of the Ni-IMAC flow-through revealed that a fair amount of NY-ESO-1 protein did not bind to the column (**Figure 3.4 B; Lane 4)**. This may be due to (i) the formation of protein aggregates during the purification, (ii) the His-tag was "hidden" inside the protein. The NY-ESO-1 protein yield was low, around 1.7 mg/L of bacteria culture; not optimal for the use of recombinant protein in downstream *in vitro* studies.

In order to improve total NY-ESO-1 protein yield, these results indicated the need for optimisation of i) bacteria culture conditions; and ii) purification steps.

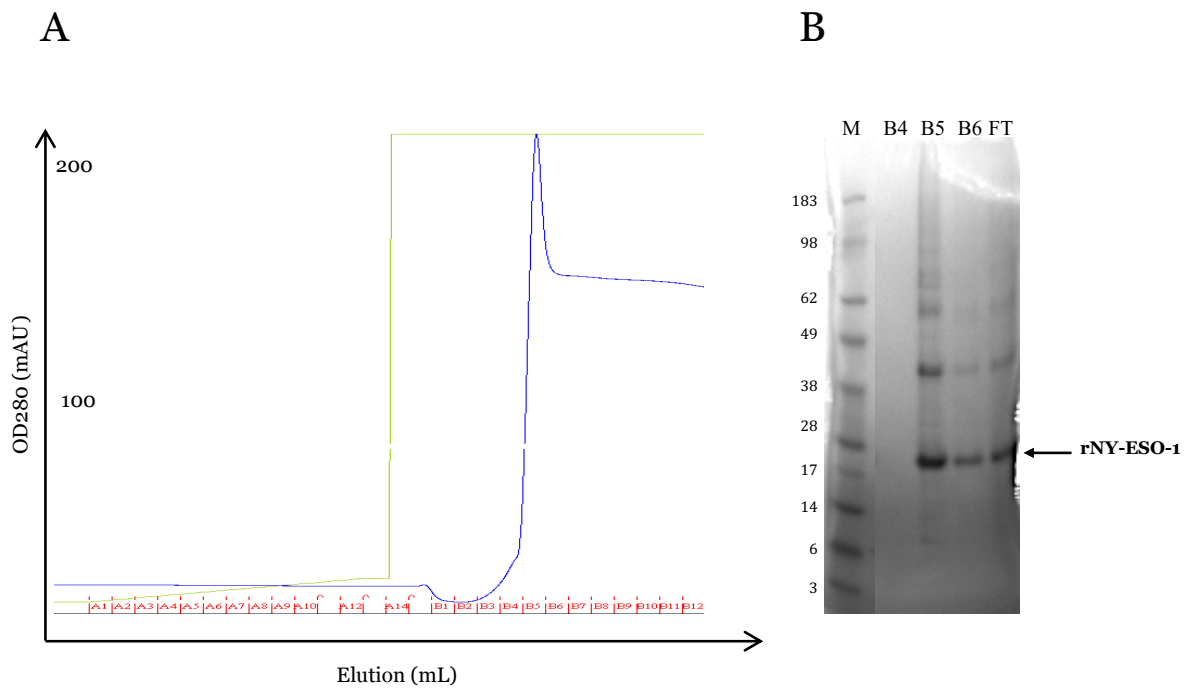


Figure 3.4. Initial purification of NYE-ESO-1 by Ni-IMAC.
(A) Representative Ni-IMAC Chromatogram of rNY-ESO-1 purified from inclusion bodies on a 5 ml HIS-TRAP column. Non-specific host proteins were washed off with 10% elution buffer (50 mM), followed by 100% elution buffer. NY-ESO-1 protein elutes as a single major peak. Fractions corresponding to the main peak (B4-B6) were collected and analysed by SDS-PAGE. The absorption at 280 nm is coloured in blue (arbitrary units), and the corresponding concentration of imidazole (%) is coloured in green. (B) Selected fractions from (A) were analysed by Coomassie-stained SDS-PAGE under reducing conditions (*Lane 1-3*, respectively). A band corresponding to NY-ESO-1 monomer can be seen at~20kDa, rNY-ESO multimers can be seen at ~40kDA and ~60kDa. The Highest concertation of protein eluted in fraction B5 also shows host protein impurities of higher MW (>62kDa) (*Lane 2*). A fair amount of NY-ESO-1 is still found in the flow-through (*Lane 4*). Lane "M": molecular weight marker. *Lane 1-3*: Ni-IMAC peak fractions B4 - B6, respectively. *Lane 4*: Ni-IMAC flow through.

3.2.3 Optimisation of rNY-ESO-1 protein production

To find out if the reason for the low total yield of recombinant protein was due to an insufficient level of protein expression or suboptimal purification method, the following conditions were tested at the level of protein expression: i) use of rare tRNA codons by maintaining pRARE plasmid in production cultures, ii) increased IPTG concentration, iii) type of medium, iv) length of the production phase, v) temperature.

RosettaBL21(DE) strain carries a pRARE plasmid, which encodes tRNAs rarely produced in *E. coli* and improves the production of proteins of non-bacterial origin. In addition, the pRARE plasmid carries the chloramphenicol resistance gene. Thus, to evaluate the impact of the presence of the pRARE plasmid on total NY-ESO-1 protein yield, bacteria were grown in the presence of carbenicillin alone or both

carbenicillin and chloramphenicol. Chloramphenicol acts by binding to the bacterial ribosome (blocking peptidyl transferase) and thus inhibits protein synthesis and slows down bacteria growth.
To minimise the variation in scale-up production, a starter culture was diluted at 1:100 to obtain an OD600 of~0.1 in a final volume of 100 ml of fresh 2TY medium. In the first four hours of the log phase, the culture with double antibiotic selection grew 2.7 x fold slower, seeing OD600 reached 0.3 vs 0.7 in bacteria cultured with carbenicillin only (not shown). Bacteria were induced with 0.5 mM IPTG, and at the time of harvesting (4 h after IPTG induction), bacteria cultured with double antibiotic selection contained a total of 0.88 mg protein in IBs, while carbenicillin supplemented culture contained 2.6mg protein in IBs. This difference, however, is not distinguishable from whole-cell lysates analysed by SDS-PAGE (**Figure 3.5 A**). After Ni-IMAC purification (**Figure 3.5 B**), a total of 0.3 mg of NY-ESO protein was recovered from IBs of bacteria grown in carbenicillin, while a total of 0.43 mg of NY-ESO-1 protein was recovered from bacteria grown in the double antibiotic selection, and recombinant protein was also found in the flow-through.
In conclusion, these results demonstrate that higher NY-ESO-1 protein production efficacy was achieved when pRARE plasmid was maintained during the protein production (11.5% recovery with carbenicillin vs 48.8% recovery with double antibiotic selection).

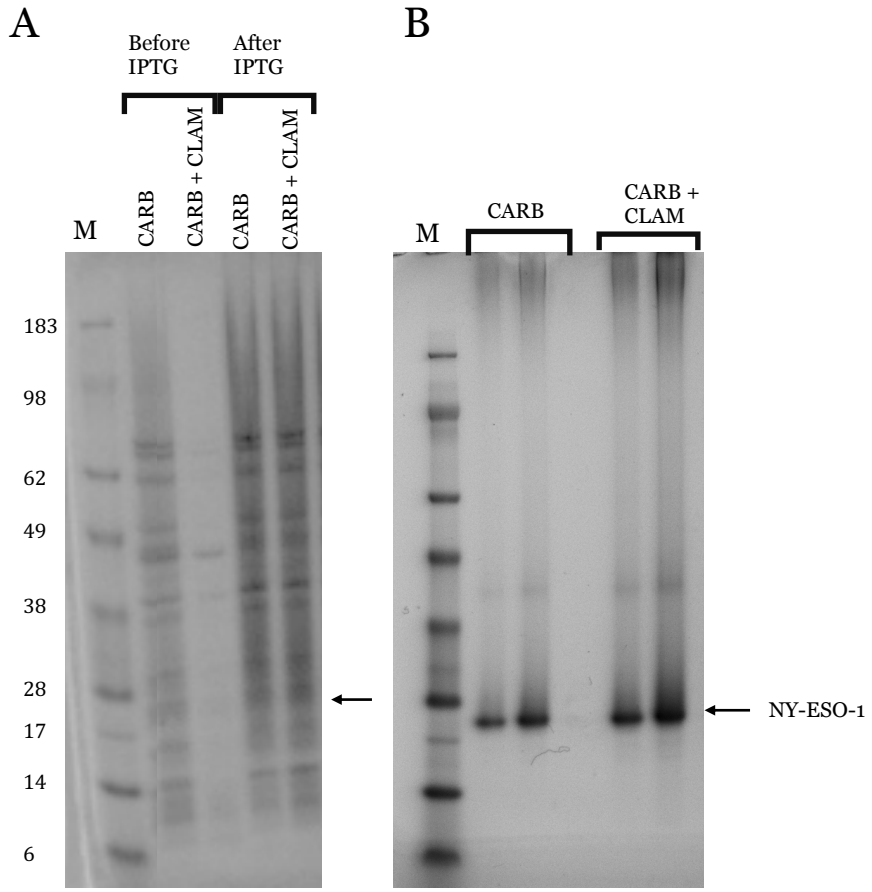


Figure 3.5. The influence of the codon usage in pRARE plasmid on production yield of NY-ESO-1 protein.

(A) SDS-PAGE analysis of whole bacteria lysates of carbenicillin alone supplemented cultures, before induction with IPTG (Lane 1) and after IPTG induction (Lane 3); and of carbenicillin/chloramphenicol supplemented cultures, before IPTG (Lane 2) or after IPTG induction (lane 4) ***(B)*** SDS-PAGE analysis of pooled Ni-IMAC fractions containing rNY-ESO-1 (see **Figure 3.5**) from bacteria cultured in the presence of carbenicillin alone (Lane 2 and Lane 3 – 1.9μg and 3.7 μg protein was loaded on the gel, respectively); bacteria cultured in the presence of carbenicillin and chloramphenicol (Lane 5 and lane 6- 6μg and 11μg of protein was loaded on the gel, respectively);

To develop a system for the production of high yields of NY-ESO-1 protein, a thorough optimisation of cultivation conditions followed since no empirical rules exist, and conditions are protein dependent (Sivashanmugam *et al.*, 2009)(Gräslund *et al.*, 2008)(Peti and Page, 2007). The following conditions were tested: i) the type of medium (TYP or TB media); ii) IPTG concentration (0.5 mM, 1 mM, 2 mM, and 3 mM), iii) length of protein production phase (5 h and 16 h) and the temperature during the protein production phase (20°C for overnight cultures and 37°C for 5 h). For all conditions, inclusion bodies were processed and purified as described in methods section 2.2.3.2 and 2.2.3.3, and the total yield of NY-ESO-1 was assessed. As expected, expression of NY-ESO-1 markedly increased with higher IPTG concentrations, either in TB or 2TY medium (Table 3.1, Table 3.2). For bacteria grown at 37°C, the highest protein yield was observed by increasing IPTG concentrations from 0.5 mM to 2 mM in TB medium (10.7 fold increase), while in 2TY medium highest protein yield was seen by increasing IPTG concertation from 0.5 mM to 1 mM (6.7 fold increase). Notably, temperature (20°C) and the length of the protein production phase (16 h) further increased total NY-ESO-1 yield; a 16.1 fold increase in total yield was observed for bacteria in TB medium, and 6.9 fold increase was observed in 2TY medium (Table 3.2, Table 3.3). Growth in TB medium resulted in a high yield of NY-ESO-1 protein, suggesting a beneficial role of glycerol as an additional carbon source. When two different media were compared, the most striking differences were seen in bacteria-induced with 2 mM IPTG at 37°C in 5h and bacteria-induced with 0.5 mM IPTG at 20°C in 16 h (Table 3.3). The total NY-ESO-1 protein yield was best in the TB medium. A substantial amount of NY-ESO-1 was also found in the flow-through (bacterial cultures with higher cell densities) (Table 3.2, Table 3.3); the flow-through was repeatedly loaded onto the Ni-IMAC column. The final protein yield increased twofold.

In conclusion, the optimal expression conditions for NY-ESO-1 was selected in TB medium, 0.5 mM IPTG induction, and protein production phase for 16 h at 20°C, which resulted in a total of 14 mg of NYE-SO-1 protein from 100 ml bacteria culture. In addition, expression in 2TY medium with 0.5 mM IPTG for 16 h, or in TB medium with 2 mM IPTG for 5 h at 37°C could also be used.

Table 3.1 Effect of culture condition on total NY-ESO-1 yield in TB medium

Growth medium	[T] & production time	IPTG [mM]	Protein yield [mg]	Protein yield -Ni-IMAC reloading [mg]	Fold difference
TB	20 °C, 16 h	0.5	14	28.02 (2 re-loads)	16.1
TB	37 °C, 5 h	0.5	0.87	/	reference
TB	37 °C, 5 h	1	3.7	7.4 (1 re-load)	4.3
TB	37 °C, 5 h	2	9.35	18.9 (2 re-loads)	10.7
TB	37 °C, 5 h	3	3.17	/	3.6

Table 3.2 Effect of culture condition on total NY-ESO-1 yield in 2TY medium

Growth medium	[T] & production time	IPTG [mM]	Protein yield -1st round Ni-IMAC [mg]	Protein yield -Ni-IMAC reloading [mg]	Fold difference
2TY	20 °C, 16 h	0.5	3.4	21 (2 re-loads)	6.9
2TY	37 °C, 5 h	0.5	0.49	/	reference
2TY	37 °C, 5 h	1	3.31	/ (1 load)	6.7
2TY	37 °C, 5 h	2	2.42	/ (1 load)	4.9
2TY	37 °C, 5 h	3	2.19	/	4.5

Table 3.3. Influence of time and culture medium type on total NY-ESO-1 yield

[T] & production time	IPTG [mM]	Protein yield in TB [mg]	Protein yield in 2TY [mg]	Relative difference
20 °C, 16 h	0.5	14	3.4	311% (4.1 fold)
37 °C, 5 h	0.5	0.87	0.49	77% (1.7 fold)
37 °C, 5 h	1	3.7	3.31	12% (0.9 fold)
37 °C, 5 h	2	9.35	2.42	286% (3.8 fold)
37 °C, 5 h	3	3.17	2.19	45% (1.4 fold)

3.2.4 Optimisation of Ni-IMAC purification

3.2.4.1 Control of protein aggregation - Optimisation of buffering system for Ni-IMAC purification

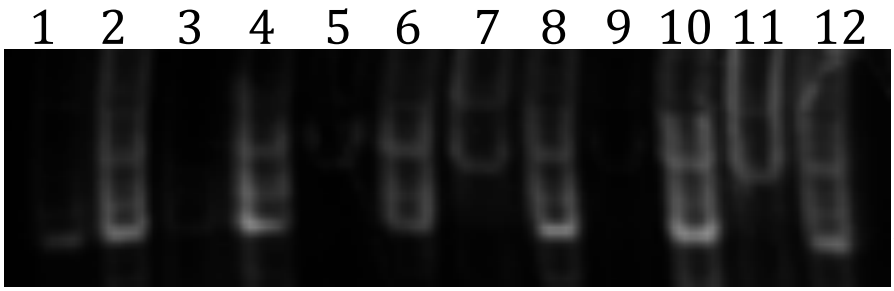
As the protein aggregation was an issue during purification steps, various cosolvents were analysed to keep the NY-ESO-1 soluble throughout the Ni-IMAC purification.

Protein solubility is a thermodynamic parameter defined as the concentration of a protein in a solution that is in equilibrium with a solid phase under a given set of conditions (Arakawa and Timasheff, 1985). Solubility can be influenced by several extrinsic and intrinsic factors. The intrinsic factors that influence protein solubility are defined primarily by the amino acids on the protein surface, while extrinsic factors that influence protein solubility include pH, ionic strength, temperature, and the presence of various solvent additives. Altering these extrinsic factors can lead to increased solubility. In theory, the solubility of a given protein is at a minimum near its isoelectric point (pI) and increases with the absolute (negative or positive) value of the net charge (Kramer *et al.*, 2012). NY-ESO-1 is overall a highly hydrophobic protein with a theoretical pI at ~8.79. In addition, it contains a C-terminal hydrophobic tail, potentially serving as a membrane-associated domain (Chen *et al.*, 1997), while the N-terminus is hydrophilic. Seeing that the pI of a polypeptide at each end differs greatly (~9.6 and 4.5, respectively), while the protein also contains many cysteine residues, determination of buffer conditions that would maintain protein soluble was expected to be difficult.

To increase protein solubility during the purification process, the solubility/aggregation test design was modified from (Bondos and Bicknell, 2003). In their report, Bonds *et al.* showed that such solubility tests can examine the behaviour of a particular protein in mixtures and crude lysate and predict the behaviour of (partially) purified protein. My general strategy was to test up to 10 solvents on whole-cell protein samples (1 ml sample of bacteria culture), which were individually resuspended in test buffers with an addition of different cosolvents. After lysis by sonication, the lysates were incubated at room temperature in the test buffer for 1 h. Then, to determine NY-ESO-1 protein solubility in the specific buffer, the soluble protein was separated from insoluble protein by centrifugation and analysed by SDS-PAGE/Western blot. Due to the large number and high concentration of other proteins in the sample, Western blotting helps to visualise the NY-ESO-1 after SDS–PAGE. The Tris lysis buffer A (**Figure 3.6, Lane 3 & 4**) was used to reference NY-ESO-1 protein aggregation, and the 8M urea buffer served as a reference for soluble protein. Cosolvents and additives concentrations that interfere with Ni-IMAC were omitted. Instead of DTT, which interferes with nickel ions, β-mercaptoethanol (2-BME) was used. Cosolvents that address a range of potential triggers for aggregation, such as exposure of hydrophobic groups, charge-charge interactions, cysteine oxidation and improper disulphide bond formation, were selected for examination.

Initial tests included MES vs Tris buffer, with the addition of natural detergents deoxycholate (1-2%) and Triton-X-100 (2%), and a reducing agent (2-BME; 5 mM). Natural detergents deoxycholate with Triton-X100 is commonly used in isolation of membrane proteins (Tao *et al.*, 2010)(Makino, Reynolds and Tanford, 1973), while it was also used in the solubilisation buffer in the protocol described by Lowe

and collegues (Lowe *et al.*, 2011). Severe aggregation was observed in the presence of 1-2% deoxycholate and Triton-X-100 (**Figure 3.6**). Accordingly, the addition of 2.5 mM 2-BME reduced aggregation but did not prevent it (**Figure 3.6**). Partial solubilisation was achieved when buffers contained a combination of 1-2% deoxycholate, 2% triton-100, and 2.5 mM 2-BME. NY-ESO-1 protein solubility was better in 2% deoxycholate containing MES buffer than in Tris buffer (**Figure 3.6**). MES is a zwitterionic N-substituted aminosulfonic acid not absorbed through cell membranes. MES buffer is active in the pH range 5.5-6.7, and it has a pKa value of 6.10 at 25°C (Good *et al.*, 1966). Seeing that NY-ESO-1 was performing better in MES buffer than in Tris buffer suggests that it prefers a more acidic environment. Nevertheless, Tris buffer has a pKa value of 8, which is too close to the pI of NY-ESO-1 to expect it would have a solubilising effect on rN-ESO-1. Since MES weakly binds Mg2+, Mn2+, Ni2+ and Cu2+ ions, it is not recommended for use in Ni-IMAC. Thus, in all subsequent experiments, all buffers contained 20 mM Na2HPO4-2H2O, 5 mM 2-BME and 20 mM imidazole (pH 7) since the primary goal was to find a suitable IMAC binding buffer.



Cosolvents tested in lysing buffers							
Lane number	**Buffer [mM]**	**Salts**	**Chaotropes**	**Detergent**		**Reducing agents**	**Polyhydric alcohol**
1 & 2	50 mM MES	300 mM NaCl	10 mM MgCl2			2.5 mM 2-BMe	10% glycerol
3 & 4	10 TRIS	150 mM NaCl	10 mM MgCl2				10% glycerol
5 & 6	50 TRIS	300 mM NaCl	10 mM MgCl2	1% deoxycholate	2% Triton-100		
7 & 8	50 tris	300 mM NaCl	10 mM MgCl2	1% deoxycholate	2% Triton-100	2.5 mM 2-BMe	
9 & 10	50 MES	300 mM NaCl	10 mM MgCl2	2% deoxycholate	2% Triton-100		
11 &12	50 MES	300 mM NaCl	10 mM MgCl2	2% deoxycholate	2% Triton-100	2.5 mM 2-ME	

Figure 3.6. NY-ESO-1 aggregation/solubility test: Western blot.
Bacteria pellets were thawed and resuspended in MES or TRIS buffers containing different concentrations of salts, detergents, reducing agents, with or without polyhydric alcohol (table in the bottom panel) and lysed by sonication. The solubility of proteins was analysed by western blotting after the cell lysates were fractioned into soluble protein (odd Lane numbers) and insoluble protein (even Lane numbers) fractions. Partial solubilisation was achieved when buffers contained a combination of 1-2% deoxycholate, 2% triton-100, and 5 mM 2-BME (*Lane 7 and Lane 11*). The black arrow indicates a recombinant NY-ESO-1 protein. Lanes 1-12, refer to table above.

Next, different additive combinations of kosmotropes and chaotropes were tested. As NYESO-1 is highly hydrophobic, with a theoretical pI at ~8.79, it was prudent to choose a pH of a buffer at least one unit below/above its pI. One can stabilise native intramolecular protein interactions by adding kosmotropes because they increase the surface tension of the solvent. They are excluded from the protein–solvent surface, and therefore they increase the energy cost for hydration of denatured states (or intermediate states) so that the native state is favourable. On this note, different concentrations of NaCl (0.3-1 M) were tested. Previous reports utilised mid-to-high range urea concentration, or CHAPS and N-lauroylsarcosine, to solubilise and purify CTAs from inclusion bodies (Yang *et al.*, 2004; Huang *et al.*, 2007; Lowe *et al.*, 2011). Chaotropic species destabilise protein-protein interactions by interacting with the protein's peptide group, hence replacing or preventing the intermolecular/electrostatic interactions that lead to aggregation. As expected, complete solubilisation was achieved in 8 M urea, and good solubility was

achieved with 4 M urea. As lower urea concentrations were preferred, the addition of deoxycholate, Triton-X-100 and CHAPS were tested. The previous report for purifying GMP grade NY-ESO-1 included 2% deoxycholate and 8 M urea in their formulation of IMAC binding buffer (Lowe *et al.*, 2011). Addition of 2% deoxycholate and 2% Trion X-100 with urea below 6M however did not greatly contribute to protein solubility. However, adding 2% deoxycholate and 2% Triton X-100 with urea below 6 M did not greatly improve protein solubility. However, the addition of 2 % Triton X-100 with 20-40 mM CHAPS improved solubility (not shown).

In conclusion, the optimal IMAC binding buffer was selected to include at least 4 M Urea, 500 mM NaCl, and 5 mM 2-BME. Other detergents Triton X-100, N-lauroylsarcosine and deoxycholate and CHAPS, were opted out since their complete removal from the protein could have been a challenging process (Makino, Reynolds and Tanford, 1973), requiring multiple steps of either protein precipitation, extensive dialysis and/or at least one additional chromatography step (Allen *et al.*, 1980; Ohlendieck, 1996). Furthermore, an unsuccessful Ni-IMAC purification of another N-lauroylsarcosine solubilised CTA antigen has been reported (Yang *et al.*, 2004), which was thought to be attributed to N-lauroylsarcosine engulfing the His-tag and preventing it from binding to the nickel resin.

3.2.5 Optimised conditions for purification of recombinant NY-ESO-1 protein from inclusion bodies

In preparing inclusion bodies, the most effective purification occurred with agents that improve solubilisation of unwanted host proteins, that is, with low concentrations of chaotropic agents and detergents, and during the removal of cell debris (**Figure**. **3.7**). Protein production in cultures with increased cell culture densities also led to more viscous lysate with more DNA. Hence the double concentration of DNAse and extensive washing of inclusion bodies reduced the viscosity and allowed a better purification process. Solubilised and clarified inclusion bodies were dialysed into the IMAC binding buffer containing 8 M urea and 5mM 2-BME with pH 7. The addition of 5 mM B-ME helped keep cysteines reduced; however, some dimer formation was still observed (**Figure 3.7**, lane 6). Processed inclusion bodies were applied onto the His-TRAP nickel column, and additional wash step with high ionic strength buffer and extensive washes with wash buffer removed almost all host impurities. It has been shown before that extensive washes with multiple detergent solutions can also drastically reduce the endotoxin levels (Lowe *et al.*, 2011). However, these measurements were not performed in this study. A minimal amount of NY-ESO-1 was lost in the flow-through while most host proteins passed through the column (not shown).

The protein eluted in one major peak (**Figure 3.8**), and all peak fractions were analysed by SDS-PAGE (**Figure 3.8**). As seen from the SDS-PAGE, all fractions contained a band corresponding to NY-ESO-1; fractions with minimal impurities were pooled and dialysed against storage buffer. After concentrating the pooled fractions by ultrafiltration centrifugal concentrators, SDS-PAGE analysis of NY-ESO-1 indicated that the protein preparation was virtually pure except for six minor contaminations (**Figure 3.8**). MALDI-TOF mass spectroscopy confirmed a NY-ESO-1 monomer and multimer (~62

kDa). While the proteins in the range of ~38-49 kDa could not be identified, they could represent a NY-ESO-1 dimer. The protein of lower MW (~14 kDa) may be a product of proteolytic degradation of NY-ESO-1. The proteins of higher MW represent chaperone proteins bound to the multimeric NY-ESO-1. Previously those higher molecular weight proteins have also been identified as DNA J, DNA K, and GroEL bound to NY-ESO-1 and were impossible to remove despite various chromatography methods, and tailoring of buffer composition with various ranges of reducing and kosmotropic agents (Lowe *et al.*, 2011)

In conclusion, using the optimised protocol, the total protein yield of NY-ESO-1 was drastically improved, from about 0.49 mg/100 ml obtained with the initially described protocol to up to 28 mg/100 ml of bacteria culture. In conclusion, this improved protocol yields a sufficient amount of relatively pure NY-ESO-1, suitable for downstream in-vitro antigen presentation studies.

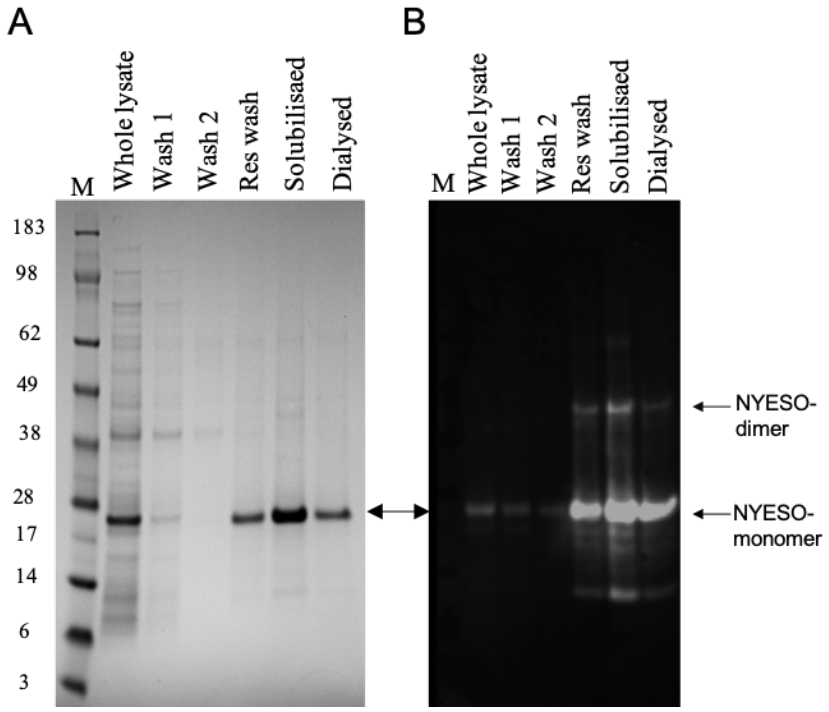


Figure 3.7. Processing of inclusion bodies.
(**A**) SDS-PAGE. *M*- see blue ladder; *Lane 1* whole lysate; *Lane 2* – wash 1; *Lane 3* – wash 2; *Lane 4* – in Resuspension buffer ; *Lane 5*- solubilised in Urea buffer; *Lane 6* – solubilised IBs dialysed into IMAC binding buffer A. (**B**) Corresponding western blot, using anti-human NY-ESO-1 monoclonal antibody.

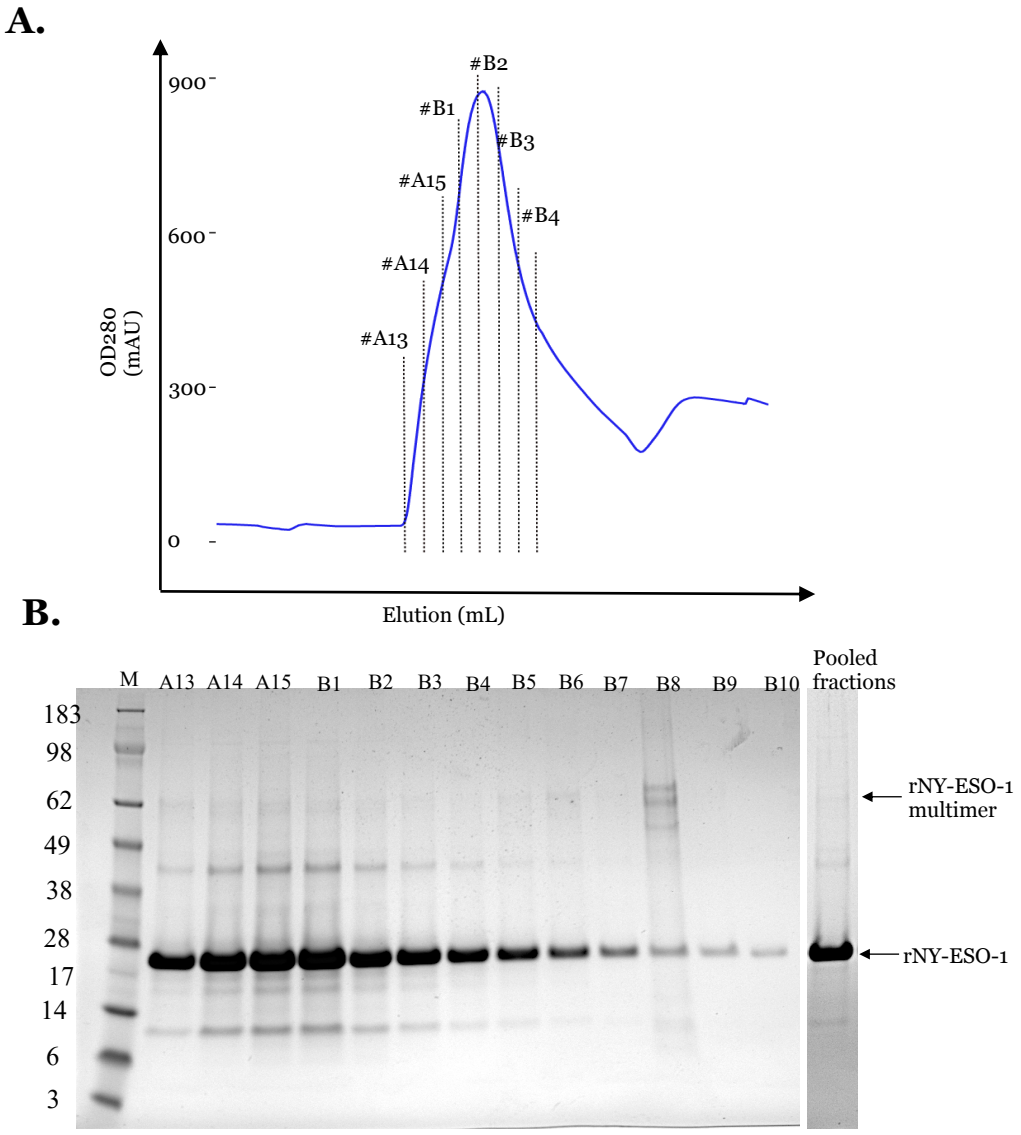


Figure 3.8. Optimised Ni-IMAC Purification of NYE-ESO-1: Chromatogram

Representative Ni-IMAC Chromatogram of NY-ESO-1 purified from inclusion bodies on a 5 ml HIS-TRAP column. First, non-specific host proteins were washed off with 10% elution buffer (50 mM), followed by a high ionic strength buffer to remove any remaining DNA in the sample, and finally, the NY-ESO-1 was eluted by 100% elution buffer. NY-ESO-1 protein elutes as a single major peak. The absorption at 280 nm is coloured in blue (arbitrary units). Fractions corresponding to the main peak were collected and analysed by SDS-PAGE (bottom panel), and fractions with minor contaminants were pooled (A13-B4) and analysed by MALDI/TOF mass spectroscopy.

3.3 Expression, isolation and purification of recombinant 5T4 protein

3.3.1 Construction of 5T4 expression plasmids

(As mentioned before, 5T4 is highly expressed on placental trophoblast cells, in many common epithelial tumours and patients with more advanced disease, including breast, colorectal (CRC), cervical, ovarian, gastric, renal cell carcinoma (RCC) and non-small cell lung cancers (Hole and Stern, 1988; Starzynska, Rahi and Stern, 1992; Griffiths *et al.*, 2005; Stern *et al.*, 2014). However, 5T4 expression was not found on most healthy tissues (Hole and Stern, 1988; Southall *et al.*, 1990), and thus it was used as a model protein in this study. 5T4 is a ~1.3 Kb long gene that codes for a 420 aa membrane protein. To produce a protein, first 5T4 gene was cloned from cDNA generated from a fast growing breast cancer cell line - MCF-7, since among breast cancer cell lines available in our laboratory, MCF7 shows highest 5T4 mRNA levels according to Human protein atlas (https://www.proteinatlas.org/ENSG00000146242-TPBG/cell#rna). **Figure 3.9** illustrates the cloning strategy for the generation of expression plasmids caring recombinant 5T4 gene. Briefly, to use one-step Ni-IMAC purification, a recombinant 5T4 (5T4) gene with C-terminal 6xHis-tag and Bam HI and Eco RI restriction sites at each end of the 5T4 coding sequence was created by PCR from the cDNA generated from the MCF7 cell line. The resulting 5T4 amplicon (1.3 Kb) was double digested and ligated into high copy plasmid vector pBlueScript-SK(-). Colony PCR and diagnostic restriction double digests with Bam HI/Eco RI endonucleases confirmed the correct size of the 5T4 insert (not shown). In addition, DNA sequence analysis of all confirmed clones revealed a silent mutation AC**G** → AC**A** at the position 1236 bp of the 5T4 gene CDS (**Appendix 1**). This silent mutation was not a product of DNA polymerase in the PCR as it is also found in the database of common SNPs (rs700494; NM_001166392.1:c.1236G>A)

The 5T4 gene was subsequently cloned into two low copy number expression plasmids: pGMT7 and pET32a, respectively. In both plasmids, the expression of target genes is driven by a strong bacteriophage T7 promoter that works well in *E. coli* (see also NY-ESO-1 production above). The choice of pGMT7 was due to its excellent success rate in expressing other difficult proteins (NY-ESO-1) in my hands. The pET32a system was selected to apply advantages of thioredoxin (TRX) fusion partner at the N-terminus. The concept of utilising 5T4-TRX fusion was to employ the benefits of the several inherent characteristics of TRX fusions (Edward R. LaVallie *et al.*, 1993), such as providing better protein yields, while instead of accumulating in IBs, the protein can be expressed in a soluble form.

Furthermore, thioredoxin as a fusion partner could also contribute to the better thermal stability of the protein, while it is small (11,675 Da) and therefore constitutes only a minor proportion of the total protein mass (Edward R. LaVallie *et al.*, 1993). This vector also conveniently includes two cleavage sites (thrombin and enterokinase), S Tag (Raines *et al.*, 2000), and 6xHis-Tag at the C-terminus of TRX tag; contributing 17 kDa to the total recombinant protein mass. Moreover, the pET32a plasmid carries the *lacI* gene with lac operator located before the T7 promoter for better control of basal level of

recombinant protein expression, which can be beneficial if the protein is toxic for bacteria. A schematic representation of the constructs is shown (**Figure 3.10**).

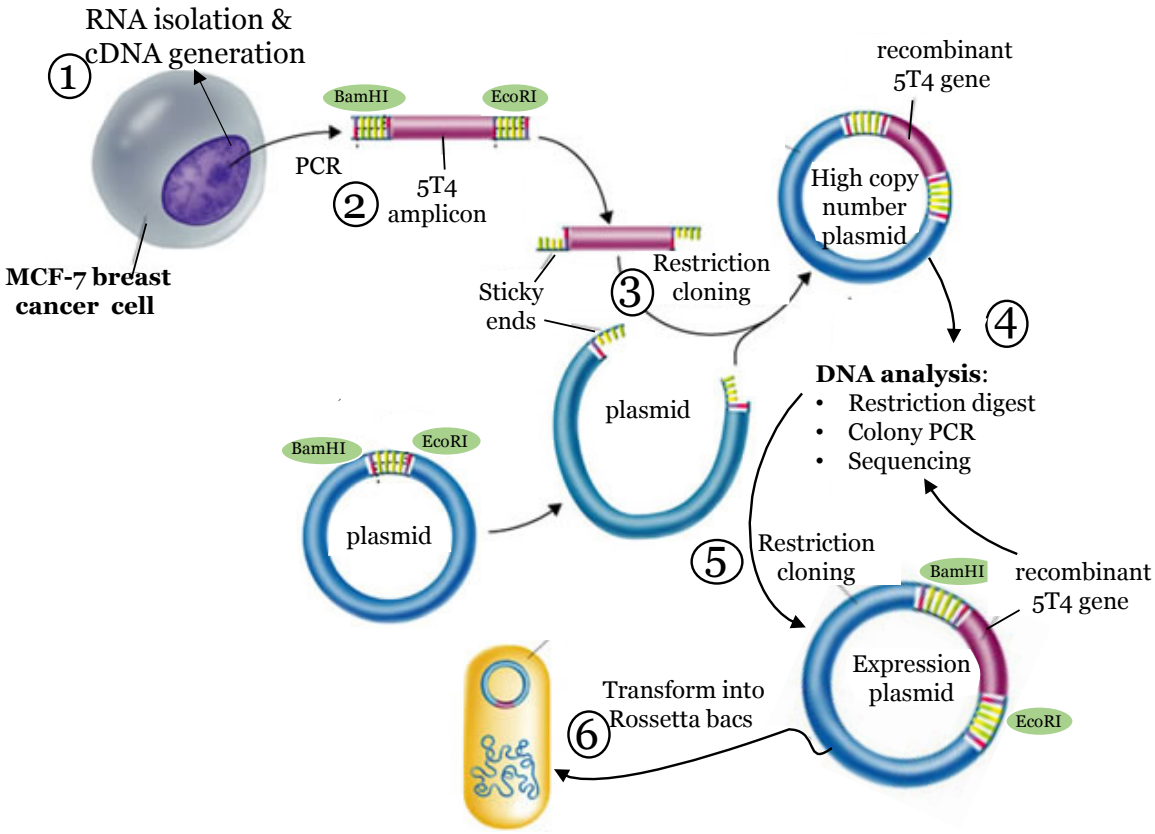
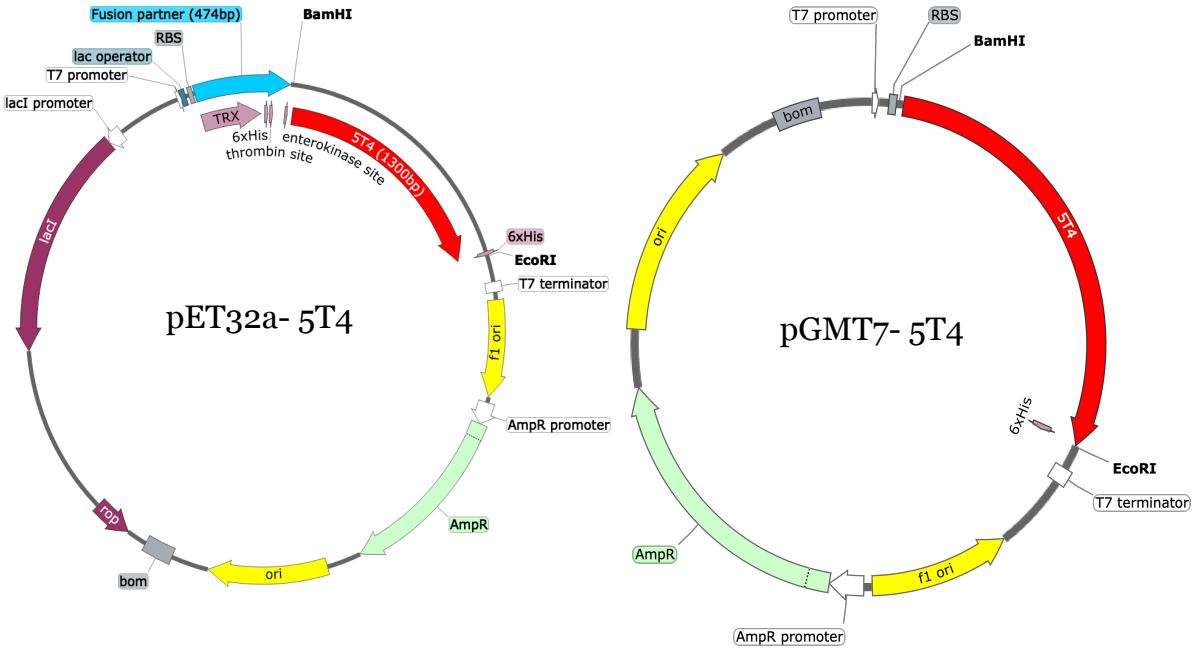


Figure 3.9. Simplified illustration of 5T4 expression vectors construction.
5T4 CDS was cloned from the cDNA of MCF-7 cells (1). Recombinant 5T4 CDS was amplified by PCR using adding Bam HI restriction site at the N-terminus, and 6 x His sequence followed by Eco RI restriction site at the C-terminus (2). The 5T4 amplicon was digested with Bam HI and Eco RI restriction enzymes and cloned into a high copy number plasmid (3). Clones were analysed by restriction digestion/colony PCR and Sanger sequencing (4). Plasmids with correct orientation and no point mutations were cloned out by restriction cloning into expression plasmid (5), sequenced again, and transformed into Rossetta bacteria strain (6).



Feature	description
T7 promoter	promoter
RBS	ribosom biniding site
5T4	5T4 coding sequence
6xHis	6xHis affinity tag
T7 terminator	T7 terminator
f1 ori	f1 bacteriophage origin of replication
AmpR promoter	promoter
AmpR	ampicilin resistance gene
ORI	high-copy-number ColE1/pMB1/pBR322/pUC origin of replication
lacI	lac repressor
lacI promoter	Promoter
Lac operator	operator
bom	basis of mobility region from pBR322
Rop	Rop protein, which maintains plasmids at low copy number

Figure 3.10. 5T4 expression vectors.
5T4 expression vectors. MCF7 breast cancer cell line served as a source for the 5T4 gene. The 5T4 CDS was amplified by PCR to generate recombinant 5T4 gene (1.3kb) with the C-terminal His-tag and flanking Bam HI and Eco RI restriction endonuclease sites. The recombinant 5T4 PCR product was first cloned into pBSKK-plasmid via Bam HI and Eco RI sites, and the sequence was verified by Sanger sequencing and cloned into pGMT7 and pET32a expression plasmids via Bam HI and Eco RI restriction sites (top panel). pET32a vector includes TRX fusion partner together with cleavage sites and additional His-tag at the N-terminus of the 5T4. The bottom panel shows a list of plasmids features.

3.3.2 Optimisation of protein expression conditions for recombinant 5T4

In order to optimise 5T4 protein expression, preliminary small scale expression tests were performed in 5 ml bacterial cultures. To employ the advantage of codons for amino acids that are rarely expressed in bacteria, Rosetta BL21 (DE) *E. coli* was chosen as an expression system for both vectors pGMT7-5T4 and pET32a-5T4-TRX. Since *E. coli* are not equipped with machinery for protein posttranslational modifications, such as glycosylation, the expected size of recombinant TRX-5T4 fusion expressed from pET32a was ~65kDa, while the expected size of 5T4 expressed from pGMT7 was ~45kDa..

To obtain a high 5T4 protein expression level but at the same time improve protein solubility, the TRX fusion partner was added at the N terminus. In the tertiary structure of TRX, both the N- and C-termini of the molecule are surface accessible, while TRX has a very compact fold, with >90% of its primary sequence involved in strong elements of secondary structure; these robust folding characteristics can contribute to its success as a fusion partner protein in recombinant protein production. TB medium was chosen for production cultures because it is generally used for high yield protein production cultures (Sivashanmugam *et al.*, 2009), and because it also resulted in high yields of NY-ESO-1 (**Figure 3.8**/Table 3.1). On the other hand, the solubility of the protein can also be altered by bacterial growth conditions: 1) lower rate of protein expression, and hence slower error rate during translation or 2) prolonged time allowed for refolding processes; both can result in a lower probability of aggregate formation (Schein, 1991). In addition, it is known that temperature can play a critical role in cellular processing; for example, some proteases can function as chaperones ("helper" proteins) at low temperatures but act as proteases at elevated temperatures (Ryan *et al.*, 2013).

Thus, with an attempt to express recombinant 5T4 protein, the expression was induced with two IPTG concentrations (0.5 mM and 1 mM) at low temperatures 28°C and 20°C for a duration of 5 h and 16 h. At the induction, culture density was 1.9 fold higher in bacteria carrying pET32a (OD600= 1.9) than in bacteria carrying pGMT7 (OD600 = 1.). However, it was not possible to determine whether these growth differences could be accounted to the influence/toxicity of the plasmid vectors on bacterial growth or were due to differences between individual transformants colonies (Sivashanmugam *et al.*, 2009). Surprisingly, no 5T4 expression was detected from either pGMT7 or pET32a plasmid (not shown). Nevertheless, at this point, it was not possible to determine if this was due to protein toxicity/plasmid instability or due to suboptimal expression conditions. In the following set-up, protein production phase temperature and IPTG concentrations were increased. When the protein production phase was maintained at 33°C or 37°C and either 1 mM or 2 mM IPTG concentrations, successful 5T4-TRX expression was achieved from pET32a plasmid in all conditions tested, while no detectable 5T4 protein expression was observed from pGMT7 plasmid (figure 3.11 A). This observation suggested that the 5T4 protein itself may be toxic for the bacteria, which is generally true for membrane proteins (Montigny *et al.*, 2004). However, it cannot be excluded that 5T4 without a fusion partner was highly susceptible to proteolytic attack and degradation during the protein production phase. Best 5T4-TRX expression levels were achieved at 33°C and 1 mM IPTG or 37°C and 2 mM IPTG (**Figure 3.11**; lanes 7 and 8, respectively).

Contrary to expectations, the protein was found in the insoluble fraction (IBs) of cell lysates (**Figure 3.11 B**). This may, however, have been due to a high rate of protein production and higher production culture temperature. Additional tests at 33-37°C but with low IPTG concentrations (≤ 0.5 mM) could confirm that. Verifying 5T4 expression by Western blot using mouse monoclonal antibody for 5T4 antibody was unsuccessful, resulting in high unspecific background staining (not shown). However, successful verification was achieved with an anti-His-tag antibody, which confirmed the expression of 5T4-TRX protein but not 5T4 from the pGMT7 expression plasmid (**Figure 3.11. C**).

In conclusion, these data suggest that recombinant 5T4 protein with no fusion partner may be toxic to bacteria or a target of proteases during the expression time and that the expression of 5T4 protein from pET32a is optimal at 33°C and 1mM IPTG. Minor differences in the expression levels of the 5T4-TRX fusion protein were observed at 37°C and 2 mM IPTG and 33°C and 1 mM IPTG. Therefore, the latter was selected for scale-up protein production..

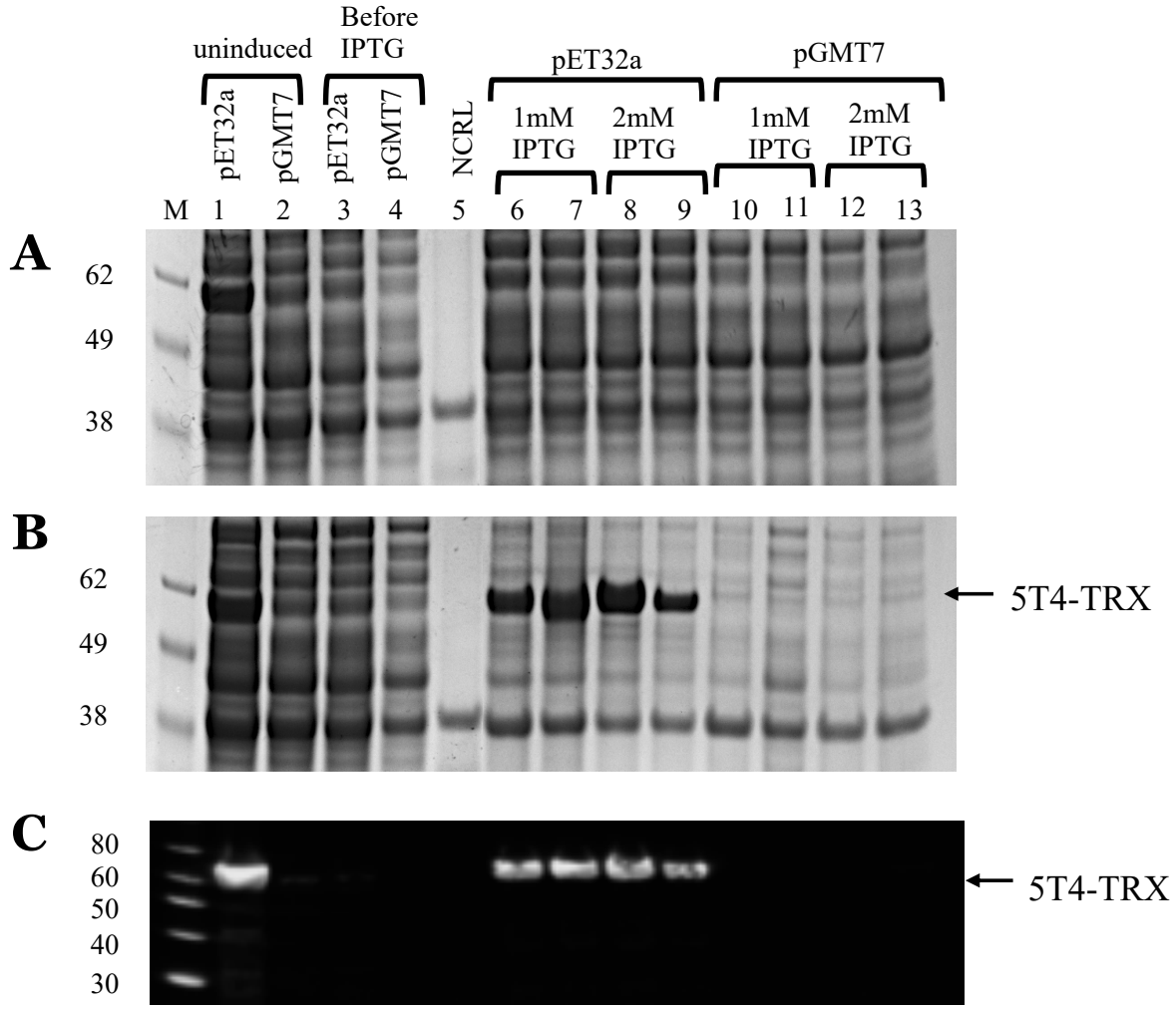


Figure 3.11. Optimisation of 5T4 protein expression from pGMT7 and pET32a plasmids.
An overnight starter culture was diluted 10x in 5 ml TB medium. Protein expression was examined after 3 h production phase at 33°C and 37°C, two IPTG concentrations (1 mM or 2 mM), and in uninduced bacteria. A substantial amount of TRX-5T4 protein was found in the whole cell lysate of uninduced cultures carrying pET32a plasmid (*Lane 1*), but none was detected from pGMT7 (*Lane 2*). No 5T4 protein was detected from either pET32a (*Lane 3*) or pGMT7 (*Lane 4*) in cultures before IPTG induction. Harvested lysates were

fractioned into (A) soluble protein and (B) insoluble protein/inclusion bodies fractions. A band corresponding to 5T4-TRX (~65 kDa) was detected in inclusion bodies from pET32a in all conditions tested: 1mM IPTG at 33°C or 37˚ (*Lane 6 & 7*); 2mM IPTG at 33°C or 37˚ (*Lane 8 & 9*). No 5T4 expression was detected from pGMT7 plasmid in any conditions tested: 1 mM IPTG at 33°C or 37˚ (*Lane 10& 11*); 2 mM IPTG at 33°C or 37˚ (*Lane 10 & 11*). Corresponding Western blot with anti-6-histidine antibody confirmed 5T4-TRX expression in inclusion bodies (C). NCTRL, negative control of unrelated protein; M, protein ladder (kDa).

3.3.3 Purification of 5T4 from inclusion bodies

The human 5T4 protein consists of 420 aa, with a short cytoplasmic sequence, a single-pass transmembrane region, and the extracellular domain, which contains leucine-rich repeat (LRR) segments. The addition of N-terminal thioredoxin fusion partner and C-terminal 6xHis-tag of 176 aa resulted in a fusion protein of 596 aa and a theoretical pI of ~6.2. Even though 5T4-TRX and NY-ESO-1 differ in their biophysical-biochemical properties, they are both single-pass membrane proteins; hence it was reasonable to adopt the purification protocol developed for the purification of NY-ESO-1. Lysis by sonication was performed on ice followed by 2h treatment with DNAse at 37 °C, further processing of inclusion bodies with Triton X-100 washes required for removing bacterial impurities resulted in more severe 5T4 protein degradation, resulting in two additional major bands of ~40 kDa and ~23 kDa appearing on SDS-PAGE and western blot (**Figure 3.12 A**). After dialysis, the Ni-IMAC purification was performed according to the protocol described for purification of NY-ESO-1 under denaturing conditions. However, the buffers were at pH 8, which gave the protein a negative charge, and hence the protein should have a better capacity to bind to positive nickel ions. The 5T4-TRX eluted in two major peaks (**Figure 3.12 B**), and the fractions A10 – 14 were pooled and analysed by western blotting (**Figure 3.12 A**, last line). Almost all 5T4-TRX was degraded into four smaller fragments of ~23, 30, 40 and 55 kDa (**Figure 3.11 B**). Even though the Rosetta strain is deficient in OmpT and Lon proteases, these data suggested that inhibition and removal of proteases before/and during purification is essential to prevent proteolytic degradation of 5T4.

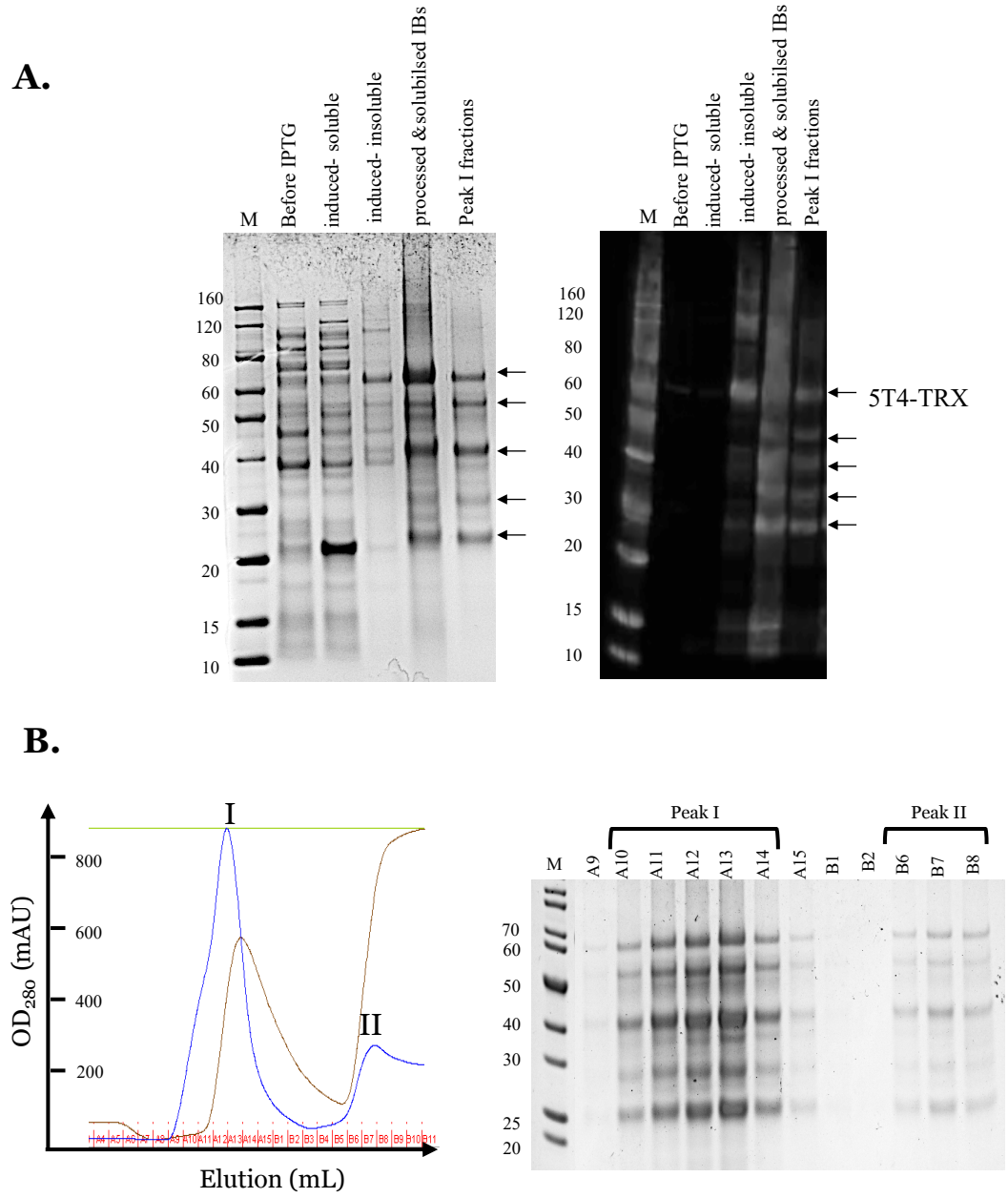


Figure 3.12. Purification of r5T4-TRX from inclusion bodies without protease inhibitors. Bacteria were grown at 37˚ C and induced with 1mM IPTG. The inclusion bodies were collected and processed as described in methods (**A**). After dialysis into IMAC binding buffer, the protein was loaded onto the HisTrap column, and 5T4-TRX eluted in two major peaks (**B**). The absorption at 280 nm is coloured in blue (arbitrary units), and the corresponding concentration of imidazole (%) is coloured in green; the brown line shows conductivity. Fractions of the two peaks were analysed by Coomassie-stained SDS-PAGE. *The Peak I* Fractions A10-A14 were pooled and analysed on SDS-PAGE (A, last line). Arrows show degraded 5T4-TRX protein. M- His-tagged protein marker (kDa); *Lane 1* uninduced, before IPTG; *Lane 2* – IPTG induced, soluble fraction; *Lane 3* – IPTG induced, insoluble fraction. *Lane 4* – processed, solubilised & dialysed inclusion bodies (IB); *Lane 5*- Peak I Pooled fractions of purified 5T4-TRX, dialysed into a storage buffer.

Thus, in the subsequent purification attempt, a protease inhibitor cocktail of five protease inhibitors with broad specificity for the inhibition of cysteine and serine proteases and metalloproteases was

added to the bacteria lysis buffer before sonication, which seemed to almost entirely prevent proteolytic degradation (**Figure 3.13**, left panel). A protein band of higher MW detected by Western blot may have represented fragments of a cleaved TRX fusion partner alone bound to a host protein or a protein aggregate. Unfortunately, the addition of protease inhibitor cocktail during lysis alone did not completely prevent their occurrence. The 2 h DNAse treatment at 37°C, and further processing of inclusion bodies, even at low temperatures (0˚-4 °C) and in the presence of EDTA, resulted in some degradation of 5T4. A full length 5T4-TRX at ~65 kDa and breakdown products of ~40-50 kDa were noted by western blot of a concentrated, purified 5T4-TRX, which have also reacted to anti-His antibody (**Figure 3.12**, line 6). These most likely represented 5T4 lacking the TRX and linker residues/accessory sequences (~42 kDa). Since the protease inhibitors used in this study exhibit reversible binding to proteases, it is possible that they also re-gained activity during the 2hr DNAse treatment at 37°C, since it seems that after solubilisation, no further degradation occurred. Although some 5T4 protein was lost during the column load, most host proteins and fragmented 5T4 products passed through the Ni-IMAC column (**Figure 3.12**: FT lane). In conclusion, purification of full-length 5T4 protein required protease inhibitors and yielded ~7 mg of purified protein from 200 ml production cultures.

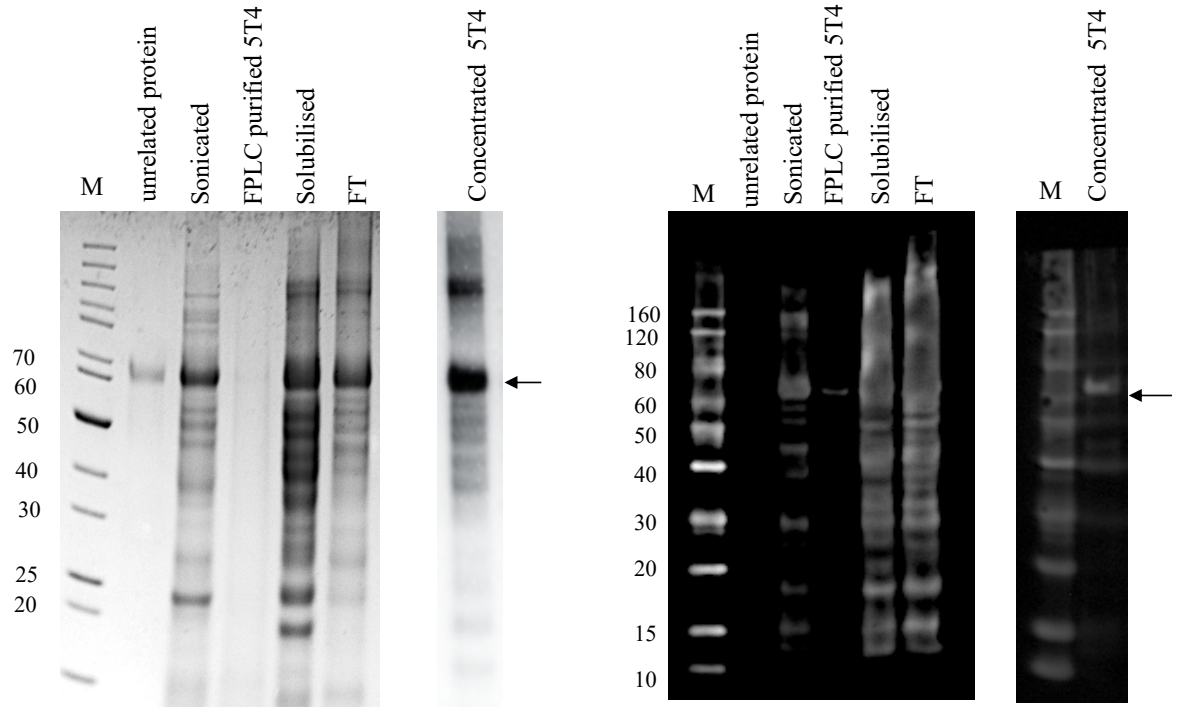


Figure 3.13. 5T4-TRX purification with protease inhibitors.

Inclusion bodies (IBs) were collected and resuspended in buffer containing protease inhibitor cocktail before being sonicated and processed as described in methods. 5T4-TRX contained within solubilised IBs was purified by nickel column FPLC. Purified fractions containing 5T4-TRX were pooled. Shown are Coomassie-stained SDS-PAGE and corresponding western blot of samples collected during different stages of purification. M- protein ladder/marker (kDa); *Lane 1* unrelated protein; *Lane 2* – sonicated pellets (SONI), insoluble fraction; *Lane 3* – pooled FPLC purified fractions containing 5T4-TRX. *Lane 4* – processed and solubilised IBs; *Lane 5*- FPLC flow-through (FT); *Lane 6*; concentrated 5T4-TRX.

3.4 Discussion

This chapter focused on the generation of purified tumour antigens to study tumour antigen processing by γδ T-APCs. The two human tumour antigens I chose were NY-ESO-1 and 5T4 because of their known immunogenicity, absence in healthy adult tissues, and current use in various cancer immunotherapy trials. In addition, my proposed studies with human γδ T-APCs require mg quantities of purified tumour antigens, which would be too costly if obtained from commercial sources.

Thus, this chapter describes improved protocols for high-level production of NY-ESO-1 and 5T4 in *E. coli* and their purification by chromatography. While cost-effective, *E. coli* offers many other advantages: it is a relatively simple procedure that allows more rapid optimisation and production of high yields of custom-designed proteins. However, it is essential to note that designing, expressing, and purifying target proteins is not always straightforward, even in a simple organism such as *E. coli*.

Although the production and purification of NY-ESO-1 were described before (Lowe *et al.*, 2011), this protocol was developed for large scale automated, bioreactor-driven NY-ESO-1 protein production at a GMP grade. While the report did not describe the production/purification of the NY-ESO-1 in detail, it was also not suitable for bench-scale protein production because specialised laboratory equipment is required throughout the procedure. In addition, several reports described purification protocols of full length, truncated wild-type and mutant 5T4 protein from mammalian cell systems (Hole and Stern, 1990; Zhao *et al.*, 2014). However, the production and purification of full-length 5T4 protein in *E. coli* or other expression systems have not yet been reported.

Here, I developed a practical protocol for bench-scale production and purification of high yields of recombinant NY-ESO-1, which was also implemented for 5T4 protein. NY-ESO-1 and 5T4 do not share similarities in biochemical or biophysical properties; however, they are both membrane proteins (Zhao *et al.*, no date; Schultz-Thater *et al.*, 2000). Despite potential challenges associated with the production of membrane proteins in *E. coli* by optimisation of protein expression and Ni-IMAC purification protocol, the total NY-ESO-1 protein yield was increased up to 28 mg/100 ml of bacteria culture with a purity of about ≥95% in a single chromatography step (**Figure 3.9**). On the other hand, the final yield of 5T4 was much lower, only ~2.7 mg/100 ml, with lower purity mainly due to the presence of fragmented 5T4 protein.

Both proteins were successfully expressed in *E. coli* Rosetta strain BL21(DE); however, the choice of expression vector played a pivotal role in the success rate. The expression of NY-ESO-1 was successful from the pGMT7 expression vector, and after optimisation of cultivation conditions, very high protein yields were achieved. On the other hand, expression of 5T4 from the pGMT7 vector failed, most likely due to its toxic effect on bacteria. The pGMT7 vector lacks the *lacI* gene and repressor binding site, which positively contributes to higher protein expression yields, but the basal expression of recombinant protein is not prevented. It is well known that the expression of membrane proteins leads to bacteria death, or protein degradation occurs instantly as a protective mechanism (Smith, 2011). Cell death is frequently observed for BL21(DE) strains, which also explains the observation of low-density cultures carrying pGMT7-5T4 plasmid. The selection of different mutant host C41 (DE) or C43(DE)

could have overcome this issue (Hattab *et al.*, no date; Miroux and Walker, 1996). Nevertheless, a successful 5T4 expression was achieved with the N-terminal fusion partner TRX from the pET32a vector. A strong influence of the fusion partner on protein solubility has been described repeatedly (LaVallie *et al.*, no date)(Hammarström *et al.*, 2002)(Peti and Page, 2007), but the initial attempt to produce soluble r5T4-TRX was unsuccessful even under cultivation in low temperatures and low IPTG concertation.

Several expression parameters were investigated to obtain sufficient amounts of recombinant proteins. In terms of altering intrinsic bacterial features to facilitate a higher yield of recombinant protein production, the supply of tRNA codons that are rarely used by *E. coli* improved the efficacy of total NY-ESO-1 protein production by 37%. To further increase NY-ESO-1 yield, several guidelines for producing very high yields of recombinant proteins in bacteria were followed (Sivashanmugam *et al.*, 2009). Numerous cultivation parameters were investigated, and all contributed to improved total NY-ESO-1 protein yield. The most vital improvement stage in NY-ESO-1 protein production was replacing the 2TY medium for TB medium, while the protein production phase was increased to 16 h at low temperature. This resulted in 4.1 fold increase in total NY-ESO-1 yield compared to the yields obtained from the initial protocol. In contrast, the optimal conditions for the highest 5T4-TRX production were achieved in the TB medium during the 5 h production phase. Temperature influenced the protein expression level under specific IPTG concentration; under lower temperature, higher IPTG concertation was required for increased protein expression, while lower IPTG concertation was sufficient under higher temperature.

NY-ESO-1 falls in the group of CTA antigens famous for their extreme hydrophobic nature, but for which crystal structures have not yet been reported. Due to its insolubility in aqueous solutions, challenging purification cannot be avoided. Therefore, it was no surprise to see NY-ESO-1 expressed in inclusion bodies. While 5T4 is a membrane protein, comprising of a secretion signal (residues 1–31), serine-rich motif (32–53), LRRs (60–344), a transmembrane region (356–376), and a short cytoplasmic tail (Zhao *et al.*, 2014); all together contributing to the protein's slightly acidic nature (pI= ~ 6.2). In contrast, insoluble 5T4-TRX expression was probably attributed to the bacterial defence mechanism protecting itself from the toxic effects of this membrane protein. The recombinant protein production in IBs has clear advantages in purification methods, allowing easy removal of the unwanted bacterial host protein. Removal of the host proteins with detergent/salt washes was more efficient for NY-ESO-1 IBs than for 5T4 IBs, where at least 80% purity of NY-ESO-1 was achieved at this stage.

The addition of a His-tag aids in the purification of a protein, and it does not impede with the use of the protein in downstream applications or even clinical trials (Gnjatic *et al.*, 2006). Approximately 60% of NY-ESO-1 was lost in the flow-through and washes from Ni-IMAC during the initial purification method. This loss was not due to exceeding the binding capacity of this column since the initial production method did not provide enough protein to over saturate the column since the column capacity exceeds 40 mg of protein/ ml of resin. Nevertheless, 10% loss was still noted even under an optimised buffering system that included reducing agents and denaturing co-solvents. The composition and conformation of protein dictate the binding affinity in Ni-IMAC. While the His-tag has been placed

at the highly hydrophobic C-terminal end of the protein, it could be speculated that was due to the Hi-tag being "hidden", preventing efficient binding. This is to some extent supported by the observation that a buffer with increased reducing-denaturing strength enhanced the protein binding to the column, and less protein was detected in the flow-through. The binding was greatly improved with at least 6 M urea, while 5 mM β-Mercaptoethanol was essential to avoid the formation of multimers; however, it did not prevent it entirely. Since NY-ESO-1 consists of a glycine-rich N-terminal region, placing a His-tag at the N-terminus instead could potentially improve binding to the nickel column. However, the same phenomenon has been reported for several CTAs including in a previous report describing the generation of GMP grade NY-ESO-1, where the authors decided to place the His-tag at the N-terminus (Lowe *et al.*, 2011). Keeping the protein soluble throughout the purification has been challenging. Even though stringent denaturing conditions (8 M urea buffer) were utilised, it may be that the protein was still aggregating during the Ni-IMAC process. Finally, optimising purification protocol based on initial detergent/chaotropic conditions for IBs processing with an improved buffering system for NI-IMAC increased the total NY-ESO-1 yield to up to 28 mg/100 ml with a purity of about ≥95% in a single chromatography step. Crucial to the improved purity was also the addition of high ionic wash step and extensive low imidazole washes during Ni-IMAC purification. The removal of the remaining impurities was practically impossible in previously reported publication, even with specialised laboratory equipment (Lowe *et al.*, 2011).

In contrast, purification of 5T4-TRX under identical conditions as for NY-ESO-1 was not successful. The biggest bottleneck represented prevention of the proteolytic attack and degradation of the recombinant protein. Even though the Rosetta stain is deficient in lon and ompT proteases, which are known to participate in physiological disintegration of inclusion bodies and can be active even in highly denaturing environments (White *et al.*, 1995; Vera *et al.*, 2005), fragmentation of the 5T4-TRX protein occurred. However, the implementation of protease inhibitors during the initial purification steps did not prevent the reoccurrence during the IBs processing steps, and the final purity of full-length 5T4-TRX was less than 50%. Several lines of evidence indicate that protein aggregation as IBs does not represent complete protection from cell proteases. It has been shown that while protein is removed or loosened from IBs, the insoluble polypeptides in loose aggregates are sensitive to site-limited digestion (Corchero *et al.*, 1997). This may explain why 5T4-TRX protein degradation was observed during the initial steps of the IB processing. While the protease inhibitors used here do not bind irreversibly to their targets, their implementation during the initial purification steps was not sufficient to prevent their reoccurrence and fragmentation of 5T4-TRX. To circumvent the proteolytic degradation throughout the 5T4-TRX protein purification, further modification of the protocol is needed. Implementing instant stringent denaturing conditions during cell lysis and incorporating protease inhibitors throughout the purification steps may allow for greater final purity of the full-length 5T4-TRX protein. Nevertheless, the biggest worry lies in the observation that in partially soluble IB-forming proteins, proteolysis can take place during the transit of recombinant protein between soluble and insoluble cell fractions (Carrió, Corchero and Villaverde, 1999), where a cascade of proteolysis has led to complete protein digestion, and some degradation intermediates, accounting for more than 50% the total recombinant protein.

As for every other protein, the optimal purification conditions depend on many factors and need to be adjusted following more in-depth experiments than those I was able to conduct within the scope of this chapter.

In summary, I have implemented an *E. coli* based expression system for high yield production and purification of NY-ESO-1 providing sufficient amounts for studying antigen (cross) presentation in professional APCs. The protocol for 5T4 production and purification has also been developed; however, the method described requires further tailoring to circumvent proteolytic degradation of the 5T4 at the early stage of purification. Nevertheless, these studies gave me enough 5T4 material to continue with my studies.

Chapter 4
Generation of tumour-antigen specific CD8 T cells from preripreal blood

4.1 Introduction

The generation of peptide-specific T cells is not only important for adoptive T cell therapies, but it provides useful experimental tools to address research hypotheses. Generally, the generation of T cell clones is favourable for TCR structural studies and studies examining T cell activation as it eliminates the uncertainties associated with polyclonal T cell populations. Nevertheless, tumour-specific T cell isolation is often challenging and time-consuming. Furthermore, many factors can influence a successful generation of a T cell clone: sample size, clonotype frequency in peripheral blood, and culture conditions.

One of the great challenges in identifying, isolating and expanding tumour peptide-specific CD8 T-cells is that not only do these cells occur at naturally low frequencies in PBMC but also a stronger antigen stimulus is required to invoke detectable responses and expansion. In contrast, even at low peptide concentrations of foreign (pathogenic) origin, robust T cell responses and rapid proliferation can be achieved, thanks to the presence of memory T cells and highly responsive naïve T cells (Wang *et al.*, 2001; Wong and Pamer, 2003; Zehn, Lee and Bevan, 2009; Schmidt and Varga, 2018). Thus, regardless of their low frequency in the peripheral blood of healthy individuals, expansion of Flu Matrix protein-specific CD8 T-cells by the peptide-stimulation method is relatively easy (M. Brandes *et al.*, 2009; Meuter, Eberl and Moser, 2010). The ideal method for expanding antigen-specific T cells should be brief and involve minimal manipulation of the cells. Many T cell cloning methods involve several peptide-stimulation strategies of the bulk CD8 T cells to obtain cell libraries consisting of a noticeable percentage of tumour antigen-specific cell populations prior to cloning. For instance, peptide-pulsed mo-DCs or PBMC have frequently been used to stimulate unselected T-cells to expand tiny populations of antigen-specific T cells *in vitro* (Croft *et al.*, 1994; Brodie *et al.*, 1999; Fonteneau *et al.*, 2001; O'Beirne, Farzaneh and Harrison, 2010; Pufnock *et al.*, 2011; Pollack *et al.*, 2014).

Whilst these procedures facilitate easier identification and isolation of rare T cells of interest, prior *in vitro* culture can have many undesirable effects. Phenotype and function of expanded cells can be affected by choice of cytokine combination, source of antigen and antigen dose - all of which can promote selective out-growth of particular T cell subpopulations (M A Alexander-Miller *et al.*, 1996; Geginat, Sallusto and Lanzavecchia, 2003; Leggatt *et al.*, 2004; Kim *et al.*, 2006; Zehn, Lee and Bevan, 2009). Likewise, soluble or bead coated monoclonal antibodies that bind the TCR and co-stimulatory molecules such as CD3/CD28 and CD49 expressed by T cells have been used to mimic the presence of DCs. However, these approaches are often time-consuming and require ample amounts of donor material, while repeated exposure to peptide and extended *in vitro* culture can often lead to terminal differentiation and exhaustion of T cells. It has been demonstrated that the use of human serum can reduce the alterations in TCRVβ expression in expanded T cells and, in addition to IL-2, can help improve the T-cell expansion efficiency *in vitro* (Block et al 2008). Other approaches involve non-

specific expansion of bulk T cells using CD3/CD28 beads which are then enriched by the INFγ– capture technique prior to cloning of peptide reactive T cells (Theaker *et al.*, 2016). To avoid prior expansion of T cells, Neller and colleagues developed a method for direct cloning of effector T cells by capturing cytokine-secreting cells after short peptide stimulation of PBMC (Neller *et al.*, 2014). Both methods, however, have to deal with some well-recognised problems with the cytokine capture technique, such as enrichment of contaminants.

Furthermore, a great challenge represents the detection/isolation of T cells specific for self-antigens due to their poor responsiveness/ low INFγ production in short-term stimulations and low peptide concentration. Since self-antigen specific responders in healthy and young individuals are found predominantly in the naïve pool, and whilst direct cloning relies on effector function, it is unsuitable for expanding naïve precursors. More recently, the cytokines IL-15, IL-7 and IL-21 have gained considerable interest for their potent effect on the activation and expansion of cytotoxic CD8 T cells. In addition, the use of pMHC multimers has been shown useful in both strategies, when peptide-specific T cells are enriched prior to cloning (Pollack *et al.*, 2014), and when sorted peptide-specific T cells are cloned directly from PBMC (Dunbar *et al.*, 1999; Yee *et al.*, 1999). However, low T cell frequency, low-affinity TCRs, and *in vivo* activation-induced downregulation of TCR can constrain successful T cell isolation via multimer staining. However, a couple of reports described helpful advances for tackling such limitations (Wooldridge *et al.*, 2009; Tungatt *et al.*, 2015; Rius *et al.*, 2018).

In this chapter, I explored methods for expanding 5T4 and NY-ESO-1 tumour peptide-specific CD8 T cells. Finally, this chapter describes an improved method for rapid expansion of a large number of 5T4 specific CD8 T cell lines and clones originating from low-frequency T cell populations in healthy individuals in as little as four to six weeks.

Aims

The overall aim of this chapter was to generate 5T4 and NY-ESO-1 specific CD8 T cells reactive to peptides 5T4p$_{17\text{-}25}$ (RLARLALVL), 5T4p$_{97\text{-}105}$ (FLTGNQLAV), NY-ESO-1p$_{157\text{-}165}$ (SLLMWITQC), and NY-ESO-1p$_{159\text{-}167}$ (LMWITQCFL) from HLA-A2+ donors, donors for their subsequent use as a tool to measure successful antigen cross-presentation function of γδ-T-APCs (Chapter 5). In order to achieve this in the shortest time, different approaches to T cell clone and T cell line production were first investigated and assessed; conventional methods utilising TCR specific and non-nonspecific stimulations of whole PBMC or bulk CD8 T cells. Then, a strategy involving pMHC tetramers and fluorescence and/or magnetic-activated T cell sorting (FACS or MACS) was established to simplify and speed up the methodology.

.

4.2 Generation of 5T4 antigen specific CD8 T cells by cell-sorter assisted T cell library approach

A literature search was performed to find methodologies with proven multiple successes in generating tumour antigen-specific CD8 T cells. A method involving a cell sorter was adopted from Prof Edus H. Warren lab at the University of Washington, Seattle (Warren, Greenberg and Riddell, 1998; Tykodi *et al.*, 2012; Neller *et al.*, 2014; Pollack *et al.*, 2014) and optimised to facilitate relatively fast generation of large number of functional tumour antigen specific CD8 T cells.

Several factors had to be optimised to develop the optimal method for generating large numbers of tumour antigen-responsive CD8 T cells for their subsequent use in APC assays in Chapter 5. Owing to the thymic central tolerance mechanism that regulates peripheral auto-reactive T cells, tumour-reactive T cells are found at a naturally low frequency in peripheral blood (Kelin *et al* 2014, Sharpe and mount 2015). Due to a very low number of tumour peptide-specific CD8 T cells found in the PBMC of healthy individuals, the conventional small-scale optimisation approach was not feasible for finding the optimal culture conditions. Thus, with one pair of hands, finding the optimal method and culture conditions was only possible by changing one factor at the time. By applying the one-factor-at the time experimental design, four factors were optimised and are described as seperate methods. Within these, stimulation type, timing, concentration and combination of some common γ chain cytokines assisting the optimal expansion of the selected tumour antigen-specific CD8 T cells were explored. Selected cytokines, the frequency of cytokine administration, and their concentrations were explored according to previously published data. It should be noted that combinations of specific cytokines used, and their relevant concentrations were not optimised in detail here due to the extensive study in the literature describing the effective combinations. The CD8 responders specific for influenza matrix protein 1 (FluM1) were generated in parallel since these should be present in each individual's CD8 T cell memory pool, thus serving as a positive control.

4.2.1. Generation of peptide-specific CD8 T cell clones from pMHC-enriched libraries by peptide stimulation

The first strategy for the generation of CD8 T cells is illustrated in **Figure 4.1**. Briefly, CD8 T cells were first isolated using anti-CD8 magnetic beads (Miltenyi), and then M158-, 5T4p17 - and 5T4p97-peptide-specific CD8 T cells were sorted using peptide-MHC tetramers (pMHC) and fluorescence-activated cell sorting (FACS). Sorted cells were seeded in 96U plates and stimulated with peptide-pulsed autologous irradiated PBMC (irPBMC) or with HLA-matched irPBMC, and allowed to expand for 2-3 weeks in the presence of IL-2 and IL-15, while re-stimulation was carried out every two weeks or as needed. Practically, this strategy would provide easier identification/visualisation of growing peptide-specific CD8 T cells of interest using light microscopy (Neller *et al.*, 2014), and thus reduce the number of wells to be screened by pMHC-tetramer and flow cytometry. When clear pMHC positive cell populations are identified, these cells are then FACS sorted and cloned at the single-cell level or expanded as enriched cell lines. Monitoring and expansion of CD8 T cells are then carried out until

sufficient cell numbers are achieved, at which point functional analyses are performed using ELISpot assay or IFNγ- intracellular cytokine staining (IFNγ-ICS) assay upon peptide challenge. The functional cells are then further expanded phenotyped.

Serum affects cell expansion and proliferation through various factors, such as providing growth factors, cytokines, vitamins and small-molecule metabolic intermediates, antioxidants and toxin neutralisers, carrier molecules and enzyme modifiers. On the other hand, human T cells cultured in foetal calf serum (FCS) can be primed by xenogeneic antigenic epitopes to HLA class I- and HLA class II-restricted FCS-specific T cells. A previous report has shown that in the presence of autologous irPBMC, human serum (HS) supplemented culture medium is superior to medium FCS regarding T-cell number and TRBV repertoire in long-term cultures (Block *et al.*, 2008). Thus, the two were compared in separate experiments using T cells from the same. Briefly, isolated peptide-specific CD8 T cells were seeded at 20-50 cells per well together with autologous irradiated PBMC pulsed with both 5T4 tumour peptides (5T4p17 and 5T4p97) or with M158 peptide alone (Table 4.1). Cells were then cultured in priming medium in the first week, followed by culturing in expansion medium in following weeks, and were re-stimulated until pMCH-tetramer-positive cells were identified. When the FSC-supplemented medium was used, two rounds of stimulation resulted in 32/192, 16/192 and 4/96 growing wells for cells stimulated with M1p58, 5T4p17 or 5T4p97, respectively. Of those, 3/32 growing wells scored >1% TET+ for M1p58 (>95%, ~5.6% and 3.2%), while all growing wells scored <0.7% TET+ for 5T4p17, and <0.5% TET+ for 5T4p97 (Table 4.1). In contrast, when cells were expanded in HS supplemented medium, all growing wells identified by microscope also scored positive for M1p58-TET; of those 21 wells were >83% TET+. While only 3/63 growing wells scored 1 - 4.6% TET+ for 5T4p17, all 30 growing wells scored <0.8% TET+ for 5T4p97 peptide (Table 4.1). Thus, these data suggest cultures may have consisted of contaminating, unspecific CD8 T cells where the FCS supplemented medium readily contributed to selection bias in expanded T-cell populations. Although the experiment was not repeated to achieve statistical significance, the HS supplemented medium greatly contributed to expanding peptide-specific CD8 T cells.

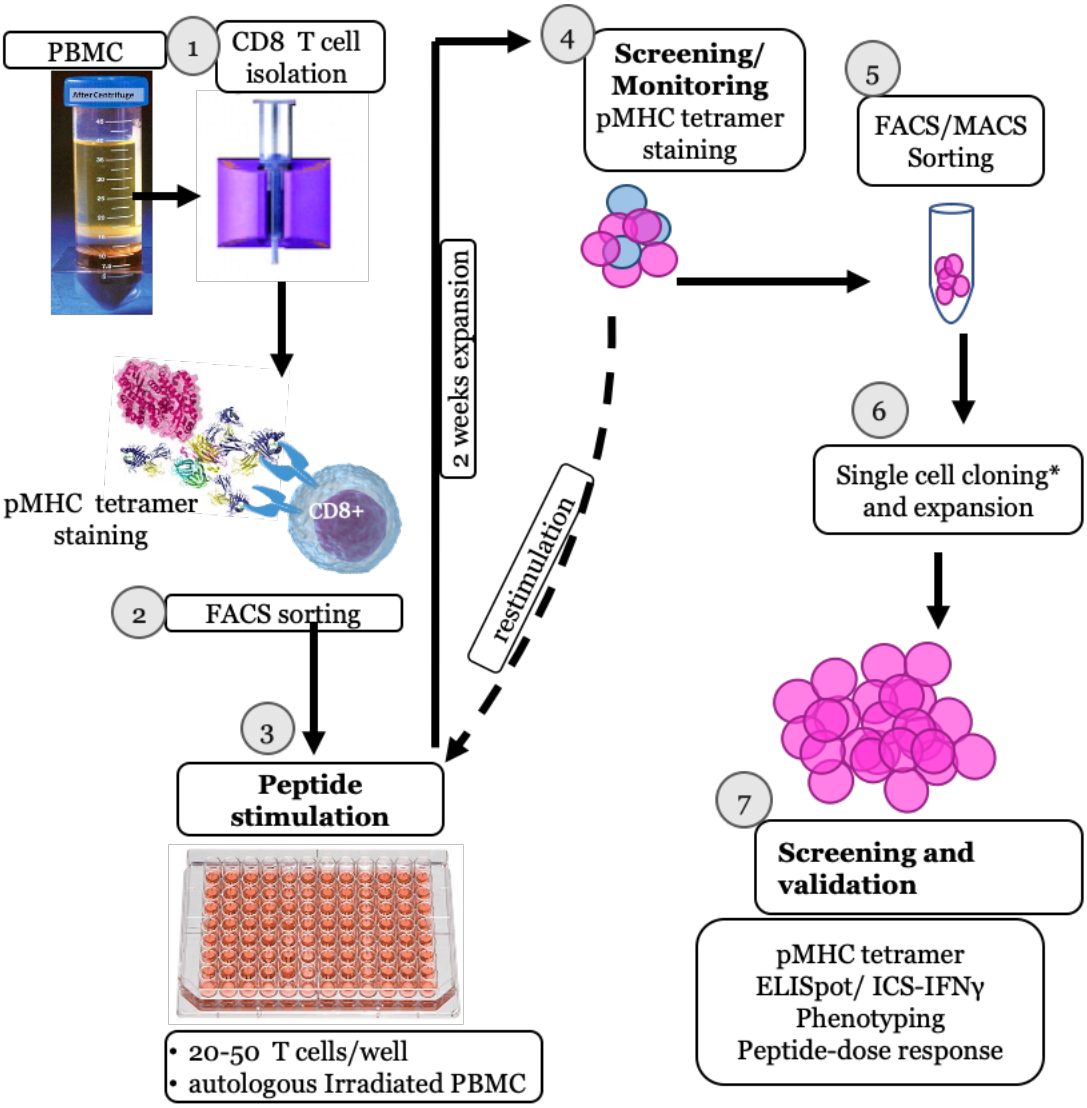


Figure 4.1. Generation of tumour peptide-specific CD8 T cell clones from pMHC-enriched libraries and peptide stimulation.

The procedure for CD8 T cell isolation, peptide-specific T cell enrichment, single-cell cloning, clone maintenance and characterization is outlined. (1) CD8 T cells were first isolated from PBMC, using negative selection magnetic microbeads (MACS), and (2) peptide-MHC (pMHC) tetramers were used to identify and enrich peptide-specific CD8 cells. (3) FACS enriched cells were seeded into 96U-well plates (20-50 cells per well) with peptide-pulsed - irradiated (35 Gγ) autologous PBMC. After two weeks of expansion in the presence of cytokines, (4) T cell libraries were screened by pMHC tetramer staining for the presence of peptide-specific CD8 T cells. (5) Identified pMHC positive cell libraries are then pooled, and FACS sorted using pMHC tetramers. Alternatively, cells are re-stimulated until pMHC positive cells are identified. (6) Enriched T cells are subject to single-cell cloning by dilution in 96U-well plates or are expanded as enriched cell lines. (7) Peptide specific CD8 T-cells cells are identified by pMHC tetramer staining, and when enough cells are obtained, functional analyses are performed by ELISpot assay or IFNγ intracellular cytokine staining assay (ICS-IFNγ) and a peptide dose-response assay. Selected functional clones are further expanded and phenotyped as part of the validation process.

Table 4.1. Composition and efficiency of peptide-stimulation based method for generation of MHC restricted CD8 T cells

donor (age)	**donor #3(60+)**	
method	FACS sorting + peptide stimulation	
frequency among CD8 T cells	**M1p58= 0.3%** **5T4p17 = 0.73%** **5T4p97 = 0.45%**	
serum	10% FSC	5% HS
peptide [µM]	5 (5T4); 1 (M1p58)	1
cytokines;	week 1: priming medium week 2(+): expansion medium	week 1: priming medium week 2(+): expansion medium
sorted cells	M1= 12532 p17 = 11178 p97 = 5469	M1= 1806 p17 = 1990 p97 = 1699
# cells/ well	~50-100	~20
# rounds stimulations	2	2
visualised growing wells	M1= 32/192 p17 = 16/192 p97 = 4/96	M1= 63/96 p17 = 30/96 p97 =30/96
M1p58 CD8/TET+ (%)	95.6; 3.24; 5.69	21 wells: 83-99 4 wells: 50-75 5 wells : 1-23
5T4p17 CD8/TET+ (%)	< 0.7	0.3 - 4.6
5T4p97 CD8/TET+ (%)	< 0.5	0.04 - 0.78

Since this chapter aimed to generate tumour peptide-specific cells in a relatively short period, while a low percentage of tumour peptide specific T cells were identified after two rounds of peptide stimulation, it was concluded that a sufficient number of clones would not be obtained promptly. In addition, as persisting antigen stimulations drives cells to exhaustion and terminal differentiated phenotype (Wölfl *et al.*, 2011), these cells could have proven unsuitable for downstream functional studies. Thus, the proceeding of this method was terminated.

4.2.2 Generation of tumour peptide-specific CD8 T cells from pMHC-enriched libraries by non-specific stimulation

Our successful strategy for relatively fast production of tumour antigen reactive CD8 T cells (CD8 responder cells) from healthy donors is illustrated in **Figure 4.2**. It involves isolation of "untouched" CD8 T cells from PBMC by negative selection using magnetic anti-CD8-microbeads, which are then stained with pMHC tetramers to facilitate the enrichment of tumour peptide-specific cells by FACS, or by magnetic cell sorting (MACS) using magnetic anti-PE beads (Miltenyi) directed to PE-conjugated pMHC complex. This isolation of peptide-specific CD8 T cells removes unwanted cells that can compete for media components and regulatory CD4+ Treg cells. Then, the enriched cells undergo one round of unspecific stimulation with PHA in the presence of irradiated allogeneic "feeder cells" comprising PBMC from three donors and Epstein-Barr virus-transformed B cells (EBV-LCLs), and γ-chain cytokines. The PHA providing non-specific signals for amplifying T cells offers improvements over the basic T cell line generation, whereby repeated peptide stimulation can lead to faster T cell exhaustion-dysfunction. Although the exact mechanism of PHA on T cell activation and proliferation has not been resolved, PHA exerts its action by binding to complex branched glycans on glycoproteins containing at least one N-acetylglucosamine (GlcNAc) (Lindahl-Kiessling, 1972; Cummings and Kornfeld, 1982; Hammarström *et al.*, 1982).

That means PHA binds and cross-links multiple cell surface receptors and molecules not only on T cells (such as CD25, CD28, TCR etc.) but also on innate cells (such as PRRs) and activates them. The presence of innate cells is essential for imposing its full activating potential on T cells (Epstein, Cline and Merigan, 1971) together providing all three key activation signals to the T cells (Pereira *et al.*, 2018). So, in our method, the first amplification occurs in 96U-well plates with a relatively limited number of cells (3-15 per well), introducing a degree of clonality from the start of the culture. Moreover, using human serum reduces the alterations in TRBV expression during the initial priming stages and of the expanded T cells and helps improve the T-cell expansion efficiency *in vitro* (Block *et al* 2008, **Table 4.1**). Following two weeks of expansion, T cell colonies are screened by flow cytometry using pMCH tetramers (TETs). Positive T cell colonies were then pooled and sorted as before and subject to single-cell cloning (at 0.3 - 3 cells/well) and expanded as before. Around two weeks later, T cells are monitored for clonality using pMHC multimers. At this point, wells containing identified TET positive cells are further amplified prior to screening with cognate antigen via IFNγ- ELISpot and/or IFNγ- intracellular staining assay (ICS) to identify responding cells. Peptide reactive T cells were then subjected to peptide dose-response analysis. Re-stimulation/expansion was carried out every two weeks as needed to obtain the desired number of cells. Alternatively, pMHC-tetramer sorted T cells can be expanded as enriched cell lines to generate a large number of cells. Below are described three variants of this strategy (method # 1, #2, and #3) that differ in cytokine supplementation..

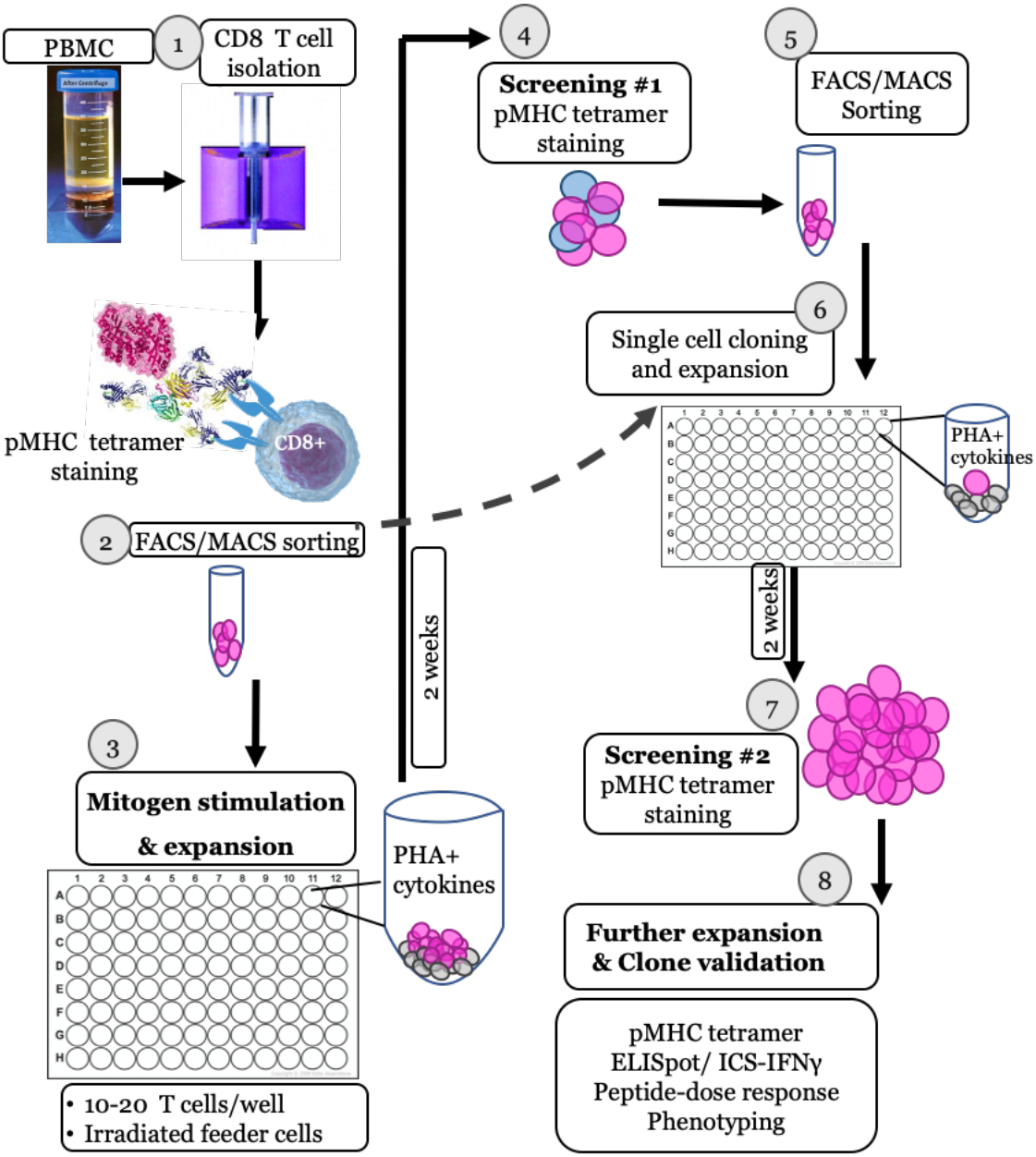


Figure 4.2. Generation of tumour peptide-specific CD8 T cell clones from pMHC-enriched libraries by non-specific stimulation.

The procedure for CD8 T cell isolation, peptide-specific T cell enrichment, single-cell cloning, clone maintenance and characterization is outlined. (1) CD8 T cells are first isolated from PBMC, using negative selection magnetic microbeads (MACS), and (2) pMHC tetramers are used to identify and enrich peptide-specific CD8 cells. (3) FACS enriched cells are seeded into 96U-well plates (500–1500 cells per plate) with irradiated (35 Gγ) allogenic PBMC from three donors plus irradiated (80 Gγ) EBV-LCLs as 'feeder cells', stimulated with PHA (1 µg/mL) and incubated in the presence of cytokines for two weeks. (4) Cell libraries are screened by pMHC tetramer staining and/or ELISpot for the presence of peptide-specific T cells. (5) Identified pMHC positive cells/peptide reactive cell libraries are pooled, and FACS/MACS sorted using pMHC tetramers. (6) Enriched T cells are subject to single-cell cloning by dilution on 96U-well plates and are expanded as before or are expanded as a line. (7) Two weeks later, peptide-specific T-cell clones are identified by pMHC tetramer staining. (8) Selected clones are functionally assessed by intracellular cytokine staining assay (ICS-IFNγ) or ELISpot, Functional clones are further expanded, and a peptide dose-response is used as part of the clone validation process.

4.2.2.1 Generation of 5T4 specific CD8 T cells: Method #1

Usually, limited cell availability of tumour peptide-specific C8 T cells is a critical obstacle in identifying and isolating these from healthy donor PBMC. To our surprise, donor #2 peripheral CD8 T cells consisted of 1% of 5T4p17 -tetramer-positive cells, which was as many as FluM1 specific cells, while there were only 0.1% 5T4p97-tetramer-positive cells (**Figure 4.3 A**). The strategy described in **Figure 4.2** was followed. These cells were sorted to establish CD8 T cell libraries of 10-14 cells per well on 96U plates, consisting of either M1p58 CD8/TET+ cells or a mixture of both 5T4p17 and 5T4p97 CD8/TET+ cells. Cells were stimulated with PHA and cultured in the presence of irradiated pooled PBMC from three donors plus irradiated Epstein-Barr virus-transformed B cells (EBV-LCLs), hereafter collectively called irradiated feeder cells (or-feeders). In the initial two weeks, cells were cultured in a priming medium in the first week and an expansion medium in the second week.

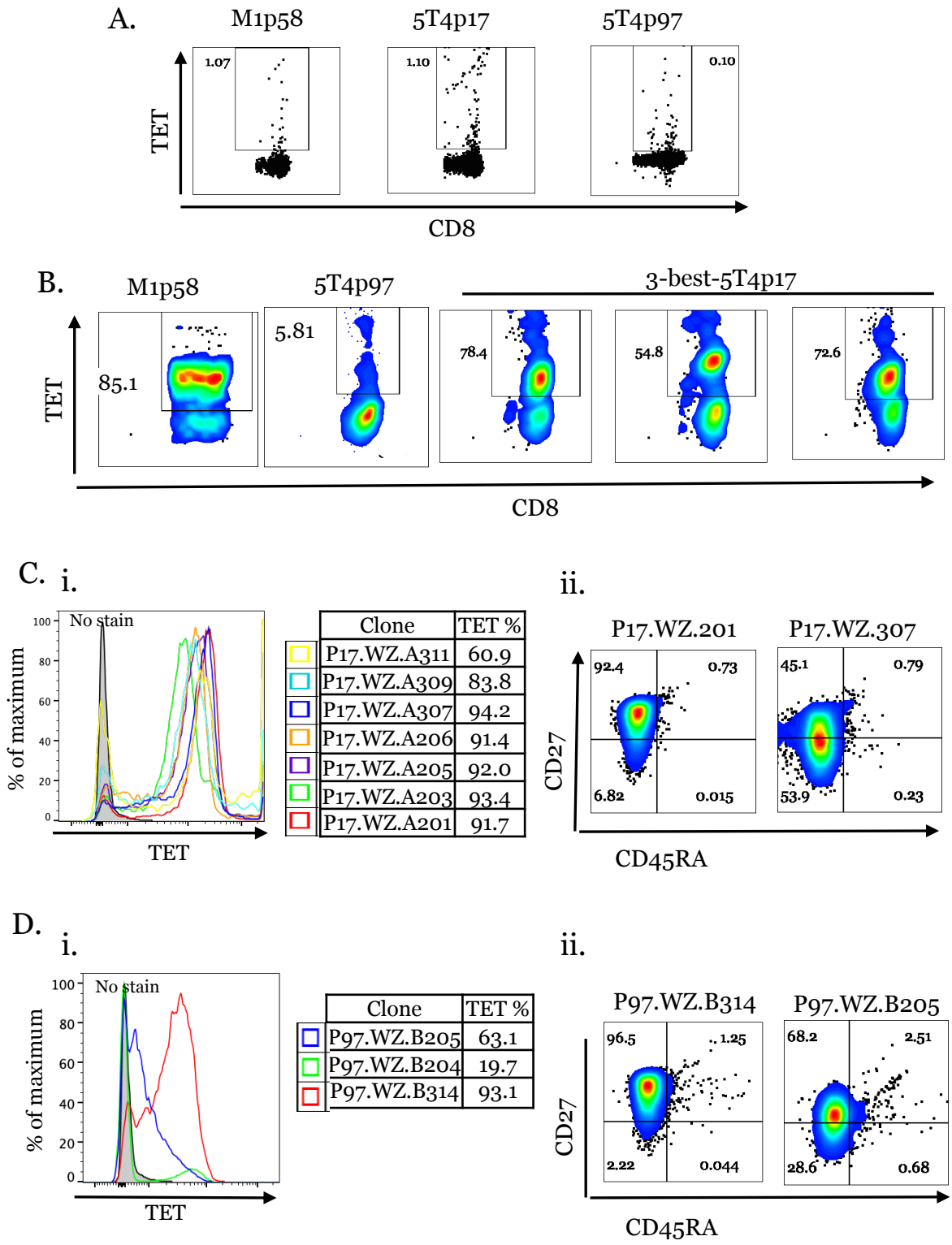


Figure 4.3. Generation of 5T4 -antigen specific CD8 T cell clones from pMHC-enriched libraries and non-specific stimulation: method #1.

The procedure for CD8 T cell isolation, pMHC-tetramer T cell enrichment, single-cell cloning, maintenance and characterization was followed as described in figure 4.2. (**A**) Frequencies of CD8 T cells specific for peptides $5T4p_{17\text{-}25}$ (5T4p17), $5T4p_{97\text{-}105}$ (5T4p97) and $FluM1p_{58\text{-}66}$ (M1p58) in PBMC in a heathy donor are shown. Cells were gated on single, live, CD3, CD8 positive cells. (**B**) CD8 T cell library consisting of a mix of both 5T4 peptide specific cells was initiated in 96 wells at 10-14 cells per well, and a separate CD8 T cell

library was generated for M1p58 specific cells. The first screening of expanded CD8 T cells was carried out by pMHC-tetramer (TET) staining. Shown is one representative well per peptide-library containing pMHC-TET positive cells after the first round of non-specific stimulation. Positive wells that showed a clear CD8/TET+ population were pooled and were FACS sorted. Three wells showed a high proportion of 5T4p17 –specific cells (3Hi-5T41p7) and were sorted as a separate pool. (**C**) (i) Four 5T4p17 –specific CD8 T cell clones were generated via single-cell cloning with similar pMCH tetramer intensities and purities. (ii) Memory phenotype of expanded clones examined by CD45RA and CD27 expression. Three clones showed similar $CD45RA^{-}/CD27+$ profile (T_{CM}-like phenotype); thus a representative plot of one clone (P17.WZ.201) is shown. Clone P17.WZ.307 showed $CD45RA^{-}/CD27^{LO/-}$ (T_{CM}/T_{EM} phenotype). Gated on single, live, CD8+, TET+ cells. (**D**) (i) Three 5T4p97 –specific clones with variable pMHC-tetramer intensities and purities were generated via single-cell cloning. (ii) Two clones (p97.wz.B314, p97.wz.B204) showed similar CD45RA-/CD27+ expression, and one clone (p97.wz.B205) showed $CD45RA^{-}/CD27^{LO}$ profile (ii).

So, after an initial two weeks of cell expansion, 38/96 positive libraries were identified for M1p58 CD8/TET, and 17/96 positive libraries for 5T4p17 and 5T4p97 CD8/TET positive cells. The frequency of CD8/TET+ cells varied among cell libraries (Table 4.3, **Figure 4.3**). One of the M1p58 specific CD8 cell libraries already represented a TET purity of 85% after the first stimulation (**Figure 4.3 B**). This particular cell library was enriched again and expanded as a cell line, and after three stimulations represented 99% of M1p58 CD8/TET+ cells (**Figure 4.4 A**). This signifies the robustness of CD8 T cell responses specific for antigens of pathogenic origin. The libraries positive for 5T4p17 showed 3 - 80% TET+ cells, while libraries positive for 5T4p97 represented only up to 10% of TET+ cells (**Figure 4. 3 B**, Table 4.2). The libraries with the highest CD8/TET+% score were pooled and subjected to FACS sorting. Three libraries scoring >50% 5T4p17 -tetramer staining were pooled and sorted as a separate pool (called "3Hi-5T4p17 cell pool," hereafter) (**Figure 4.3 B**), and the remaining 5T4p17-TET positive libraries were sorted as a separate second pool ("#2 - 5T4p17 cell pool"). Sorted cells were then cloned at a single cell level by dilution and expanded as before but received the cytokines every 3-4 days.

In two weeks of clonal expansion, cells did not proliferate well and gave only ~ 10 - 30,000 cells per clone; thus, all cells underwent another round of stimulation and expansion as before. By week 8, clones expanded only about 2-3-fold, as most cells were dying (5 -20% live cells) (data not shown). 4/576 growing clones were identified from the 3-best-5T4p17 pool, 15/576 clones from the second 5T4p17 pool, and 13/576 growing wells from the 5T4p97 pool. However, only seven 5T4p17-clones and five 5T4p97 clones produced enough cells for analysis; none of the 3-best-5T4p17 clones expanded sufficiently. All 5T4p17 clones presented 60-94% tetramer positive cells (**Figure 4.3 C**), while of 5T4p97-clones 3 clones showed 19% -93% tetramer positive cells (**Figure 4.3 C**). Table 4.2 displays details of the cloning method and details of individual clones. It was previously demonstrated that naive, memory, and effector cells CD8 T cells can be identified by surface expression of either CD27 or CD28 and CD45RA marker using flow cytometry (Tomiyama *et al.*, 2004; Takata and Takiguchi, 2006). All 5T4p17 -specific and 5T4p97-specific clones showed $CD45RA^{neg}$ phenotype while CD27 varied among clones (**Figure 4.3 C and D**). No other markers (such as CCR7, CD62L, CD95, CCR4) (Sallusto *et al.*, 1999; Geginat, Lanzavecchia and Sallusto, 2003; Romero *et al.*, 2007) were included in this analysis; thus, it was not possible to determine the precise phenotype. These clones were cultured in

priming medium in the first week, and in expansion medium during the following weeks, both containing IL-2 and IL-15, and the cultures were re-stimulated fortnightly, yet under these culture conditions, our 5T4 specific T clones did not expand well, and insufficient numbers of cells were obtained even after 12 weeks. Due to too low cell numbers, further functional analysis (cytokine production upon peptide challenge) was not possible. With CD27 being expressed, these cells would be expected to possess proliferative capacity, yet testing CD28 expression would add another layer to understanding cells' proliferative potential. More frequent administration and higher concentrations of cytokines could potentially yield a larger number of cells. Notwithstanding, Neller *et al.* reported the generation of a large number of T cell clones specific for CMV, LCL and melanoma peptides, using only half the concentration of IL-2 while no IL-15 was administered in their expansions (Neller *et al.*, 2014). However, the report did not disclose the number of melanoma-specific clones generated and the time required to achieve such expansion.

On the other hand, it should also be noted that the frequency and quality of CD8 T cells specific for MELAN-A in PBMC is about 10-fold higher than of any other tumour-associated antigens (Troutt and Kelso, 1992; Pittet *et al.*, 1999). Thus, even with a lower concentration of cytokines, it is not surprising that Neller and colleagues had better success in the expansion of MELAN-A specific T cells than I did of 5T4-specific T cells. As the lifespan of human T lymphocytes is limited, T cells can only proliferate for a limited number of cell divisions *in vitro*, after which they stop proliferating and senesce. Another explanation for the low number of clones generated from this donor is that these cells may have come from a more differentiated compartment and thus had minimal replicative potential. In general, T lymphocytes cannot be expanded beyond 20-30 population doublings, and in such cases, further subcloning usually fails or yields subclones with poor expansion potential. On the other hand, seeing that CD27 expression was still present on some of these T cell clones suggests that these cells require a higher concentration of cytokines for optimal expansion.

In parallel to establishing 5T4 specific clones, I generated enriched cell lines as described in **Figure 4.2**. Two cell lines specific for 5T4p17 (p17.wz.3B-LN1 and p17.wz.LN2) were generated from "left-over" sorted T cells (not used in cloning), either from (1) the 3-best-5T4p17 cell pool or (2) the second 5T4p17 cell pool. These two cell lines scored 75.5% and 89% TET+ respectively. The cell line generated from 5T4p97-pool showed 32.6% tetramer positivity (p17.wz.3B. ln) (**Figure 4.4 A**; Table 4.3). Six weeks after initial stimulation, 8 million cells of p17.wz.3B-LN, 22 million of p17.wz.LN2 and 12 million of p97.wz.LN were obtained. The p17.wz.3B-LN cell line was mostly $CD45RA^{neg}/CD27^{neg}$, while p17.wz,LN2 line and p97.wz.LN predominately showed $CD45RA^{neg}/CD27^{lo}$ phenotype. As mentioned before cells specific for M1p58 peptide showed TET purities of 99% and $CD45RA^{neg}/CD27^{neg}$ phenotype, yet these cells expanded to 100 million in six weeks (**Figure 4.4 C**, not shown).

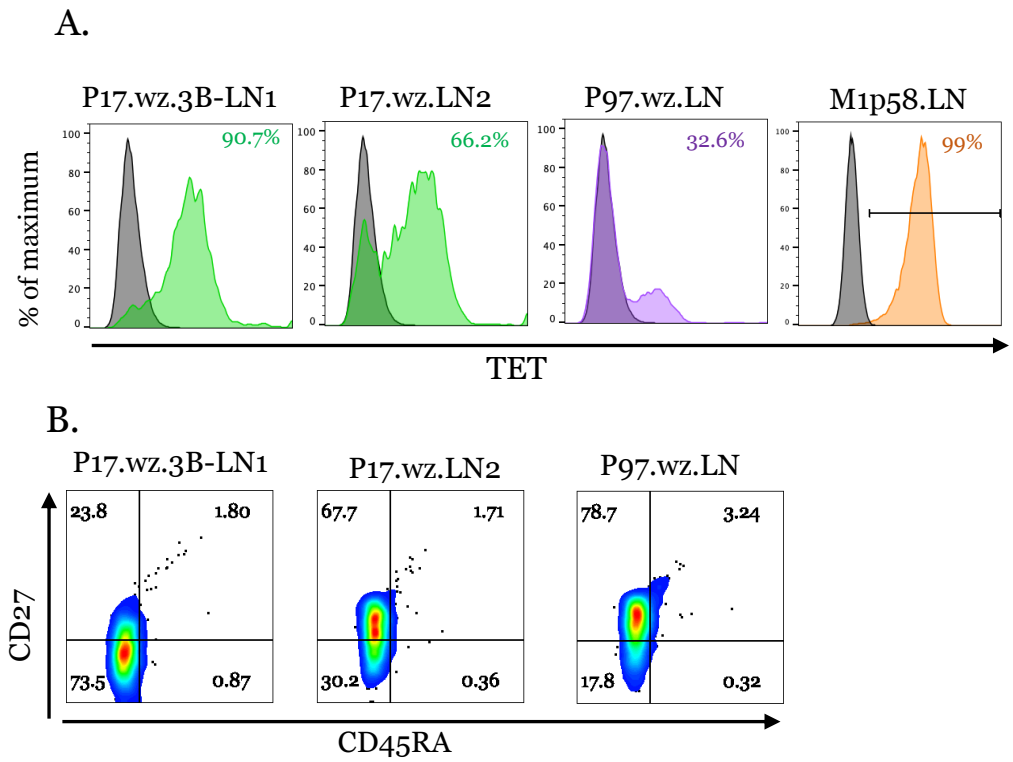


Figure 4.4. Characterisation of 5T4- and FluM1 specific CD8 T cell lines from pMHC-enriched libraries generated by method #1.

The procedure for CD8 T cell isolation, peptide-specific T cell enrichment, and expansion as a cell line was followed by the strategy described in figure 4.2. Initial CD8 T cell libraries consisting of 96 wells of enriched 5T4 peptide-specific cells or a library consisting of Influenza matrix protein 1 (FLuM1) peptide-specific cells were generated from a healthy donor. After the first expansion, wells consisting of pMHC- CD8-TET positive cells were pooled, enriched by FACS sorting, and expanded as peptide-specific polyclonal cell lines. From enriched HLA-A2/$M1_{58\text{-}66}$ (M1p58), HLA-A2/$5T4_{17-25}$ (5T4p17) and HLA-A2/$5T4_{97-105}$ (5T4p97) cell pools, polyclonal cell lines were generated by three rounds of non-specific stimulation with PHA. (**A**) Shown is tetramer (TET) staining of end products for two cell lines specific for 5T4p17-pMHC tetramer (p17.wz.3B-LN1, p17.wz.LN2), 5T4p97-pMHC tetramer (p97.wz.LN), and M1p58-pMHC tetramer (M1p58.LN). Three cell libraries showed a high proportion of 5T4p17 –specific CD8 T cells at the first screening (**Figure 4.3**; 3-best-5T41p7); these were sorted separately and expanded as a separate cell line (P17.wz.3B-LN1). (**B**) Memory phenotype examined by surface expression of CD45RA and CD27 in expanded peptide-specific polyclonal cell lines, P17.wz.3B-LN1, P17.wz.LN2, P97.wzLN. Gated on single, live, CD8, TET positive cells.

When tested for reactivity to their cognitive peptide in IFNγ- intracellular staining (IFNγ-ICS) assay without APCs, no responses were observed in 5T4 specific lines while M1p58 specific cell line produced a great response (**Figure 4.5 A**). Briefly, T cell lines were pulsed with their cognate peptides without APCs, and after washing, cells were further incubated in the presence of Brefeldin A for 4 h. The IFNγ production was then determined by intracellular staining with anti-IFNγ antibody and analysed flow cytometry. In contrast, when the CD8 T cell responses were re-assessed in ELISPOT assay using T2 cells as APCs, both 5T4p17 specific T cells produced IFNγ upon stimulation with 5 µM peptide (**Figure 4.5 B**). Again, p17.wz.M1p58 specific CD8 T cells produced a strong response in the absence of APCs, which

was as strong as seen in 5T4 specific cells in the presence of APCs, while the response to peptide presented by APCs was almost two-fold higher (**Figure 4.5 B**). These results signify the importance of costimulatory receptors provided by APCs for induction of successful activation and IFNγ response in tumour-antigen specific CD8 T cells. Together, in a total of six weeks, 133.163-fold expansion of functional 5T4p17 specific cell lines was achieved, showing up to 90% tetramer specificity.

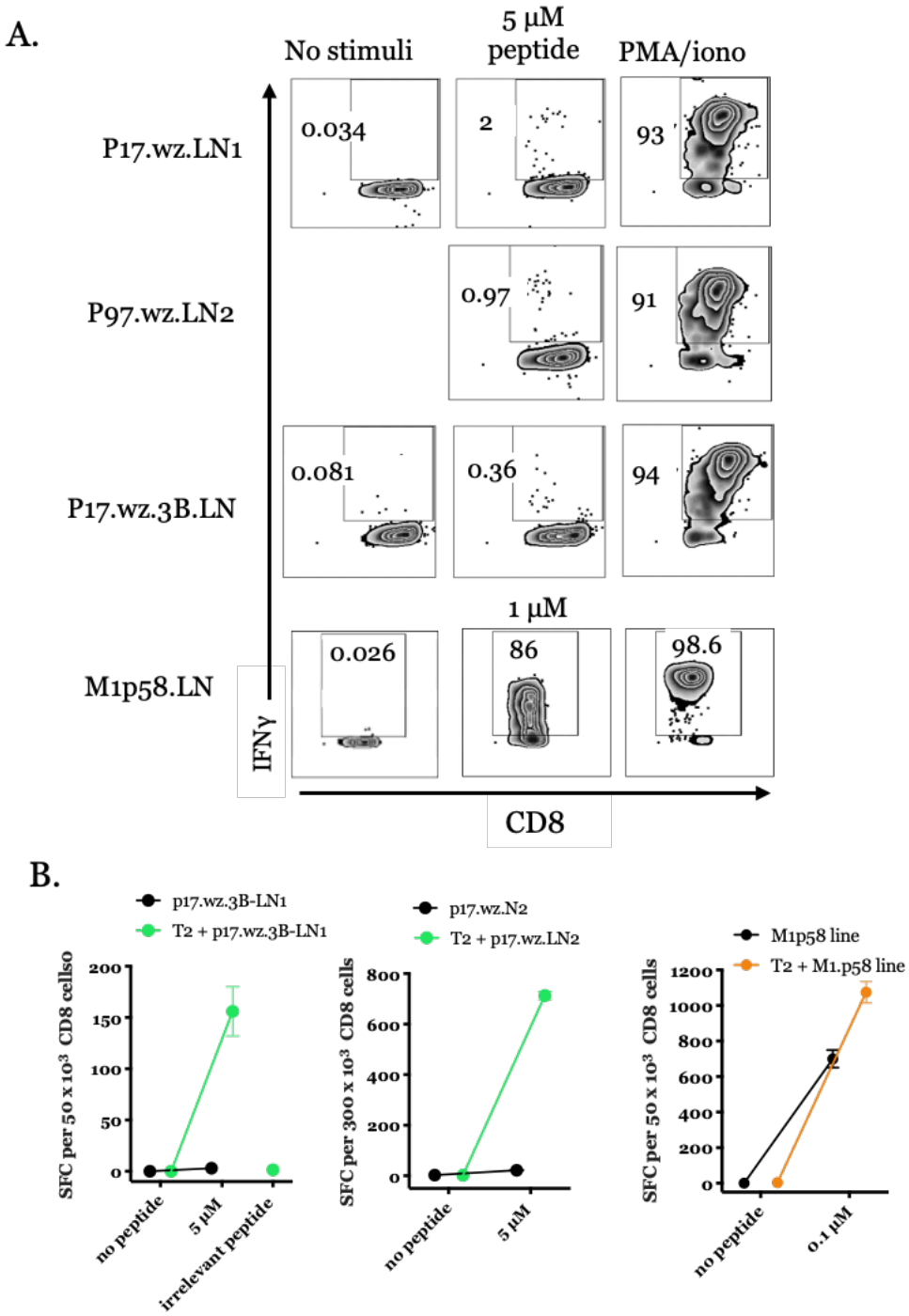


Figure 4.5. Functional characterisation of 5T4-specific and FluM1-specific CD8 T cell lines generated from a heathy donor by method #1.

Polyclonal cell lines described in FIG 4.4 were examined for the reactivity against their cognate peptides. (**A**) An IFNγ –ICS assay (Interferon-gamma -intracellular cytokine staining assay) was used to determine peptide specificity of polyclonal cell lines. 5T4p17.wz.3B-LN1, 5T4p17.wz.LN2, 5T4p97.wz.LN and

M1p58.line were pulsed either with $5T4p_{17\ -25}$ (5T4p17), $5T4p_{97\ -105}$(5T4p97) or Flu matrix protein 1 (FluM1)p_{58-66} (M1p58), BRE A was added for 5 h, and the outcome was measured as an intracellular accumulation of IFNγ by flow cytometry. No antigen-presenting cells (APCs) were used in this assay. Stimulation with PMA/ionomycin (PMA/IONO) represents a positive control, and no stimuli a negative control. Unstimulated sample with 5T4p17.wz.LN2 cells was spoiled; thus is not shown. High IFNγ response was observed in M1p58 peptide stimulated M1p58.LN cells, no response in 5T4p97 peptide stimulated 5T4p97.wz.LN, and only 1-2% response in 5T4p17 peptide stimulated 5T4p17.wz.3B-LN1 & 5T4p17.wz.LN2 cells. Gated on single, live, CD8, TET+ cells. (**B**) Polyclonal cell lines 5T4p17.wz.LN1, 5T4p17.wz.LN2, and M1p58.LN were screened for reactivity to their cognate peptides by IFNγ-enzyme-linked immunospot assay (ELISpot). T2 cells were used as antigen-presenting cells, or CD8 cell lines were incubated with the cognate peptide alone. 5T4p17.wz.line1 and 5T4p17.wz.line2 showed similar responses to peptide presented by T2 cells; almost no cells responded to peptide alone. M1p58.LN cell line showed high responses to either peptide alone or presented by T2 cells. Spot forming cell (SFC) per indicated cell number is shown for each cell line. Data points represent mean values of duplicate samples; error bars show ±SD.

In conclusion, method #1 successfully generated peripheral 5T4-antigen-specific CD8 T cells; however, cells did not show good proliferative potential. Furthermore, the signalling provided by APCs was crucial for the induction of IFNγ responses in 5T4 specific CD8 T cells. Therefore, it was concluded that further method optimisation requires an alteration in cytokine concentration and administration frequency to improve expansion efficiency and the total yield of functional 5T4 antigen-specific CD8 T cells.

Table 4.2. Comparison of cloning method details and efficiency of method #1 & #2

cloning method	sorter assisted method #1				sorter assisted method #2	
donor (age)	30-40				50-60	
pMHC	5T4p17		5T4p97	M1p58	5T4p17	5T4p97
frequency among CD8 cells	1%		0.1%	1%	0.73%	0.45%
# sorted cells (CD3/CD8/TET+)	1348 (p17+p97)			1014	1960	2647
# initiated library plates	1		1	1	2	2
# initiated cloning plates	na		na	na	3	3
cell culture, frequency	priming medium (week 1) 1 x week; expansion medium (week 2), @3 days				priming medium (week 1); expansion medium (week 2); @ 3days	
SCREEN 1: # CD8/TET+ wells	17/96		17/96	38/96	0/288 clones; 16/192 libraries	0/288 clones; 21/192 libraries
SCREEN 1: CD8/TET+ (%)	5.4 - 80		3.9 -7.3	>50	0.3 - 23	1.8 -27
2nd SORTING: # cells (x 10^3)	201	62 (3Hi pool)	77	255.3	35.72	8.887
# of cloning plates	6		6	NA	5	5
SCREEN 2: # growing wells (microscope)	20	4	18	NA	30	39
SCREEN 2: # wells; CD8/TET+ (%)	7/576 (22.8 - 95)		4/576 (7.6 - 87)	NA	5/480 (12 – 89%)	8/480 (8- 40%)
Clones used for expansion to final product: cells/ well-clone	5		1	NA	4	8
FINAL SCREEN: % CD8/TET+	60 – 95		90	NA	50- 90	18 - 90
final cell count/ clone	100– 400,000			NA	10 - 45 million	
Specific clones*	na		na	NA	3	2
total weeks**	12		12	NA	7	7
Notes	* Expanded clones that subsequently stained strongly with tetramer and were specific for the cognate Ag in IFN -ELISpot assay or INF-ICS assay. ** Total time required for expansion and functional analysis					

Table 4.3. Details and efficiency of CD8 T cell line generation

Method	sorter assisted method #1			sorter assisted method #2	
donor (age)	30-40			50-60	
pMHC	5T4p17	5T4p97	M1p58	5T4p17	5T4p97
frequency among CD8	1 %	0.1	1 %	0.71%	0.45%
# sorted cells (%CD8/TET+)	1348 (p17+p97)		1014	1960	2647
cell culture frequency	priming medium (week 1):1 x week; expansion medium (week 2): 3 days			priming medium(week 1); expansion medium (week 2); every 3 days	
SCREEN 1: CD8/TET+ (%)	5.4 - 80%	3.9 -7.3	>50	0.3 - 23	1.8 -27
2nd sorted cells (x 10^3)	201 & 62 (3Hi pool)	77	255.3	35.72	8.887
Total # rounds of expansion	3	3	2	2-3	2-3
FINAL SCREEN: # CD8/TET+ (%)	66.2 -90.1	32	99	72 -89	>36
final cell count: lines;	30 mio	12 mio	50 mio	24 mio	15 mio
Functional cells*	yes	NA	yes	yes	no
total weeks**	6	6	4	5	5
fold increase from 2nd sorting	**133-162.8**	**155.8**	**195.8**	**704**	**562.6**
Notes	* Expanded cells that subsequently stained strongly with tetramer and were specific for their cognate Ag in IFN -ELISpot assay or INF-ICS assay. ** Total time required for expansion and functional analysis mio, million				

4.2.2.2 Generation of functional 5T4 specific CD8 T cell clones: Method #2

The method illustrated in **Figure 4.2** was followed to isolate and expand CD8 T cells specific for 5T4p17 and 5T4p97 epitopes from a donor in the age group 50-60 years. To improve the expansion efficacy, sorted 5T4p17 - and 5T4p97 – specific CD8 T cells received cytokine supplementation every three days. A proportion of sorted T cells were cloned by dilution at 1 cell/well (in three 96-well plates), but no cell growth was observed after the initial two weeks of expansion (data not shown). The remaining sorted CD8 T cells were established as 5T4p17 - or 5T4p97- specific T cell libraries, each initiated at 10 cells/well in 192 wells. After the initial two-week expansion, 16/192 wells were positive for 5T4p17 (1.2 -31% TET+) (Table 4.2, **Figure 4.6 A-B**). These TET+ wells were pooled, sorted by FACS, and cloned at a single cell level by dilution. In two weeks of clonal expansion, 30/480 growing wells were identified, while of those five clones scored positive for 5T4p17 tetramer staining above 30% as assessed by pMHC tetramer staining. Four of these 5T4p17 -TET+ T cell clones (*p17.BM.A01, p17.BM.A11 , p17.BM.A33, p17.BM.A03)* were subjected to another round of stimulation and expansion. Finally, these clones produced 30-45 million cells with 49-87% TET+ (**Figure 4.6.C**) and showed a heterogeneous population of predominately T_{CM} - early T_{EM} cells (Tomiyama, Matsuda and Takiguchi, 2002; Tomiyama *et al.*, 2004; Takata and Takiguchi, 2006; Romero *et al.*, 2007) as depicted by $CD45RA^{neg}/CD27^{LOW}$ expression and with about 80-90% of cells expressing CCR7 (**Figure 4.4.D**).

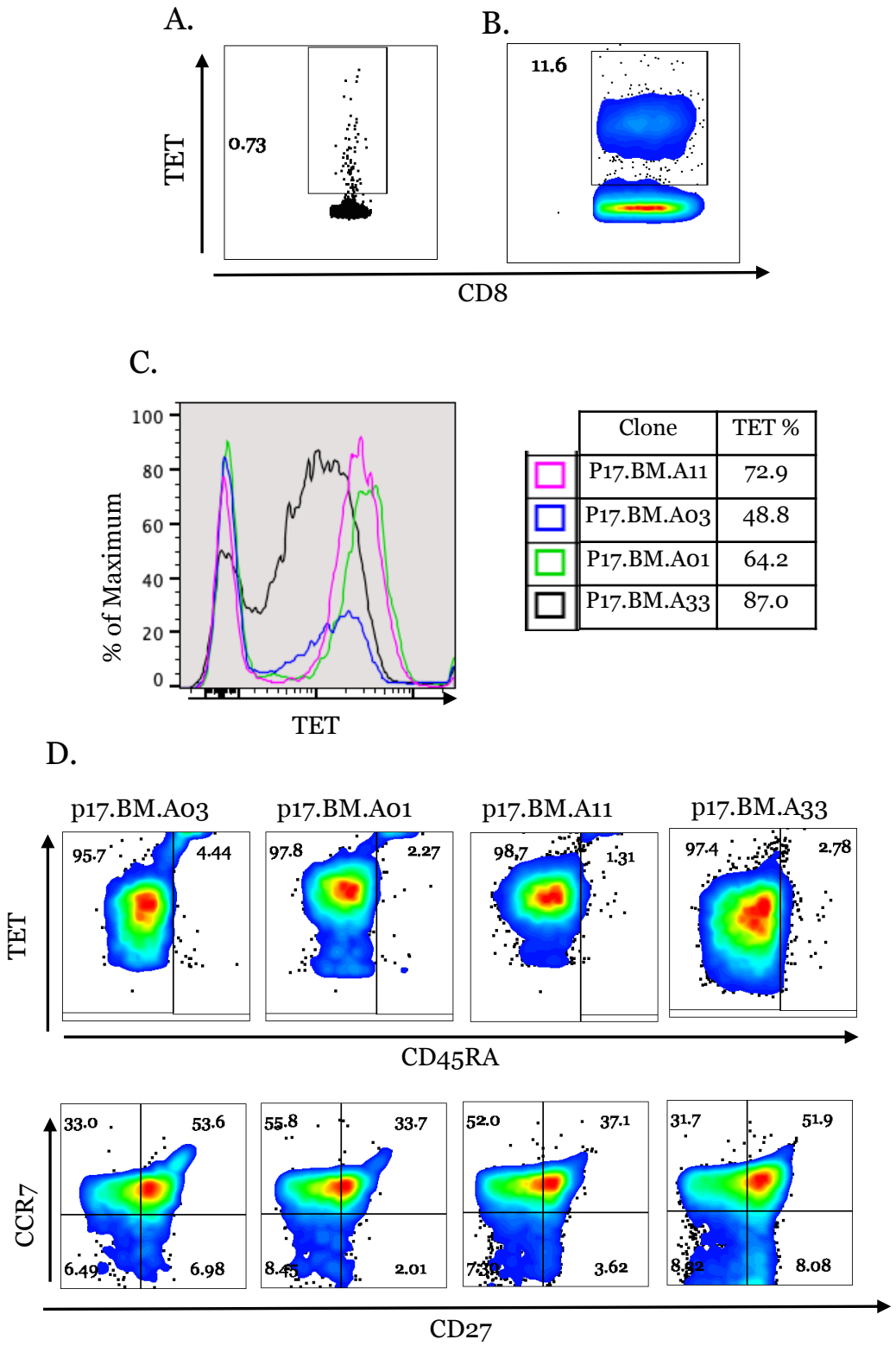


Figure 4.6. Generation and characterisation of $5T4_{17-25}$ -specific CD8 T cell "clones" from pMHC-enriched libraries using non-specific stimulation (method #2).

Cell clones were generated from a healthy donor via strategy described in figure 4.2; (**A**) Shown is the initial frequency of 5T4p17 –specific CD8 T cells from PBMC of an HLA-A2+ healthy donor. (**B**) Enriched 5T4p17-specific CD8 T cell libraries were established (192 wells at 10 cells/well), and cell cultures were supplemented with 200U/mL IL-2 and 20ng/mL IL-15 every three days. After two weeks of expansion,

pMHC tetramers were used to screen and identify wells containing 5T4-p17 peptide-specific T cells. Shown is a representative library containing 5T4p17 –TET+ cells. All tetramer-positive wells were pooled and enriched by FACS sorting prior to single-cell cloning. (**C**) Four 5T4p17 - specific CD8 T cell clones (p17.BM.A33; p17.BM.A01, p17.BM.A03 and P17.BM.A11) were established, all showing 48-87 % tetramer positivity, with varying tetramer staining intensities. (D) Analysis of memory phenotype by CD45RA, CD27, and CCR7 surface expression. All clones have shown T_{CM}/T_{EM} ($CD45RA^{neg}/CCR7+/CD27^{+/-}$) predominantly. Cells were gated on single, live, CD3+, CD8+, TET+ cells.

IFNγ-ELISpot assay was performed to screen cells' reactivity to their cognate peptide. p17.BM.A01, p17.BM.A11 "clones" scored a higher number of IFNγ spot forming cells upon challenge with 5T4p17 peptide than clone p17.BM.A33 (**Figure 4.7 A**). p17.BM.A01 and p17.BM.A11 cells were further assessed in a dose-response assay for intracellular production of IFNγ and membrane expression of degranulation molecule CD107a. Briefly, 5T4p17-peptide-pulsed T2 cells were incubated with CD8 T cells in the presence of brefeldin A and monensin for 16 hr, and production of IFNγ and CD107a was determined by flow cytometry. As expected, a positive correlation was found with increasing peptide concentration and increasing IFNγ production in the p17.BM.A11 clone (**Figure 4.7 B**). Stimulation with 10μM peptide-induced IFNγ response in >60% CD8 T cells, while 100μM peptide-induced nearly 90% response. Compared to cells stimulated with PMA/IONO, which represents a maximal response, the response to 10μM peptide was about seven-fold weaker, while 100μM generated around 3.5-fold weaker response (**Figure 4.7 B**).

In contrast, expression of CD107a was detected in 20% of CD8 T cells when cocultured with 5 – 20 μM peptide-pulsed T2 cells (**Figure 4.7 B**), and it decreased in cocultures with 50 -100 μM peptide-pulsed T2 cells. The MFI of CD107a was also very low (**Figure 4.7**). Likewise, low CD107a expression was noted in p17.BM.A11 cultures stimulated with PMA/IONO. This is in line with previously reported observations by Aktas and colleagues (Aktas *et al.*, 2009).). Of note, the p17.BM.A11 clone exhibited a relatively high background activation in the form of IFNγ (~30%) and CD107a (~8%) release in cocultures with CD8 T cells and T2 cells alone (no stimulation). Since the p17.BM.A11 cells also consisted of ~25% of CD8/TET negative cells, the most likely explanation for this observation is that these cocultures were allogeneic (Aktas *et al.*, 2009) and may have been reactive to T2 cells. However, the background cytokine production was also visible in unstimulated CD8 responders alone. This observation suggests that a 5% serum-supplemented medium may still possess a too high amount of cytokines/growth factors impacting the behaviour of CD8 T cells. Using media with 2% serum could have provided 'cleaner' results (Tan *et al.*, 2015). Functional analysis of clone p17.BM.A01 showed a similar response profile (not shown). In conclusion, p17.BM.A01 and p17.BM.A11 expanded well and proved responsive to their cognate peptide and were thus ready for downstream research.

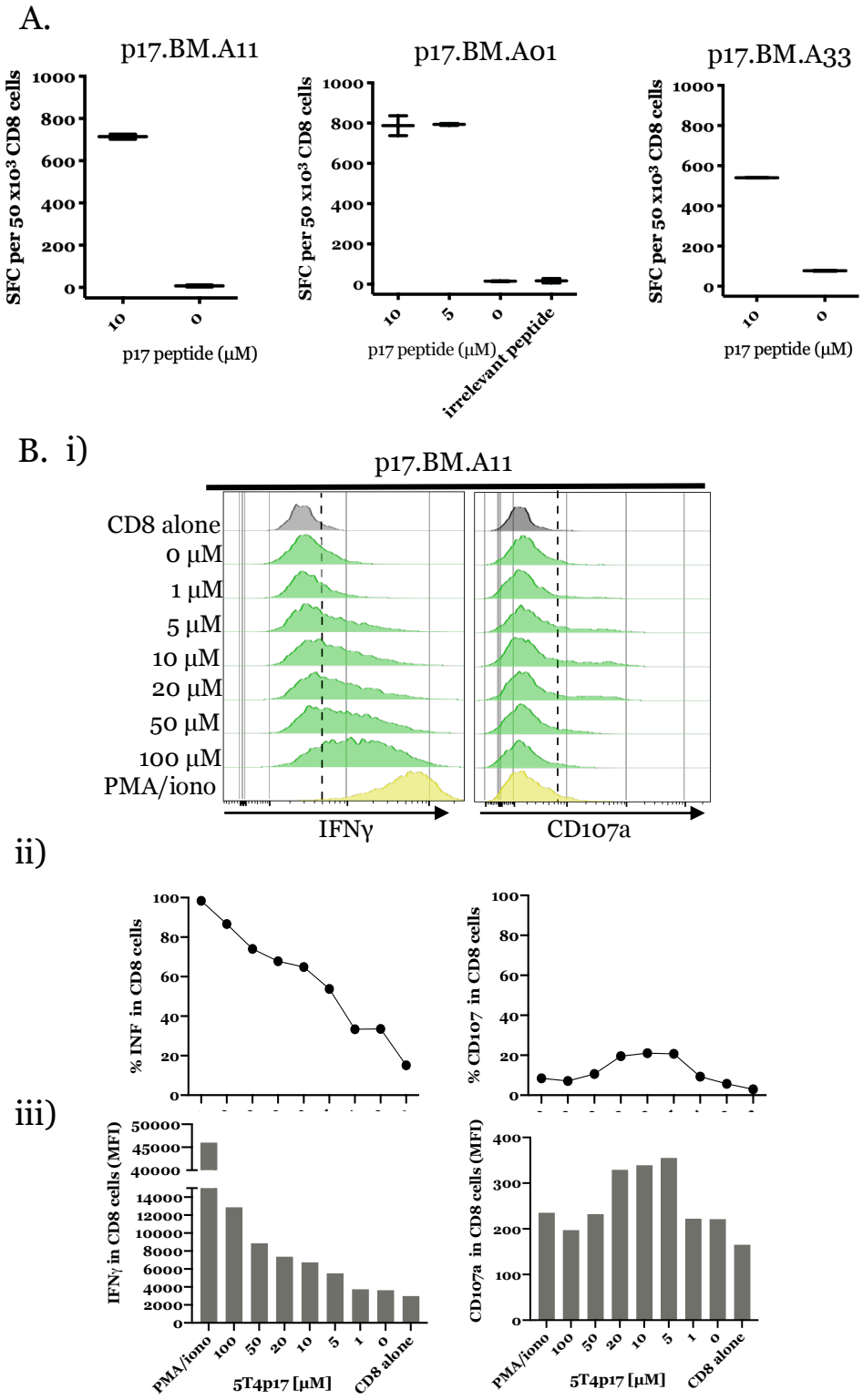


Figure 4.7. Functional characterisation of 5T4p17 -specific CD8 T cell "clones" generated from a healthy donor by method #2.

Three 5T4p17-specific CD8 T cell clones (p17.BM.A33; p17.BM.A01 and P17.BM.A11) described in figure 4.6 were selected for functional analysis. (**A**) IFNγ-enzyme-linked immunospot assay (ELISpot) was used to determine the peptide specificity of expanded clones. All three clones showed responses to 5T4p17 pulsed T2 cells. An 'irrelevant' (5T4p97) peptide was used to determine singular specificity for P17.BM.A11. Spot forming cell (SFC) per 50 x 10^3 cells is shown for each clone. Data points represent mean values of duplicate

samples; error bars show ±SD. (**B**) (i) Dose-response ICS-assay for IFNγ and CD107a confirmed sensitivity to 5T4p17 but suggested a low cytotoxic potential of the clone p17.BM.11. Stimulating the CD8 clone with PMA/ionomycin (PMA/IONO) represents maximal response; cocultures consisting of CD8 T cell clone and T2 cells without stimuli (0 μM), and cultures of CD8 T cell clone alone represent negative controls. The percentage and MFI values of IFNγ and CD107a producing CD8 T cells are shown in (ii) and (iii), respectively. Similar data were obtained for clone p17.BM.A01. Clone p17.BM.A33 was not characterised in detail. Gated on single, live, CD3+, CD8+ cells.

Within the initial CD8 T cell libraries of 5T4p97-MHC sorted cells, 18 out of 192 were positive for 5T4p97-tetramer (4.8 – 25.9%) two weeks after initial expansion (**Figure 4.8 A**, Table 4.2). These TET+ libraries were pooled, enriched by FACS sorting, and cloned at a single cell level by dilution. After two weeks of clonal expansion, cell growth was identified in 39 out of 480 wells, and 8 of these showed positive 5T4p97-tetramer staining and were further expanded. So, in an additional two weeks, these clones showed 18-48.8% tetramer positivity (**Figure 4.8 B**) and very different expansion potentials, fruiting from 10 – 30 million cells depending on the clone. Two "clones" (p97.BM.B15 and p97.BM.B02) were found to contain two distinct CD8$^{/Hi}$ populations, of which p97.BM.B15 cells showed very contrasting TET staining **(Figure 4.9 A**).

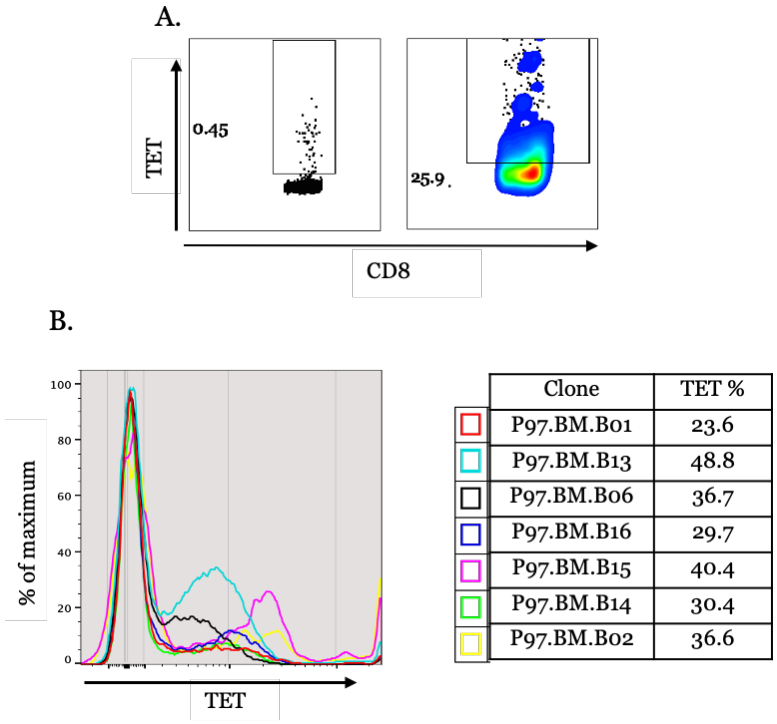


Figure 4.8. Generation of $5T4_{97\text{-}105}$ specific CD8 T cell clones by pMHC-tetramer assisted T cell library approach and non-specific stimulation (method #2).

Cell clones were generated from an HLA-A2+ healthy donor via strategy described in figure 4.2; (**A**) Shown is the initial frequency of 5T4p97 –specific CD8 T cells in PBMC of a healthy donor (left). Enriched 5T4p97-specific CD8 T cell libraries were established (192 wells at 10 cells per well), and cell cultures were supplemented with 200U/mL IL-2 and 20 ng/mL IL-15 every three days. After two weeks of expansion,

pMHC tetramers were used to screen and identify wells containing 5T4-p97 peptide-specific T cells. Shown is a representative library containing 5T4p97 –TET+ cells (right). All tetramer-positive wells were pooled and enriched by FACS sorting prior to single-cell cloning. (**B**) Seven 5T4p97 - specific CD8 T cell clones were established, showing low tetramer positivity (23-48%) and varying tetramer staining intensities. Gated on single, live, CD3+, CD8+ cells.

The $CD8^{hi}$ population consisted of 88% 5T4p97 tetramer-positive cells, while the $CD8^{lo}$ population contained only 15% tetramer-positive cells. The $CD8^{hi}$ predominately stained $CD45RA^{neg/lo}/CD27^{neg/lo}$ and CCR7+ (**Figure 4.9 B**), suggesting TCM phenotype due to the presence of CCR7 expression (Romero *et al.*, 2007). Notwithstanding, these cells had an inferior proliferative potential and did not produce enough cells for research. In a functional analysis by IFNγ-ICS assay, p97.BM.B15 cells showed moderate responses to 5T4p97-peptide-pulsed T2 cells. A high peptide concentration was required to get ~50% IFNγ-producing CD8 T cells (**Figure 4.9 C**). Likewise, the strength of the response to 50-100 μM peptide was about a third of maximal responses seen in cells stimulated with PMA/ionomycin (**Figure 4.9 C**).

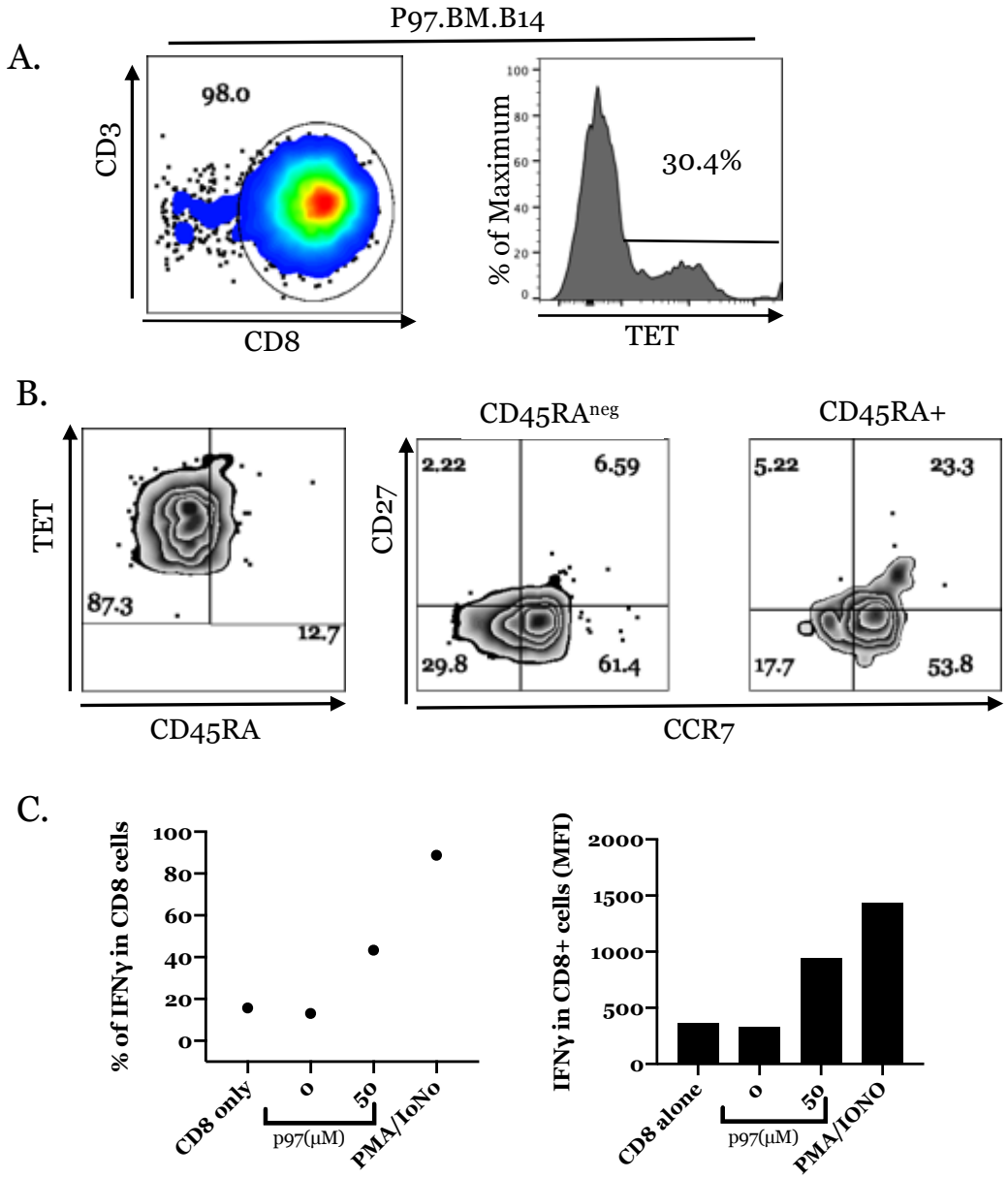


Figure 4.9. Characterisation of P97.BM.B15 CD8 T cells, specific for 5T4p$_{97-105}$ peptide (continue on next page)

Description for clone generation is illustrated and described in **Figure**s **4.2** and **4.8**. (**A**) Clone P97.BM.B15 presented two distinctive CD8 populations; 88% CD8^{HI} population showed high intensity 5T4p97-TET+; 19% CD8 population stained positive for 5T4p97-TET. (B) Tetramer positive population showed CD45RA$^{low-neg}$/CCR7+/CD27^{low} T_{CM}/T_{EM} phenotype predominately. Cells were gated on single, live, CD3+, CD8, TET+ cells. (**C**) IFNγ- ICS-assay was used to determine the sensitivity of p97.BM.B15 clone to the cognate peptide at 20 μM, 50 μM and 100 μM concentrations. T2 cells were used as APCs. Stimulation of the CD8 clone with PMA/ionomycin (PMA/IONO) represents maximal response; cocultures consisting of CD8 clone and T2 cells without stimuli (0 μM), and cultures of CD8 T cell clone alone represent negative controls. The percentage of IFNγ producing CD8 T cells are shown in (i), and MFI values are shown in (ii), and representative FACS plots are shown in (iii). Half maximal percentage of responding cells were detected at 50 μM; but only a third of maximal MFI was detected at 100 μM. Cells were gated on single, live, CD3+, CD8+ cells.

Nevertheless, this suggested that all 5T4p97-TET+ CD8 T cells (36% of all CD8 T cells) responded to a peptide challenge. p97.BM.B14 cells showed only ~30% 5T4p97 tetramer positivity (**Figure 4.10 A**), and mostly an effector/memory phenotype – most of the cells (~87%) were CD45RA^{NEG}/CD27^{NEG}, although again these cells also expressed CCR7, commonly expressed on naïve or central memory cells. In the functional analysis, ~40% of CD8 T cells produced IFNγ in response to 50 μM peptide (**Figure 4.10 C**). Seeing that this clone contained only ~ 30% 5T4p97-tetramer specific cells, this result suggested that all 5T4p97-tetramer specific cells responded to the peptide challenge while the PMA/IONO stimulation was only about a third stronger than the peptide-induced response. Despite that, background cytokine production was detected in both unstimulated cultures containing p97.BM.B14 cells only and in unstimulated cocultures with T2 cells. These cells did not proliferate well and did not produce enough cells for further research. Although production of perforin and cytotoxic molecules was not assessed here, these results suggest a late effector memory phenotype. The other six generated 5T4p97-TET+ cells were not functionally tested. Experimental details and cloning efficiency are shown in Table 4.2.

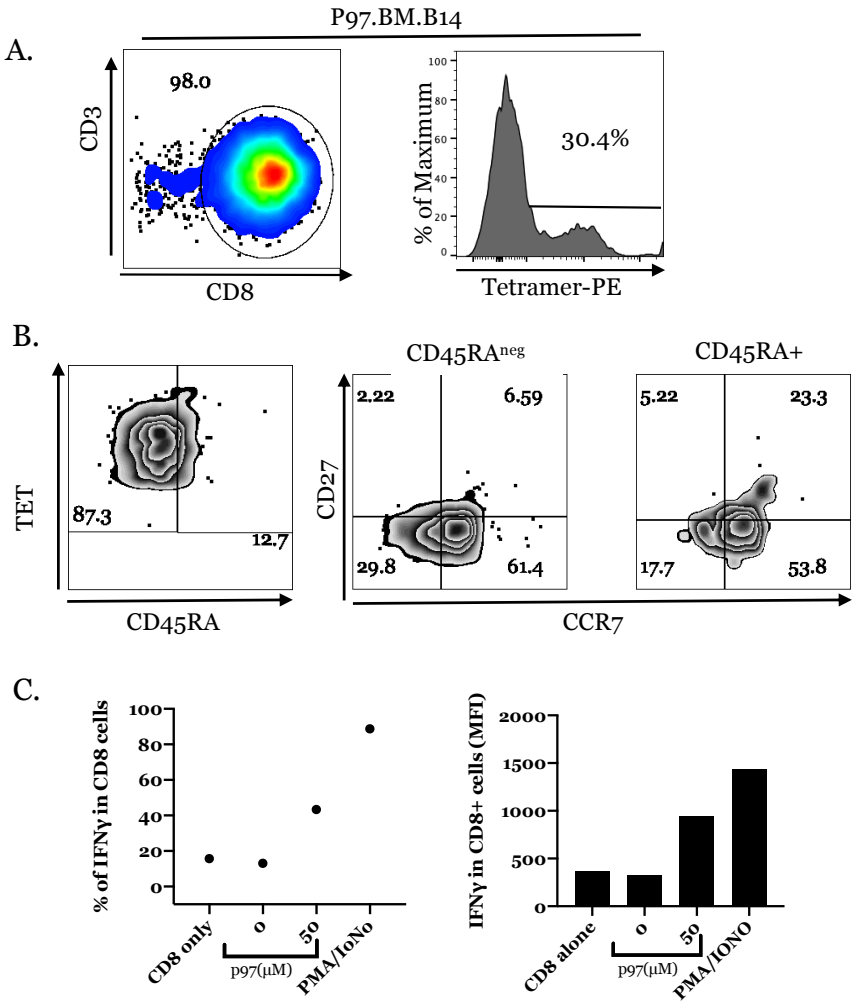


Figure 4.10. Characterisation of 5T4p$_{97-105}$ specific CD8 T cells, P97.BM.B14, generated by method #2 (next page).

Description for clone generation is illustrated and described in figures 4.2 and 4.8. (**A**) P97.BM.B14 cells show a distinct CD8 population with various staining intensities and ~30% 5T4p97-TET+ cells. (**B**) Tetramer positive population showed$CD45RA^{neg}/CCR7^{low}/CD27^{neg}$ effector-memory phenotype predominately. Cells were gated on single, live, CD3+, CD8+, TET+ population. (**C**) IFNγ- ICS assay was used to determine the sensitivity of p97.BM.B15 cells to the cognate peptide at 50μM. T2 cells were used as APCs. Stimulation of the CD8 clone with PMA/ionomycin (PMA/IONO) represents maximal response; cocultures consisting of CD8 clone and T2 cells without stimuli (0 μM), and cultures of CD8 T cell clone alone represent negative controls. The percentage of IFNγ producing CD8 T cells (left) and MFI values (right) are shown. A high basal level of IFNγ production is noted in both negative controls. At 50 μM peptide, all TET+ cells produced IFNγ. Cells were gated on live, single, CD3+, CD8+ cells.

Of interest, cells with specificity for 5T4p17 -peptide showed between 5 -25-fold higher TCR affinity than all but one 5T4p97-specific "clone" (**Figure 4.11**). p97.BM.B14 cells showed particularly low pMHC fluorescence intensity, yet all tetramer specific cells produced a high level of IFNγ in response to a peptide (**Figure 4.11**). This is in line with observations that in addition to TCR affinity for its cognate peptide, a successful and robust peptide-specific response is also orchestrated by the CD8 coreceptor and its affinity for the MHC I, and the presence of other costimulatory/inhibitory receptors expressed by the cells at the time of stimulation (Schodin, Tsomides and Kranz, 1996; Valmori *et al.*, 1999; Laugel, van den Berg, *et al.*, 2007; Park *et al.*, 2007; Robbins *et al.*, 2008; Laugel *et al.*, 2011; Stone *et al.*, 2011; Anikeeva *et al.*, 2012). Thus, to provide an exact explanation for the different responses observed among generated 5T4 T cell clones, more comprehensive analyses are required.

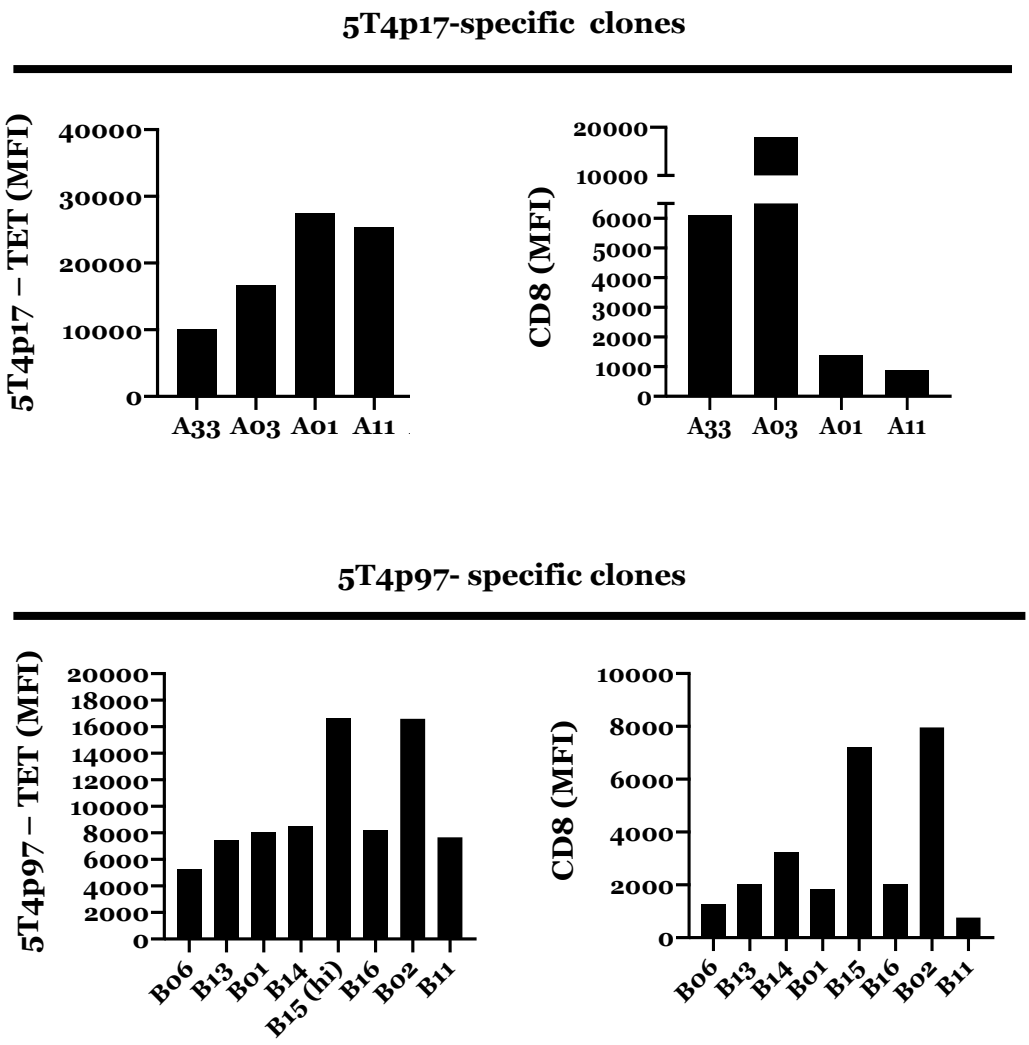


Figure 4.11. CD8 and TCR cell surface expression in 5T4p17 –specific and 5T4p97-specific T cell clones generated from a heathy donor.

Shown are MFI values of 5T4p17 –specific clones (top panel) and 5T4p97 –specific clones after 3 rounds of expansion and 6 weeks culture, stained with anti-CD8 APC-conjugated antibody and PE-conjugated pMHC tetramers. Data are representative of MFI values from one experiment. Gating was on single, live, CD8+ TET+ cells.

In addition to clones, 5T4p17 and 5T4p97 pMHC enriched cell lines were expanded from FACS sorted cells which were not used for cloning. The same cell culture method was followed as described for clones, although the enriched cells were collectively expanded on 24 well plates, in a total of two rounds of expansion, 84.9% of p17.BM.LN1 cell line cells showed specificity for 5T4p17 tetramer (**Figure 4.12 A**) and showed a 5T4p17 - peptide-specific response in IFNγ -ELISPOT assay and IFNγ-ICS assay (**Figure 4.12 A**), while the strength of the response was about -50% weaker as compared to responses seen upon PMA/IONO stimulation. In contrast, expansion of 5T4p97-pMHC enriched cells resulted in a cell line showing 69% 5T4p97-tetramer positivity, while these cells failed to respond to peptide challenge in IFNγ-ELISpot assay (**Figure 4.12 B**). Together, 704-fold expansion of functional 5T4p17

-tetramer specific cell lines with up to 85% purity was obtained in 4 weeks. Table 4.3 summarises the culture conditions and expansion efficiency.

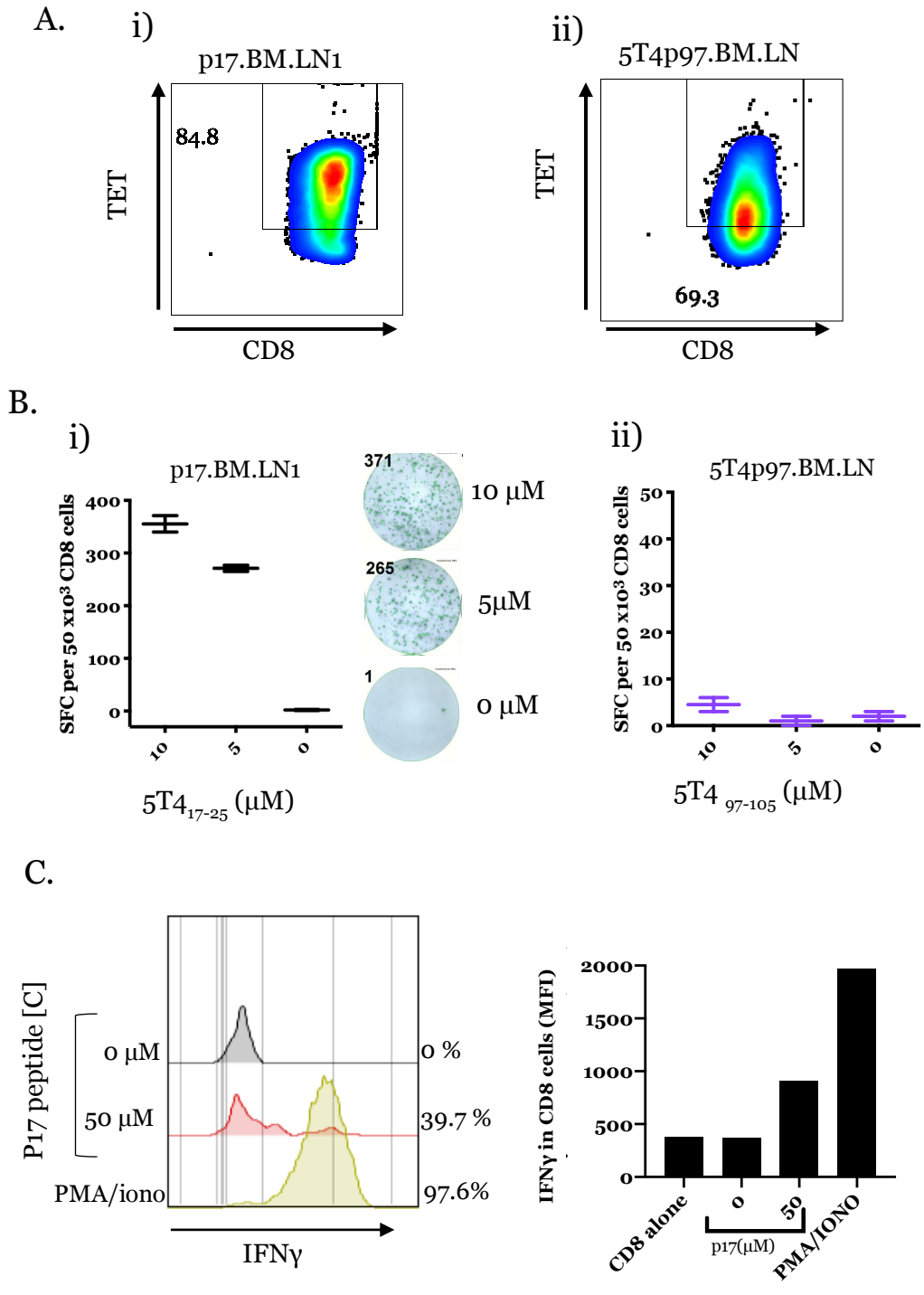


Figure 4.12. Characterisation of enriched 5T4p17 - and 5T4p97- specific CD8 T cell lines generated by method #2.

Initial CD8 T cell libraries consisting of 5T4p17 and 5T4p97 specific CD8 T cells were established from an HLA-A2+ healthy donor. The procedure for CD8 T cell isolation, peptide-specific T cell enrichment, and expansion as a cell line was followed by the strategy described in figure 4.2. Polyclonal cell lines were generated by two rounds of non-specific stimulation with PHA in the presence of IL-12 and IL-15 and

irradiated feeder cells. (**A**) Shown is tetramer (TET) staining of end products for a cell line-specific for (i) 5T4p17-pMHC tetramer (p17.BM.LN1) and (ii) for 5T4p97-pMHC tetramer (p97.BM.LN). Gated on single, live, CD3+, CD8 cells. (**B**) IFNγ –ELIspot was used to screen (i) p17.BM.LN1 cell line and (ii) p97.BM.LN cell line for reactivity to their cognate peptides presented by T2 cells. The SFC per 50 x 103 CD8 cells is shown. Data points represent mean SFC values of duplicate samples. (**C**) p17.BM.LN1 cell line sensitivity was confirmed in IFNγ - ICS assay. Stimulation with PMA/iono was used to determine maximal response; cocultures of CD8 cell line with T2 cells alone (0 μM) represents negative control. Stimulation with 50μM 5T4p17 peptide showed a half-maximal response. Gated on single, live, CD19-, CD8+ cells.

In summary, from one attempt, three functional 5T4p17 -specific "clones" were successfully established from CD8 T cell libraries of FACS sorted cells and were ready for research in a total of 6-7 weeks. In this time, two functional 5T4p97-specific “clones” were established but gave insufficient cell numbers required for further research. Future TCR-Vβ chain-typing analysis will warrant the clonality of these T cell products. A 5T4p17 -specific cell line with limited functionality was established in 4-5 weeks, and no 5T4p97-specific T cell lines were generated. In conclusion, method #2 cell sorter-assisted T cell library approach successfully generated a large number of 5T4-reactive CD8 T cells. However, to improve expansion efficiency while sustaining the functional phenotype, further method optimisation was required.

4.2.2.3 Expansion of functional 5T4 -specific CD8 T cells in the presence of IL-21: Method #3

To improve the expansion and function of difficult tumour-peptide specific CD8 T cells, including from naïve T cell repertoire, I sought to explore the potential of adding IL-21 to my strategy described in **Figure 4.2.** Growing evidence suggests that IL-21 can enhance the expansion of tumour antigen-specific T cells, increasing the total cell number and, at the clonal level, enrich for a population of high-affinity CD8 T cells with sustained elevation of CD28 levels and a helper-independent phenotype (Moroz *et al.*, 2004; Li, Bleakley and Yee, 2005; Liu *et al.*, 2007, 2015; Tian and Zajac, 2016). While IL-21 has also been described to aid in expanding peptide-specific T cells from naïve populations, Moroz and colleagues (Moroz *et al.*, 2004) reported that IL-21 enhances and sustains CD8 T cell responses to achieve durable tumour immunity. To test described benefits, I first wanted to determine the culture conditions using circulating 5T4- specific CD8 T cells from a healthy donor as a model system.

The strategy illustrated in **Figure 4.2** was followed as described. Bulk CD8 T cells isolated from fresh PBMC from donor D212 contained 0.35% of 5T4p17 specific CD8 T cells and 0.15% 5T4p97 specific cells (not shown), giving ~8000 and ~240 isolated cells, respectively. T cell libraries were initiated on 48w plate at ~200-500 cells/well. To examine the benefit of IL-21 on the expansion efficiency of functional CD8 T cells, two cytokine conditions were tested*: condition 1*, IL-21 alone; *condition 2*, IL-21, IL-15 and IL-2. I used the optimal concentration of IL-21 defined for expansion of tumour peptide-specific CD8 T cells by Li and colleagues (Li, Bleakley and Yee, 2005). The analysis included two control cultures: *control #1)* no cytokine administration (complete medium only); *control #2)* administration of IL-2 and IL-15 only as described in Method #2 above. When IL-21 was administrated alone or combined

with IL-2 and IL-15, two of four T cell libraries were identified with a distinct population of >1% 5T4p17 -pMHC tetramer-positive CD8 T cells (**Figure 4.13**). While in cell cultures under *condition 2*, two wells also contained a tiny but distinctive population of TET+ cells (0.7%), but these cells were not included for further expansion (**Figure 4.13**). In contrast, the administration of IL-2 and IL-15 (control #2) resulted in <0.5% 5T4p17 -tetramer-positive CD8 T cells in all wells, while one well showed a tiny distinctive population but scored only 0.14% tetramer positivity – that was as much as detected in *control* culture with no cytokines. Thus, none of these wells was selected for further expansion. So, the positive 5T4p17 T cell libraries from each condition were pooled, and MACS sorted, resulting in 5 x 10^3 cells from condition 1 (IL-21 only) and 3 x 10^5 isolated cells from condition 2 (IL-21-IL-15-IL-2). The enriched cells were then subject to expansion in flasks under the same culture conditions described above (Table 4.4).

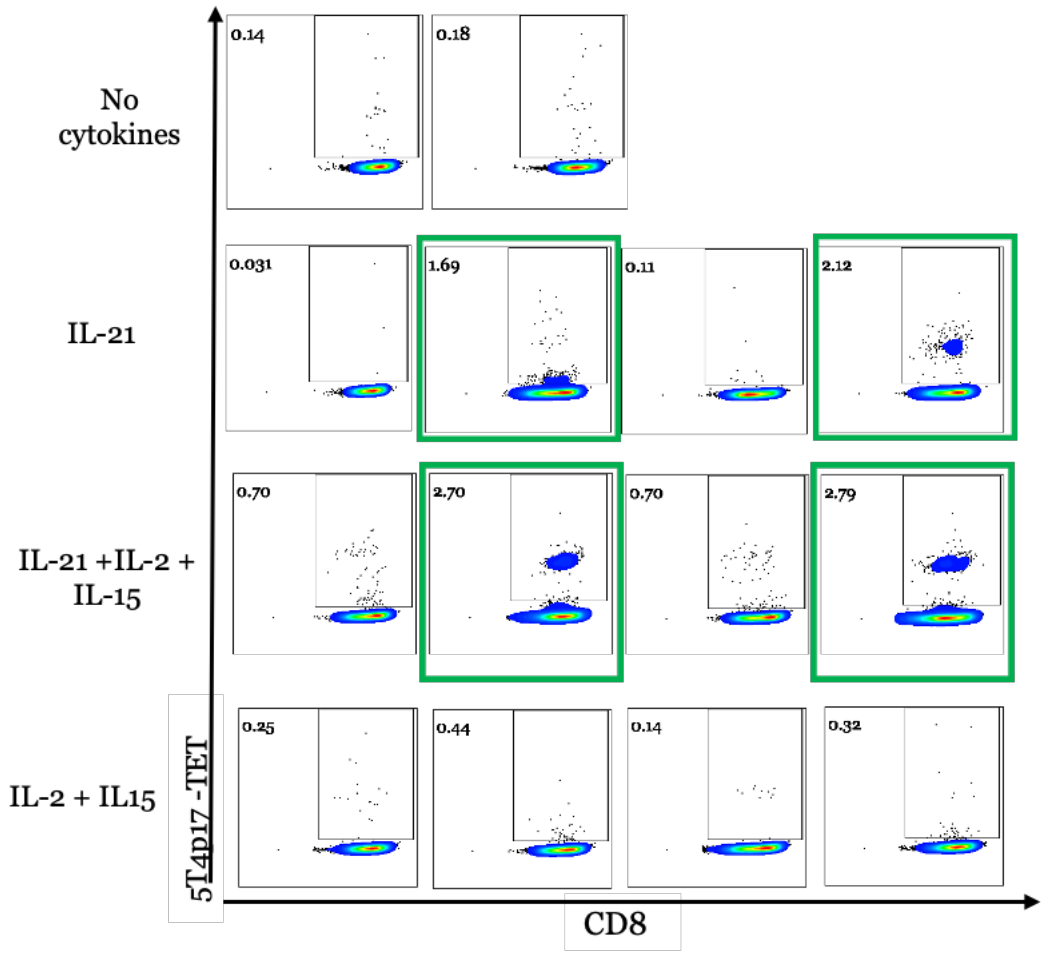


Figure 4.13. Elevated frequency of 5T4p17-specific CD8 T cells following primary mitogen stimulation and IL-21

The effect of IL-21 on the expansion of peptide-specific CD8 T cells was evaluated. The strategy for CD8 T cell isolation, pMHC-tetramer T cell enrichment by MACS, maintenance and characterization of cells was followed as described in figure 4.2. Briefly, from a healthy HLA- A2+ donor, 5T4p17 - specific CD8 T cells were isolated using tetramer and MACS and were stimulated *in vitro* with PHA in the presence of irradiated allogeneic feeder cells. IL-21 was added alone or together with IL-2 and IL-15 twice in the first four days. Controls included 5T4p17 –specific CD8 T cells cultured in the absence of cytokines or in the presence of IL-2 and IL-15 as described in previous sections. Cells were seeded on 48w plates in four wells per test

condition and two wells per "no cytokines" control. On day 14, after primary expansion, screening using pMHC tetramers was performed. Data are expressed as a percentage of 5T4p17 -tetramer-positive cells among CD8 T cells. Gated on single, live, CD3+, CD8+ cells. Wells that contained ≥1% TET+ cells were considered positive. Wells (plots) encircled with the green line were selected for further expansion; wells were pooled, and 5T4p17 –specific cells were enriched using tetramers and MACS before being expanded as polyclonal cell lines under the same culture condition as above. Cells cultured with IL-2 and Il-15 alone and the cultures expanded in the absence of cytokines did not produce ≥1% of TET+ cells and thus were not included in further experiments.

After two weeks, the three cell lines (p17.RA.LN1, p17.RA.LN2, p17.RA.LN3) under the culture condition 2 yielded a homogenous population with 80-85% 5T4p17 -pMHC tetramer-positive cells (**Figure 4.14 A**). Whereas two cell lines (p17.RA21.LN1 and p17.RA21.LN2) under culture condition 1 showed heterogeneous (multiple) populations of tetramer-positive cells with different staining intensities (**Figure 4.14 C**). The phenotype analysis revealed P17.RA.LN2 cell line predominately expressed CD45+CD27+, and the level of CD28 expression remained high but variable (**Figure 4.14 C**). The addition of other markers (CCR7, CD62L, CD95, CD122) would help to determine the differentiation phenotype more precisely but were not included in the analysis.

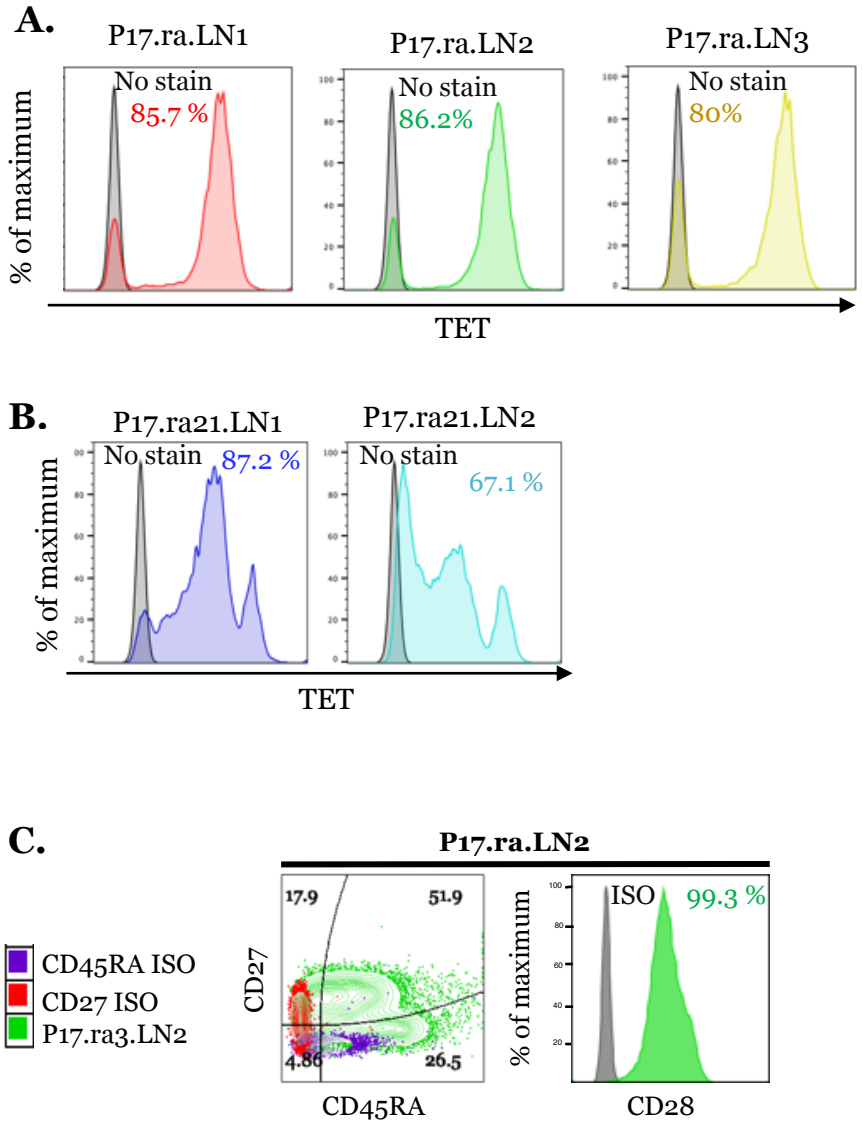


Figure 4.14. Phenotype characterisation of 5T4p17 -specific CD8 T cell lines generated in the presence of IL-21.

5T4p17 –specific T cell lines were generated in the presence of IL-21 as described in the text. Briefly, Enriched 5T4p17 –specific cell lines underwent a total of two rounds of stimulation with mitogen and were cultured in the presence of irradiated allogenic feeder cells and either IL-21 alone or together with IL-2 and IL-15. (**A**) Three cell lines (P17.ra.LN1, P17.ra.LN2, P17.ra.LN3) were established in the presence of IL-21, IL-15, and IL-2 showing a clean singular population of ≥80% TET+ CD8 T cells. (**B**) Two cells lines (P17.ra21.LN1, P17.ra21.LN2) were established in the presence of IL-21 and showed clean multiple populations of 80-90% TET+ CD8 T cells. (**C**) Memory phenotype was examined in p17.RA.LN2 cells by surface marker flow cytometric analysis. All cells expressed CD28 with variable intensities and predominant CD45RA+/CD27+ (T_{SCM} -like) and CD45RA+/CD27- (T_{CM}) phenotype. Cells were gated on single, live, CD3, CD8, TET+ population. ISO; isotype.

In the first instance, IFNγ ELISpot was employed, as described before, to examine the functionality of these cells. All cell lines generated excellent responses to 10, 5, and 2 μM 5T4p17 peptides presented by T2 cells (**Figure 4.15 A**). Two cell lines generated by culture *condition 2* (p17.RA.LN1, p17.RA.LN2)

were further examined in a dose-response IFNγ-ICS assay, induction of half-maximal response was seen at ~5 µM 5T4p17 peptide concentration (**Figure 4.15 B-C**; **Figure 4.16**) and a similar trend in the production of CD107a was noted as seen in 5T4p17 specific T cell clones generated under Method #2 conditions. Despite that, a higher percentage of CD107a producing p17.ra.LN2 cells were observed in cultures stimulated with 50 µM peptide, i.e. >50% compared to clones generated by Method #2.

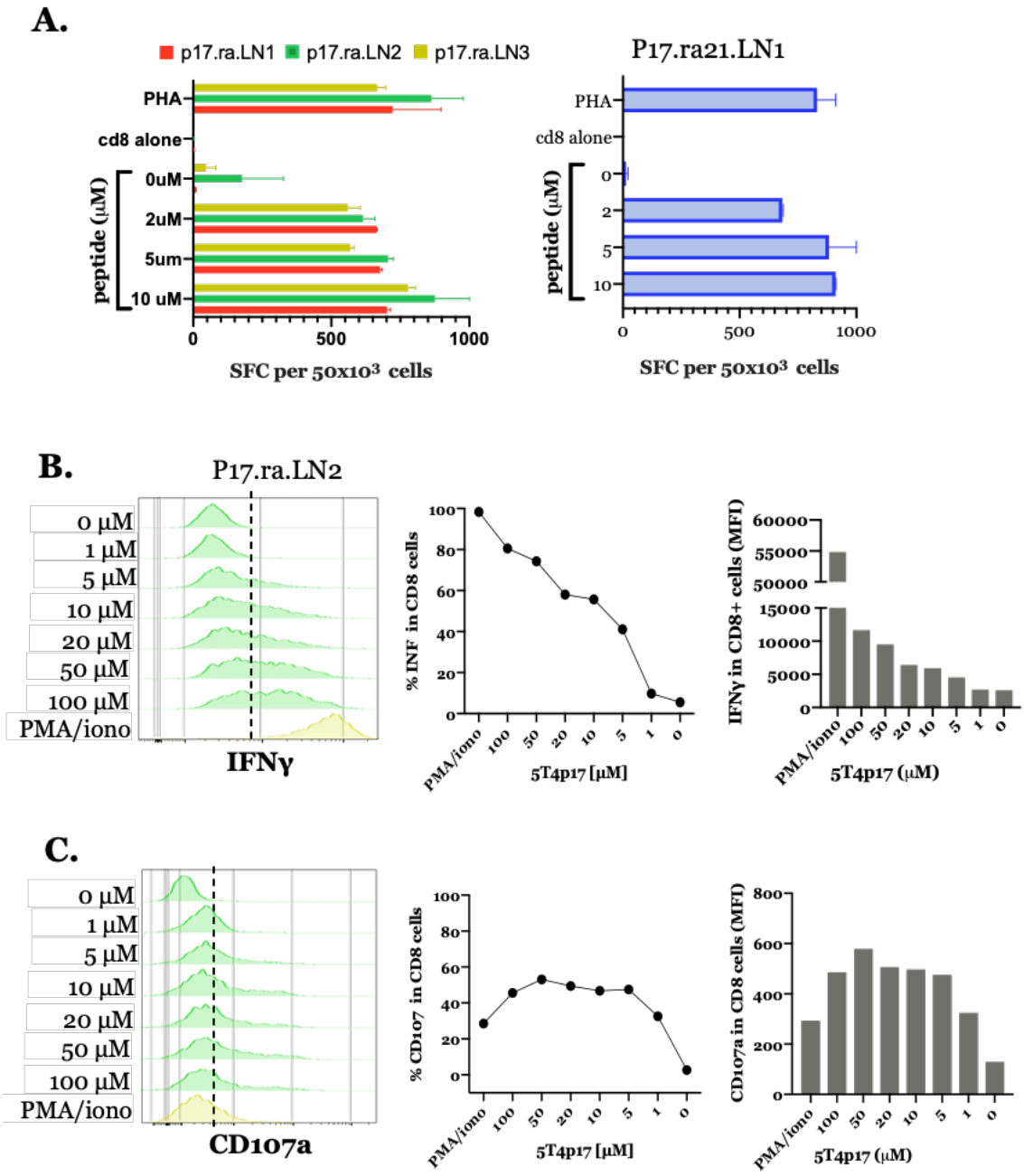


Figure 4.15. Functional characterisation of 5T4p17 specific CD8 T cell lines generated in the presence of IL-21, IL-2 and IL-15.

Description for peptide-specific CD8 T cell line generation is illustrated and described in **Figure 4.2** and 4.13. (**A**) Cell lines were examined functionally in the IFNγ-ELISpot assay. All established cell lines showed excellent responsiveness to their cognate peptide 5T4p17 -25 presented by T2 cells. SFC per 50 x 103 cells are shown. Stimulation with PHA served as a positive control. Cocultures with CD8 T cells and T2 cells without stimulation (0 µM) and culture of CD8 cells alone served as a negative control. (**B**) Sensitivity of

p17.ra3.LN2 cells to the 5T4p17 peptide were determined in a dose-response IFNγ - ICS assay. Stimulating the CD8 T cells with PMA/ionomycin (PMA/IONO) represents maximal response; cocultures consisting of CD8 T cells and T2 cells without stimuli (0 μM) cultures of CD8 T cell clone alone represent negative controls. Half maximal response was seen at 5 μM. Graphical representation of the percentage of IFNγ producing CD8 T cells is shown in the middle panel and MFI values to the right. (**C**) Cytotoxic potential of p17.ra3.LN2 T cells was determined by CD107a expression by flow cytometry after exposure to decreasing peptide concentrations presented by T2 cells. Cocultures consisting of CD8 T cells and T2 cells without stimuli (0 μM), and cultures of CD8 T cell clone alone represent negative controls. The percentage of CD107a producing CD8 T cells are shown in the middle panel and MFI values to the right. Gated on single, live, CD19-, CD3+, CD8+ cells

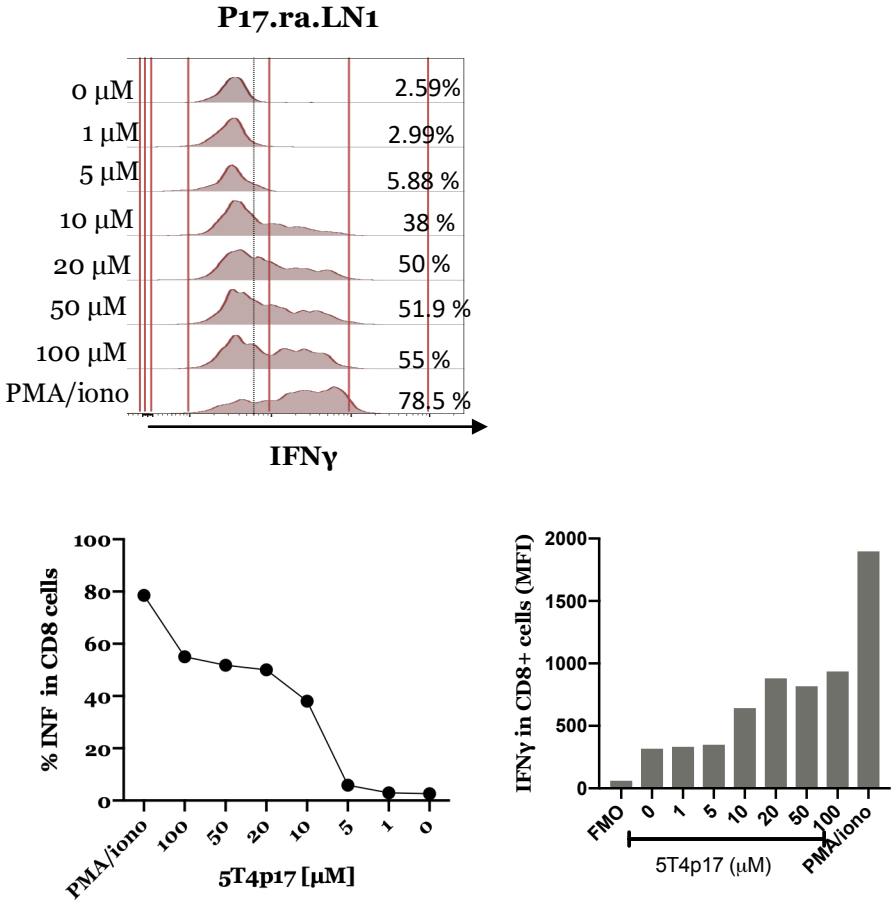


Figure 4.16. Functional characterisation of 5T4p17 specific CD8 T cell line P17.ra.LN1 generated in the presence of IL-21, IL-2-IL-15.

Sensitivity of p17.ra.LN1 cells to the 5T4p17 peptide was determined in a dose-response IFNγ - ICS assay. Stimulating the CD8 T cells with PMA/ionomycin (PMA/IONO) represents maximal response; cocultures consisting of CD8 cells and T2 cells without stimuli (0 μM), and cultures of CD8 T cell clone alone represent negative controls. Half maximal response was seen at 10 μM. Graphical representation of the percentage of IFNγ producing CD8 T cells are shown in the bottom left panel and MFI values to the right. Gated on single, live, CD19-, CD3+, CD8+ cells.

Although the T cell phenotypes were not examined prior to expansion, these cells likely came from less differentiated precursors (Pollack *et a*l. 2014) (Li, Bleakley and Yee, 2005). p17.RA.LN1 cells were not examined for the expression of surface markers to determine differentiation phenotype and showed slightly different responses to their cognate peptide (**Figure 4.16**). To reach half-maximal response, p17.ra.LN1 cells required double the concentration of peptide (10 μM).

Finally, in as little as four weeks, 620- and 1066-fold expansion was achieved in CD8 T cells specific for 5T4p17 peptide (Table 4.4). The cell culture condition details and expansion efficiencies are summarised in Table 4.4. In contrast to the report of Li and colleagues (Li, Bleakley and Yee, 2005), who show that combination of IL-21 with IL-2 and IL-15 did not have an impact on the final cell number of peptide-specific T cells, in this study, 5T4-specific CD8 T cells expanded in the presence of all three cytokines proliferated better at the experiment initiation. Finally, expanded cells then showed a favourable phenotype with improved functional responsiveness. To note, under the same culture conditions, expansion of isolated 5T4p97-specific CD8 T cells was not successful; no cell growth was detected after the initial expansion period, suggestive these cells were not viable, or the tetramer-specific isolation failed (data not shown).

Taken together, the addition of IL-21 to cultures of expanding tumour-peptide specific T cells proved beneficial not only in terms of final fold expansion rate but also in terms of the cells' functional performance.

Table 4.4. Comparison of T cell expansion culture conditions and efficiency

METHOD	pMHC-tetramer enriched T cell libraries			
Donor age	**(30-40)**			
Isolated CD8	**2.3 mio**			
pMHC	**5T4p17**			
frequency among CD8 (% TET+)	0.35			
# of isloated TET+ cells **/*	~8000			
# of initiated wells	4 wells/ condition			2 wells
Culture conditions	IL-21	IL-21, IL-2, IL15	Il-2 & IL-15	No cytokine CONTROL
SCREEN 1: p17 TET+ wells	2	4	0	0
SCREEN 1: p97 TET+ wells	0	0	0	0
Least% positive	0.05 & 0.21%	0.81 & 0.88%	0.11 & 0.32%	0.05 - 0.16
Largest% positive (used for sorting& expansion)	1.89 & 2.28%	4.62 & 3.96%	0.59 & 0.56	na
Least positive: cell count	3.52 mio/ mL; 8.8 mio	3.22 mio/mL; 8.05 mio	3.65 mio/ml; 9.13 mio	1.82 mio/ml; 4.55 mio
Largest positive: cell count	2.23 mio/mL; 7.4 mio	1.53 mio/mL; 5 mio	4.24 mio/mL; 10.6 mio	
# TET+ purified cells**	5×10^3	300×10^3	na	na
# of cells/expansion flask	50 000 cells	100 000 cells	na	na
SCREEN 2: TET+ (%)	67.1 - 87.2% (multi populations)	79- 85.4;% (homogenous populations)	na	na
Least% positive	67.1	78.9	na	na
Largest% poostitive	87.2	85.4	na	na
Functional lines	all	all	na	na
Final cell count	53.3 mio	185.85 mio	na	na
time in weeks	4	4	na	na
fold increase from 2nd expansion***	**1066**	**620**	na	na
NOTES	**** isolation/purification of TET+ CD8 T cells by MACS using antiPE-conjugated magnetic beads' (Miltenyi) and PE-conjugated pMHC-tetramers** ***** calculated from number of isolated CD8 T cells divided by frequency of pMCH-tetramer+ cells fund in CD8 T cells** **mio, million**			

4.3 Generation of CD8 T cell clones specific for NY-ESO-1 epitopes

Along with the challenge with low clonotype frequencies, another key obstacle in identifying and isolating peptide-specific T cells is limited cell availability. This is often the case when obtaining samples with T-cells recognising tumour-associated antigens from PBMC of cancer patients (Sharpe and Mount, 2015) – since due to thymic selection, the presence of self-reactive T-cells in the periphery is reduced (Klein *et al.*, 2014), while patients normally also undergo "treatments" with cytotoxic agents which tend to reduce PBMC numbers. Thus, in order to test if cell-sorter method #2 (section 4.3.2.2) can effectively produce tumour peptide-specific T cell clones from smallest starting cell numbers, frozen 20-25 million PBMC of two melanoma patients, patient 89 and patient 90, were used. 86,000 CD8 T cells were isolated from Patient 89, and 38,000 CD8s were isolated from Patient 90. While the patients were not sero-tested for the NY-ESO-1 antibody production nor biopsies were examined for NY-ESO-1 expression (Stockert *et al.*, 1998; Jäger *et al.*, 2000b), the frequency of NY-ESO-1 p157-165 (ESOp157) and NY-ESO-1 p159-167 (ESOp159) peptide tetramer stained CD8 T cells was between 0.2 -0.38 % (**Figure 4.17 A**). Targeting only cells with intense intensity tetramer staining (Yee *et al.*, 1999): (i) 1000 ESOp157 CD8 T cells were isolated from patient-89, and 700 ESOp157 CD8 T cells were isolated from patient-90; and (ii). 140 ESOp159 CD8 T cells were sorted from patient-90, and 840 ESOp159 CD8 T cells were sorted from patient-89. Sorted ESOp157 and ESO159 tetramer-positive CD8 T cells were cloned by FACS by single-cell disposition directly into 96U cloning plates and were expanded as described before (section 4.3.2.2). In two weeks of initial clonal expansion, 56 growing wells were identified from patient-89; but the tetramer screening revealed all clones scored <3 % pMHC positive CD8 T cells. While 49 growing wells were identified from patient-90, all showed <0.5 % tetramer staining (**Figure 4.17 B**). Also, no distinct TET+ populations had formed in any positive wells in either patient. All clones were subjected to another round of stimulation and one week of expansion. The second screening using pMHC-tetramers showed all CD8 T cell clones from patient-90 were between 0.5% and 4% ESOp157-tetramer-positive, whereas clones from patient-89 were up to 26.5%% Eso157-tetramer-positive (**Figure 4.17 C**). All clones specific for ESOp159-tetramer scored less than 1% (not shown). Still, none of the clones from either patient formed distinct tetramer-positive populations; some TET-positivity was noticed. Three T cell clones were derived from patient-90 (ESO157.90.6, ESO157.90.30, ESOp157.90.39) and three clones derived from patient-89 (ESOp157.89.17, ESOp157.89.25, ESOp157.89.33) (**Figure 4.17 C**), but no ESO159 clones were selected for FACS enrichment and further expansion. Low T cell numbers were obtained from patient-89 clones (140 - 280 cells per clone), while between 400 – 800 cells were obtained from clones in patient-90.

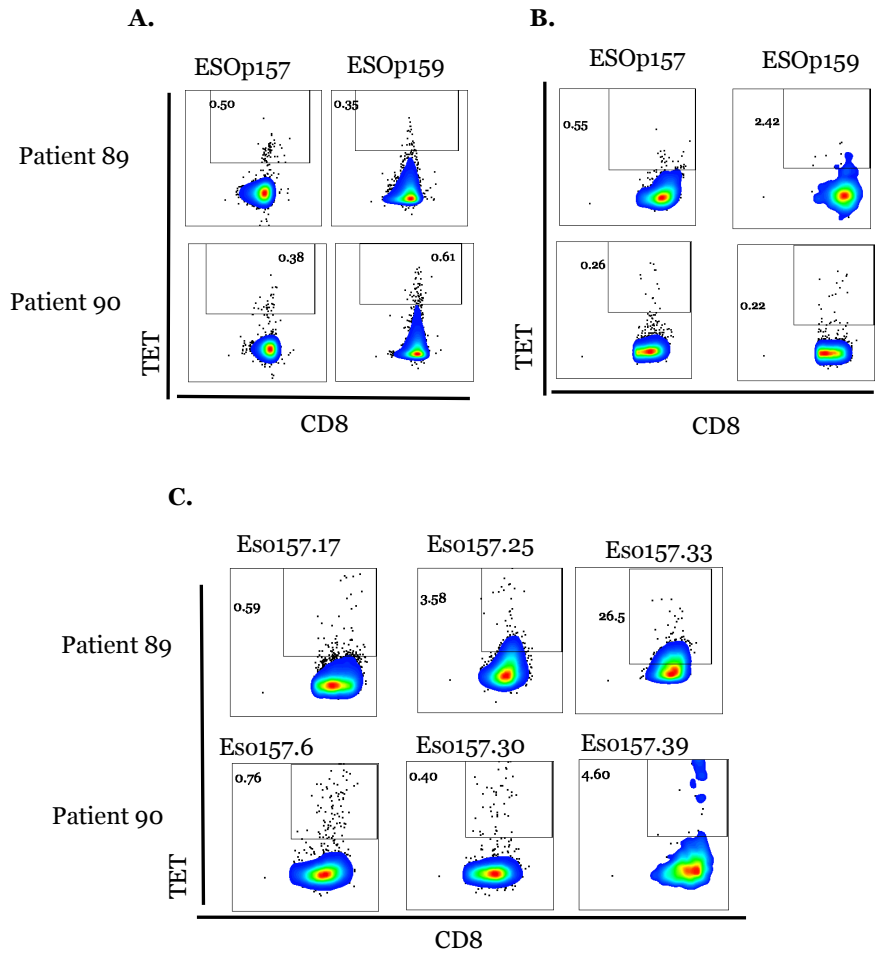


Figure 4.17. Isolation and expansion of NY-ESO-1 specific CD8 T cells by direct cloning from melanoma patients' PBMC.

The procedure for CD8 T cell isolation, pMHC-tetramer T cell enrichment, single-cell cloning, maintenance and characterization was followed as described in Figure 4.2. (**A**) Initial frequencies of CD8 T cells specific for peptides NY-ESO-$1_{157\text{ -}165}$ (ESOp157) and NY-ESO-$1_{159\text{-}167}$ (ESOp159) n patient 89 and patient 90 are shown. Cells were gated on single, live, CD3, CD8 positive cells. (**B**) MHC-tetramer sorted ESOp157 or ESOp159 specific CD8 T cells were cloned directly by FACS single cell disposition, placing one of ESO peptide-specific cells per well into 96U plates. In two weeks of clonal expansion, cells were screened using pMHC tetramers. In patient-89, 56 growing wells were identified and scored >3% ESOp157-MHC-tetramer-positive. In patient-90, 49 identified wells displayed >0.5% tetramer staining, and no distinct populations have formed. Shown is one representative "positive" well per peptide per patient. (**C**) All clones were subject to another round of stimulation and expanded for another week. Second screening using pMHC-tetramers showed all CD8 T cell clones from patient-90 scored between 0.5% and 4% ESop157-tetramer positive, whereas clones from patient 89 were up to 26.5% Eso157-tetramer positive. Three clones derived from patient 90 (ESO157.90.6, ESO157.90.30, ESOp157.90.39) and 3 clones derived from patient 89 (ESOp157.89.17, ESOp157.89.25, ESOp157.89.33) were selected for FACS enrichment and expansion. Gated on single, live, CD3+, CD8+, TET+ cells.

These enriched T cell clones were subjected to another round of stimulation. At the end of week 6, every clone contained about 50-70% ESOp157 tetramer-positive cells that showed dim CD8 expression (**Figure 4.17 B**, **Figure 4.18 A**). Control cultures consisting of stimulated feeder cells did not proliferate (data not shown); thus, it is unlikely these $CD8^{low}$ cells were outgrowing feeder cells,

although this cannot be entirely excluded. These clones were established by FACS-single-cell cloning, gating on CD3+/CD8/TET+ T cells (**Figure 4.18 A**), yet analysis of CD19, CD56, CD3, TCRVβ markers of final products would clarify this phenomenon. Clone ESOp157.89.17 did not proliferate well and was excluded from further analysis due to low cell numbers. The final expansion of the other clones (ESOp157.89.25, ESOp157.89.33, ESO157.90.6, ESO157.90.30, ESOp157.90.39) produced between 24 – 43 million cells, depending on the clone. These clones were examined for functional responses to their cognate peptide in an antigen cross-presentation assay (ACPA) measuring intracellular IFNγ (IFNγ-ICS). Briefly, APCs were pulsed with ESOp157 peptide or unrelated M1p58 and ESO159 to examine singular peptide specificity. ESO-clones and APCs were incubated at a 1:1 ratio in the presence of BRE A for 6hr, and the intracellular IFNγ production was measured by flow cytometry. Upon stimulation with their cognate peptide at 10 μM, no peptide-specific responses were detected in any generated ESOp157 clones. (**Figure 4.18**).

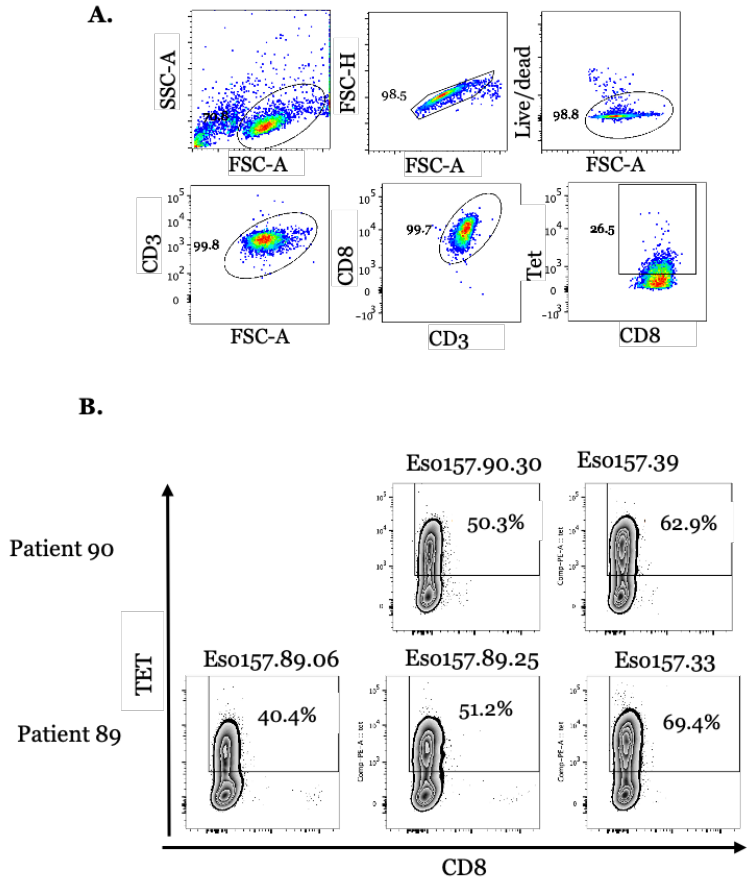


Figure 4.18. Enrichment and expansion of NY-ESO-1 $_{157\text{-}165}$ CD8 T cells.

Three clones derived from patient 90 (ESO157.90.6, ESO157.90.30, ESOp157.90.39) and 3 clones derived from patient 89 (ESOp157.89.17, ESOp157.89.25, ESOp157.89.33) were selected for FACS enrichment and expansion. (**A**) Shown is a representative gating strategy for FACS sorting. (**B**) Enriched NY-ESO-1 T cell clones were subject to another round of stimulation and expansion. Shown are final products after a total of 3 rounds of non-specific stimulation and six weeks of culture. ESOp157.89.17 clone did not produce sufficient cell quantity thus was not included in the analyses. Gated on single, live, CD3+, CD8+, TET+ cells.

Functional ESOp157 reactive CD8 T cells (tESOp157 T cells described in section 4.5) were kindly provided by Andrew Sewell laboratory and were included in the assay as a positive control. These tESOp157.1G4 T cells (called tESOp157 T cells hereafter) were generated by transduction of wt high-affinity 1G4-TCR into bulk peripheral CD8 T cells of healthy HLA-A2+ donor, and their functionality was confirmed (Chen *et al.*, 2005, 2014; Zhao *et al.*, 2005). However, only 9.8%% of TET+ cells showed IFNγ staining in ACP assay upon a challenge with NY-ESO-1p157 peptide presented by T2 cells under the same assay conditions (**Figure 4.19 A**), while no unspecific responses with irrelevant peptides were detected. On the other hand, a positive result was detected when the IFNγ-ELISpot assay was used to examine responses to ESOp157 peptide in tESOp157 T cells, but only a few cells responded in the ESOp159.90.06 clone (figure 4.19 C). Briefly, in the IFNγ-ELISpot assay, T2 cells pulsed with 5 or 10 μM ESOp157 peptide were incubated with tESOp157.1G4 responder T cells or ESOp157.90.06 T cell clone in a 1:1 ratio and were incubated for 16 h before the readout was taken. Per 50 x 10^3 tESOp157 cells >1000 IFNγ- spot forming cells (SFCs) were detected in cultures stimulated with 10 μM, and proportionally less in cocultures stimulated with 5 μM peptide. In contrast, poor responses were detected in the ESOp157.90.06 clone, which may be down to the fact that these cells showed no or very low CD8 expression (Luescher *et al.*, 1995; Laugel, van den Berg, *et al.*, 2007) – since per 1 x 10^5 cells only ~ 25 SFCs were detected. . Nevertheless, seeing poor responses in IFNγ-ICS for tESOp157 T cells suggested that the standard assay duration (4-6 h) is insufficient for the cells to produce enough IFNγ detectable by flow cytometry. In addition, the cytokine release/detection may have been influenced by the concentration and/or type of serum used in the assay medium. Thus, these results indicated that our standard assay routinely used to test the functionality of microbial antigen-specific CD8 T cells (Marlène Brandes *et al.*, 2009; Meuter, Eberl and Moser, 2010) was not optimal for examination of functional responses in tumour antigen-specific CD8 T cells. The ICS assay was then optimised for measuring cytokine responses in tumours antigen-specific CD8 T cells using tESOp157 T cells as a model system (*Section 4.5*). At this point, however, the functionality of NY-ESO-1 clones generated from melanoma patients was not further examined.

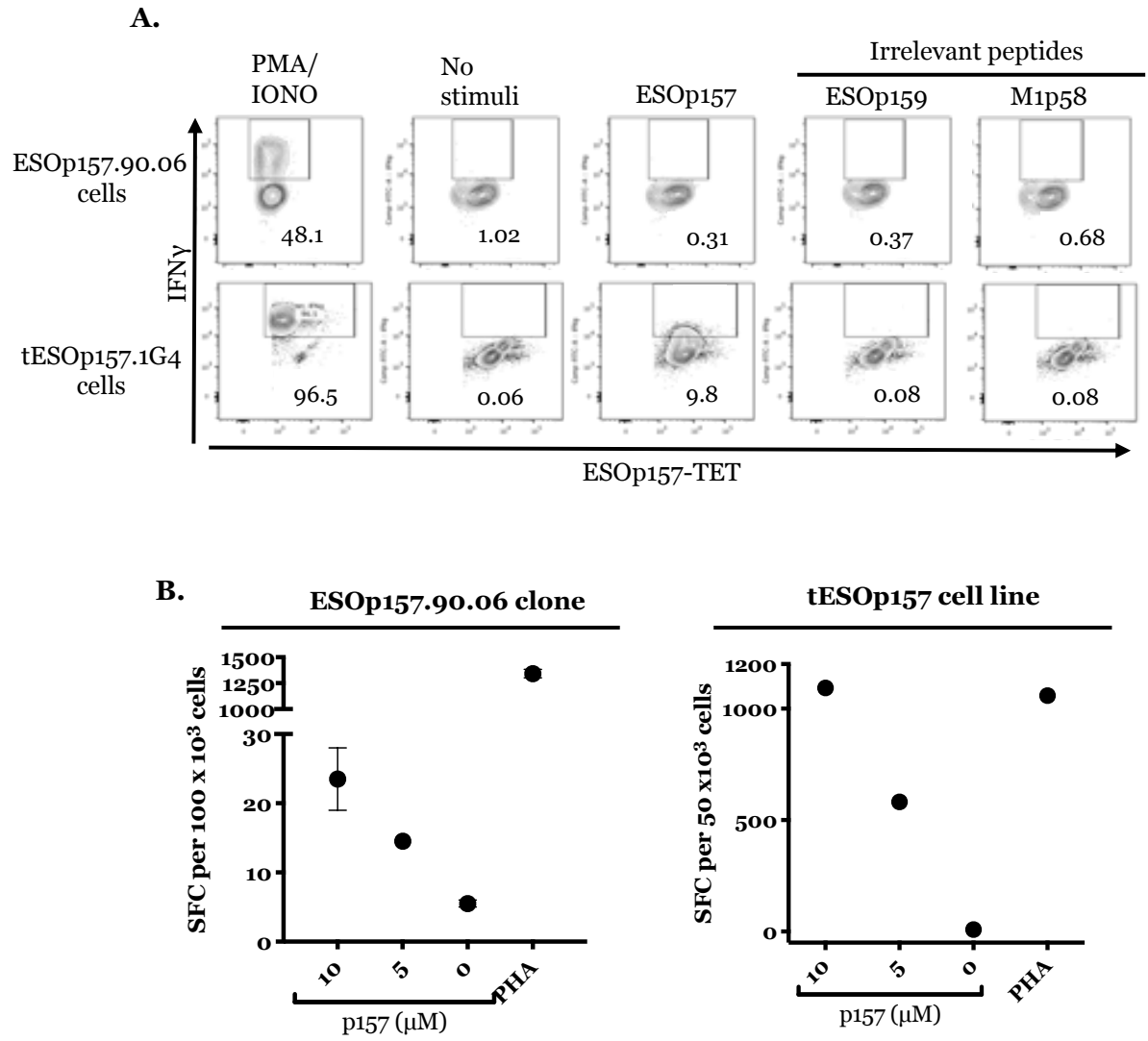


Figure 4.19. Functional characterisation of ESO $_{157\text{-}165}$ specific CD8 T cells generated from melanoma patients.

(A) Initially, the ACP-IFNγ -ICS assay was used to determine the sensitivity of established ESOp157-specific clones to the cognate presented by APCs. Stimulation with PMA/ionomycin (PMA/IONO) represents maximal response; cocultures consisting of CD8 cells and APCs without stimuli (0 μM), and cultures of CD8 T cell clone alone represent negative controls. Two irrelevant peptides were used (M1p58 and ESOop159) to determine "clones'" specificity. Representative assay results are shown for ESOp157.90.06 cells. A similar outcome was seen in all other "clones" tested. A functionally characterised ESOp157-specific CD8 T cell line generated by transduction of ESOp157 specific TCR (1G4) into bulk CD8 T cells (tESOp157.1G4 line) was used as a positive control for the assay performance. Gated on single, live, CD19 negative, CD8+, TET+ cells. **(B)** IFN- ELISpot was used to confirm the peptide sensitivity of the ESOp157.90.06 cells and tESOp157.1G4 cell line. Peptide pulsed T2 cells were used as APC. Stimulation with PHA was used as a positive control; unstimulated T2 cells and CD8 T cell cocultures (0 μM) represent negative controls. Spot forming cell (SFC) per 100 and 50 x 10^3 cells, data points represent mean values of duplicate samples; error bars show ±SD.

Taken together, pMHC-tetramer enriched T cell library Method #2 failed to generate functional NY-ESO-1 specific CD8 T cells from melanoma patient PBMC. Nevertheless, it is not clear whether the

lymphocyte compartment of melanoma patients PBMC did or did not contain HLA-A2 restricted NY-ESO-1 specific CD8 T cells. Serological testing of NY-ESO-1 reactivity in the PBMC or examination of their tumours for expression of NY-ESO-1 protein (Stockert *et al.*, 1998; Jäger *et al.*, 2000b) prior to expansion would be advantageous. While the generation/expansion of NY-ESO-1 specific cells from naïve CD8 T cell population would require a different cytokine cocktail, thus cell sorter-assisted method # 2 seem suboptimal for such task. As IL-21 has also been described to promote and aid expansion of naïve T cells, such culture conditions would be favourable and would be a method of choice for generation of NY-ESO-1 specific CD8 T cells from melanoma patient PBMC.

4.4 Intracellular cytokine staining (ICS) assay for detection of tumour antigen specific CD8 T cell responses

tESOp157 T cells T cells were provided by A. Sewell' lab and were used as a model system to optimise IFNγ-ICS assay to measure responses in tumours antigen-specific CD8 T cells using. These tESOp157 T cells were generated by transduction of wt high-affinity 1G4-TCR into bulk peripheral CD8 T cells of a healthy HLA-A2+ donor (Chen *et al.*, 2005, 2014; Zhao *et al.*, 2005). The analysis showed that these cells consisted of a heterogeneous cell population with varying CD8 expression and 75% ESOp157-MHC-TET^{HI} cells (**Figure 4.20 A**).

To determine the optimal conditions of IFNγ-ICS assay for responses in tumour-antigen specific T cells, the incubation times and the use of serum was evaluated. Briefly, T2 cells were pulsed with 10μM ESOp157 peptide and were incubated with rested responder cells in 1:1 ratio, for 14, 16, 18 and 20 h in the presence of BRE A, and cells were fixed and analysed by flow cytometry. In addition, since using 10%, FSC supplemented assay medium under controlled conditions (no stimulation) showed a stimulatory effect on CD8 T cells (data not shown), 5% serum-supplemented assay medium was used. Antibodies with excitation/emission spectra in different channels were used for an optimal distinction between APCs and responder cells. The peptide-specific responses were equally strong, showing ~50% IFNγ producing CD8 T in all-time points tested (**Figure 4.20 B**), which was not significantly different from responses detected in PMA/IONO stimulated cells. Since long term exposure to BRE A can be toxic, and the cell viability was relatively low in cultures at all time points tested, it did not significantly differ among the tested incubation times (not shown), yet a substantial activation-induced cell death has been noted. However, since this experiment was not repeated for significance, a definite conclusion on cell death/ toxicity induced by BRE A cannot be drawn. Taken together, the optimal IFNγ-ICS assay for the determination of tumour antigen-specific responses in a 5% serum-supplemented assay medium require at least 14 h incubation time in the presence of BRE A.

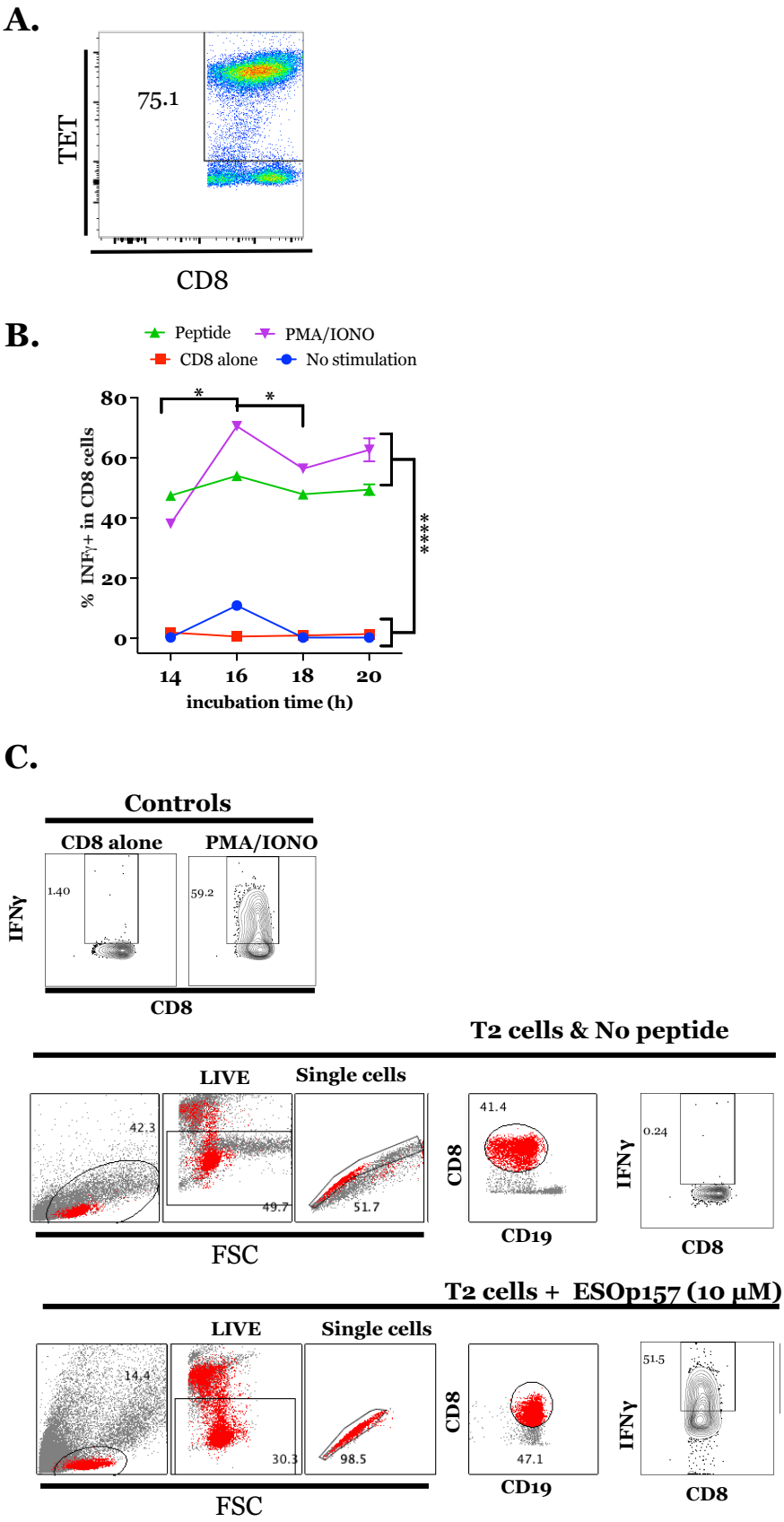


Figure 4.20. Optimisation of INFγ intracellular staining for antigen presentation assays with tumour specific CD8 T cells. (continue on next page)

(A) tESOp157 T cells T cell line generated by transduction of 1G4 TCR into bulk CD8 T cells of a healthy donor was provided by Andrew Sewell laboratory and consisted of ~75% ESOp157-tetramer-positive cells. T2-A2 cells pulsed with ESOp157-165 peptide were used as a model APCs and were co-cultured with rested and washed tESOp157 T cells T cells in the presence of BRE A for 14 – 20 hr. Negative controls included CD8 T cells alone without stimulation, T2 cells + CD8 T cells (no peptide). CD8 T cells stimulated with PMA/Ionomycin served as a positive control. Cells were stained with fluorescently labelled antibodies against surface markers and intracellularly against INFγ and analysed by flow cytometry. **(B**) Shown is the percentage of IFNγ positive CD8 cells at different time points. Statistical significance was determined by two way ANOVA followed by Tukey's multiple comparison test. Data points represent the mean of duplicated samples from a single experiment. **(C)** Gating strategy (back gating) is shown. Positivity for IFNγ was determined in cultures with CD8 responders alone (negative control). Cocultures with CD8 T cell responders and APCs alone (no peptide) served as a negative control. Gated on single, live, CD19-, CD8+ cells. NS, not significant. * = $p<0.05$; ****=$p<0.001$

4.5 Discussion

The identification of tumour rejection antigens in several cancers has initiated the development of strategies for the generation of antigen-specific tumour-reactive T cells for cancer treatment. In addition, it has opened doors to research on the biology of specific T cell subtypes. However, traditional approaches using peptide-pulsed DC or PBMC to generate antigen-specific T cells are met with variable success. These standard techniques have worked well in our laboratory for generating T cells specific for antigen (Ags) of microbial origin, yet robust tumour-Ags specific CD8 T cell clones failed to grow more often than had succeeded. Two obstacles to the practical application of this method have been known across many laboratories and clinical settings. First, the precursor frequency of T cells recognising tumour Ags is very low (for naturally occurring 5T4p17 -specific CD8 T cells is as low as 1 in 107 CD8 T-cells in responding donors (Tykodi *et al.*, 2012). Second, due to the negative selection of high avidity self-reactive T cells, most of the detectable tumour Ag-specific T cells have only a low affinity for the tumour Ag. In addition, studies have demonstrated the loss of high avidity CD8 T cells following *in vitro* stimulation with peptide-loaded APCs, making the isolation of tumour-reactive T cells from patients more difficult (Alexander-Miller, Leggatt and Berzofsky, 1996; Martha A. Alexander-Miller *et al.*, 1996).

A critical requirement for the successful generation of high avidity tumour-reactive CTL clones is the ability to rapidly identify and isolate such clones from the heterogeneous population of T cells. Modern advances in cell sorting, using pMHC multimers and fluorescence or magnetic beads, or functional detection of peptide activated T cells using antibodies have allowed the generation of T-cell clones following physical isolation from *ex vivo* samples. These tools have revolutionised the study of antigen-specific T cells by enabling their visualisation, enumeration, phenotypic characterisation and isolation. In order to circumvent the difficulties encountered with the traditional methods, I established a pMHC-tetramer enriched T-cell library approach described here. A previous study has reported a different T cell library approach to generate various peptide-specific cells but instead used CD3/CD28 beads to expand bulk CD8 T cells before physically isolating peptide-reactive cells by cytokine capture method (Theaker *et al.*, 2016). Although that methodology has been proven successful for microbial and some self-antigens, it remains possible that rare clones are lost during this expansion phase, while it is also reagent and labour intensive. Other reports have used short term peptide stimulation to isolate peptide-responsive T cells by cytokine-capture technique directly from PBMC (Neller *et al.*, 2014). Both methods, however, rely on cloning of effector/memory cells which makes it unsuitable for expanding naïve precursor, while many tumour-antigen specific responders are known to be found predominantly in the naïve pool. In addition, expansion of T cells from early precursors is favourable, especially in clinics, since these cells persist longer. By using improved pMHC tetramer staining techniques (Wooldridge *et al.*, 2009; Tungatt *et al.*, 2015; Rius *et al.*, 2018) to isolate tumour-peptide specific CD8 T cells ensured that also low -affinity but functional TCRs are selected for isolation. Thus, potentially cells from all repertoires are targeted and can be studied. On the other hand, the generation of peptide-specific T cells using pMHC multimers requires knowledge of the epitope target; thus, this method is

not suitable for antigen discovery. Removing the need for extended stimulation with antigen and antigen-presenting cells, while initial cultures of peptide-specific T cell libraries consist of <30 cells, a degree of clonality is already introduced at the culture initiation. Thus, T cell clones can be generated rapidly. Expansion in the presence of irradiated allogeneic feeder cells, PHA, selected cytokines, and HS supplemented medium in the strategy described here have proved successful for simultaneous expansion of a large number of multiple (viral and tumour) antigen-specific CD8 T-cell clones and lines in as little as 4 - 6 weeks. Both PHA and CD3/CD28 Dynabeads have been shown to distort the TCR repertoire over time during *in vitro* expansion from PBMC (Neller *et al.*, 2012). While using a pure human serum-supplemented medium can help preserve TCR repertoire while improving the final number of expanded CD8 T cells (Röth *et al.*, 2007; Block *et al.*, 2008). Here, limiting dilution cloning was utilised after the expansion/enrichment of peptide-specific T cells. Cloning by FACS single cell disposition would potentially eliminate the occasional need for additional enrichment or re-cloning of established clones, as seen for some clones (**Figure 4.6** and **4.8**), although it can be harsh for cells. In addition, by avoiding the selective outgrowth of more rapidly dividing cells, direct cloning would likely enhance the repertoire diversity of the resulting collection of clones.

For this study, PBMC from 6 healthy HLA-A2+ donors were used to generate 5T4 specific CD8 T cells, and PBMC from 2 melanoma HLA-A2+ patients were used for the generation of NY-ESO-1 specific CD8 T cells. Using PBMC of two young donors and traditional methodologies (peptide stimulation), rapid generation of 5T4-specific CD8 T cell clones was unsuccessful. Nevertheless, it cannot be excluded that this failure may have been due to no 5T4-peptide-specific T cells present in the memory repertoire of these healthy donor PBMC (Smyth *et al.*, 2006; Tykodi *et al.*, 2012). With the pMHC enriched T cell library methodology described here, a simultaneous establishment of two different 5T4-peptide-specific CD8 T-cells from the memory repertoire of healthy donors in the presence of IL-2 and IL-15 was possible. Using this methodology, CD8 T cells specific for immunodominant nonamer peptides 5T4p17 and 5T4p97 were identified and sorted from fresh PBMC of four healthy donors. I established a total of 3 functional 5T4p17 -specific CD8 T cells clones and two functional 5T4p97 -specific CD8 T clones and many polyclonal cell lines from healthy donors of age group ≥30 years. Although this is likely an underestimate of the total number of the 5T4-specific CD8 T clones generated since only selected clones were tested. The data presented here are in line with previous observations that found 5T4 specific CD8 T cells in 4/6 healthy donors, while responses were more prevalent against the 5T4p17 epitope than 5T4p97 (Tykodi *et al.*, 2012). Similarly, in response to peptide challenge, 5T4p17 specific CD8 T cells generated in this study produced up to 5 fold more IFNγ than 5T4p97 CD8 T cells.

p17.BM.A01, p17.BM.A11 "clones" scored the highest number of IFNγ spot forming cells upon challenge with 5T4p17 peptide than p17.BM.A33 cells (**Figure 4.7**). This is not surprising, since although the p17.BMA33 clone showed the highest percentage of tetramer stained cells; it was a heterogeneous cell population with lower TET-MFI than other clones (**Figure 4.11 A**), suggestive of low-affinity TCRs. In contrast, p17.BM.A01 and p17.BM.A11 cells that showed 65% and 73% 5T4p17 -TET staining, respectively, also showed the highest TET-MFI values (**Figure 4.11 A**). While there are many different mechanisms by which CD8 T cells tune their responses, these results go in line with reports that demonstrated a positive correlation between TCR affinity for its cognate peptide or the TCR-cell surface

density and the strength of response (Laugel, van den Berg, *et al.*, 2007). In agreement with a previous report (Tykodi *et al.*, 2012) these data demonstrate heterogeneous and polyclonal reactivity to the 5T417 epitope for responding individuals. Examination of the TCRVβ gene segment family expressed by all isolated and expanded 5T4p17-specific clones would explain these differences. It would be interesting to see if these compare to Tykodi *et al.*, who found three unique TCRVβ chains among all donors (Tykodi *et al.*, 2012). Since this study aimed to generate IFNγ secreting T cells to serve as a tool in downstream immunological studies, other functional abilities were not examined here, but an expression of CD107a was measured simultaneously.

There are several methods for estimating cytotoxic activity. As degranulation occurs, secretory lysosomes are released, and the lysosome-associated membrane protein-1 (LAMP-1) or CD107a is transported to the surface of cytotoxic cells, making it accessible for antibody binding. This allows the identification of CD8 T cells that have been activated for degranulation. Studies by Aktas *et al.* have demonstrated that by evaluating CD107a production, one can determine cells' cytotoxic ability (Aktas *et al.*, 2009). On the other hand, the secretion of cytokines such as interferon IFNγ can enhance their cytotoxicity but cannot induce it. It was shown that peptide reactivity resulting in cytokine release alone is not a rigorous marker for determining a true anti-tumour activity T cell activity (Yee *et al.*, 1999), thus, it cannot be used for evaluating cytotoxicity. Here, the CD107a production by 5T4p17 clones was intermediate-low, suggesting a modest cytotoxic potential of the functionally examined 5T4 clones. To further confirm this finding, lysis of the CFSE-labeled target cells or a chromium release assay would directly define 5T4-CD8 T cells' cytotoxic/lytic capacity. In addition, determining the level of perforin and granzymes would add additional information about CD8 T cells lytic/cytotoxic potential. Tykodi and colleagues reported a high cytolytic ability in a 5T4 specific clone whereby their cells exhibit poor IFNγ production. Although the authors have not examined the memory differentiation phenotype in their 5T4 specific CD8 T cells, their high lytic capacity combined with IFNγ production suggest a late T_{EM} phenotype, which is different from the 5T4 CD8 T cells generated here in this study. Like Tykoidi and colleagues, I also found only one healthy individual that responded to the 5T4p97 epitope. While I was able to establish two "clones" from this donor, these cells had minimal replicative potential, and in six weeks, did not produce enough cells for research; thus, these 5T4p97 cells were also not evaluated for CD107a production. Although I have concluded that insufficient cytokine supplementation was responsible for such an outcome, it cannot be excluded that these cells had a reduced proliferative potential. A more detailed phenotype analysis would provide a more conclusive answer.

Utilising the pMHC-enriched T cell library approach to generate 5T4-reactive CD8 T cell clones by non-specific stimulation (Method #2), investigations were expanded to see if the versatility of this method could generate clones against NY-ESO-1 targets from melanoma patients PBMC. I have targeted only CD8 T cells with intense tetramer staining, expecting expansion of high avidity tumour-reactive T cells (Yee *et al.*, 1999). Unfortunately, the same methodology failed to establish functional NY-ESO-1 peptide specific CD8 T cells from melanoma patients. Although the functional assay (IFNγ-ICS) was not fully optimised at the time of analysis, examination by IFNγ-ELISpot has confirmed inadequate responses. At the same time, it should be noted that after three rounds of stimulation and eight weeks of cell culture, all NY-ESO-1-specific clones showed a dim CD8 expression. One explanation could be that

these cells have expanded into a memory population of $CD8^{low}$ expressing cells (Littman and Singh, 2007).

On the other hand, it is also known that various signals regulate CD8 levels on peripheral CD8 T cells, allowing dynamic tuning of immune responsiveness. Furthermore, downregulation/upregulation of CD8 expression is also known to occur as a homeostatic mechanism for cellular self-regulation to "tune" responsiveness according to the affinity of their TCRs (Park *et al.*, 2007; Harland *et al.*, 2014). Thus, if downregulation occurred due to high-affinity TCR, one would expect that these NY-ESO-1-specific clones possess super high-affinity TCR and still respond to a peptide challenge. In contrast, coupled with low-affinity TCR, low CD8 expression would potentially render cells dysfunctional (Laugel, Price, *et al.*, 2007) – which seemed the case for these "clones". However, as no further investigation was carried out at this stage, solid conclusions cannot be drawn. Of note, normally NY-ESO-1 specific CD8 T cells are only found in individuals whose tumours express the NY-ESO-1 antigen or are seropositive and are not found in healthy individuals (Stockert *et al.*, 1998; Jäger *et al.*, 2000b). Although only about 10% of melanomas express NY-ESO-1 antigen, antibodies to NY-ESO-1 were found in 40–50% of patients with advanced NY-ESO-1-expressing tumours.

On the other hand, patients with soft tissue sarcomas can express very high levels of NY-ESO-1, but the often undetectable levels of NY-ESO-1 specific CD8 T cells in their peripheral blood makes it challenging to generate autologous NY-ESO-1 T cell products for adoptive immunotherapy (Pollack *et al.*, 2014). Thus, if these patients PBMC did not contain the cells of interest, it would, however, mean that the pMHC tetramers exhibited non-specific binding, and irrelevant cells were expanded. Although lineage analyses (CD45RA/^{RO},CCR7,CD62L,CD28,CD95) was not performed on freshly isolated cells to discriminate the origin of NY-ESO-1-pMHC-tetramer-positive CD8 T cells in this study, a previous study reported that some tumour antigen-specific CD8 T cells could be found in the naïve T cell compartment (Pittet *et al.*, 1999). Thus, the lack of success could have been potentially attributed to culture conditions lacking appropriate cytokines. Although together with TCR signal, IL-7 is typically required for survival of naïve T cells. While it has been shown that even in the absence of TCR stimulation, the individual common γ-chain cytokines including IL-2, IL-7 and IL-15 can increase CD8 levels on naive CD8 T cells *in vitro*, the combination of certain cytokines (Type 2) can have an opposite but reversible effect on CD8 expression in naïve CD8 T cells (Harland *et al.*, 2014). Thus, since the culture conditions here consisted of irradiated allogeneic feeder cells, the cytokine release of mismatched cells may have induced CD8 downregulation and redirected the differentiation of NY-ESO-1-specific CD8 T cells into Tc2 cells. A potentially successful establishment/expansion of functional NY-ESO-1 specific CD8 T cells from these patients PBMC would require modified culture conditions (Geginat, Sallusto and Lanzavecchia, 2003; Pollack *et al.*, 2014; Snauwaert *et al.*, 2014). In line with this, a previous report from Warren & Yee's lab described a successful *in vitro* generation of NY-ESO-1- clones from sarcoma patients using peptide-pulsed DCs in a culture medium supplemented with IL-21 (Pollack *et al.*, 2014). In line with this, and with method optimisation conducted here, future attempt in generating NY-ESO-1 specific CD8 T cells would include IL-21 as described in *section 4.3.3*.

IL-21 is a cytokine belonging to the IL-2 γ-chain receptor cytokine family (IL-2, IL-7, IL-15) and appears to share many of the properties associated with T and NK cell activation and differentiation (Li, Bleakley

and Yee, 2005; Liu *et al.*, 2015). In addition, a few studies have reported that administration of IL-21 could substantially increase the total number of antigen-specific CD8 T cells and enrich for a population of high-affinity CD8 T cells, including the pool of NY-ESO-1 specific CD8 T cells (Moroz *et al.*, 2004; Li, Bleakley and Yee, 2005; Liu *et al.*, 2007, 2015; Pollack *et al.*, 2014; Tian and Zajac, 2016).

I explored the IL-21 administration during the initial expansion period using circulating 5T4-specific T cells as a model system to define the effect in this strategy for the generation of tumour peptide-specific T cells (*Section 4.2.2.4*; **Figure 4.15 - 17**). I found that administration of IL-21 alone or in combination with IL-2 and IL-15 in the initial steps of expansion greatly improved enrichment of 5T4p17 - peptide specific CD8 T cells. While in contrast to previous studies, where Li and colleagues (Li, Bleakley and Yee, 2005) reported that IL-21 alone could increase the total number of tumour antigen (MART-1)-specific CD8 T cells by >20-fold, here IL-21 increased the total number of 5T4p17 - peptide-specific CD8 T cells for 1.4 fold. This is not surprising, seeing that the comparison was to cultures administered with IL-2 & IL-15 only, while they compared the cultures to the ones exempt from any cytokine administration (Li, Bleakley and Yee, 2005). Despite this, such drastic improvement can undoubtedly be attributed also to the fact that MART-1-specific T cells are the most frequent and accessible population of tumour-antigen specific T cells (Pittet *et al.*, 1999).

Since I have not examined the origin of 5T4-specific CD8 T cells at the culture initiation, unfortunately, the elucidation of whether IL-21 contributed to the expansion of memory or naïve T cells or both cannot be defined at this stage. While previous data suggest IL-21 substantially influences the antigen-driven expansion of naïve T peptide-specific CD8 T cells, this may be different in my culture setting with mitogen stimulation and irradiated feeder cells, as opposed to cultures containing autologous DCs and peptide stimulation (Li, Bleakley and Yee, 2005; Pollack *et al.*, 2014). Furthermore, IL-21 has been shown to enrich a population of high-affinity CD8 T cells at the clonal level with sustained CD28 levels and a helper-independent phenotype (Li, Bleakley and Yee, 2005). 5T4p17 -specific T cells generated here in this study showed variable CD28 expression and predominantly CD45RA+/CD27+ phenotype, suggesting a stem cell – memory like T (T_{SML}) cell phenotype (Gattinoni and Restifo, 2013). However, further examination of CD45RO/CCR7/CD62L/CD95 expression is required for determining a more precise phenotype.

Nonetheless, these data suggest that like the combination of CD3/CD28 stimulation with IL7 and IL-15 administration (Gattinoni and Restifo, 2013), PHA in a combination of IL-21, IL-15 is also capable of generating T_{SML} cells. Unfortunately, the differentiation phenotype was not determined on freshly isolated peptide-specific T cells at the initiation of cultures. Thus it is not possible to determine whether these cells originated from the T_N cell population or have differentiated from the memory repertoire (Zeng *et al.*, 2005). It would be interesting to set up initial cultures containing only T_N, T_{CM}, or T_{EM} peptide specific CD8 T cells and follow their linage differentiation and functional potential in the presence of IL-21.

Since polyclonal tumour-peptide specific T cells were generated, the proper approach to examine TCR affinity in these 5T4p17 specific T cell population would have been at the single-cell level using in situ TCR affinity and sequence test (iTAST) (Zhang *et al.*, 2016), and then perform experiments to

examine/correlate TCR affinity with T cell avidity. Due to lack of expertise and time for the execution, the intensity of pMHC tetramer binding was used as a measurement (Yee *et al.*, 1999). According to Yee and colleagues, the higher affinity T cells bind the tetramer better than lower affinity T cells. It was demonstrated that the staining intensity is not related to the density of TCR expression since the flow cytometric analysis using anti-pan TCR Ab revealed no difference in staining between high vs low avidity T cells (Yee *et al.*, 1999). Thus, the variation in staining intensity suggests that the dissociation rate for TCR-pMHC interaction may be sufficiently rapid for low-affinity clones; therefore, a lower proportion of tetramer-bound TCRs at a given time will result in lower fluorescent intensity. In contrast, it is assumed that the anti-TCR Abs are superior in detecting the difference in TCR density than pMHC tetramers. Thus, data here in this study suggest that a combination of IL-21, IL-2 and IL-15 is also good in mediating expansion of high-affinity/avidity T cells, seeing that all T cells lines generated under the influence of all three cytokines presented a very clean population of cells with high-intensity tetramer staining.

In contrast, the polyclonal lines generated with IL-21 alone potentially contained both high and low-affinity TCR. It should be noted that the isolation (sorting) of pMHC tetramer specific cells was carried out by MACS technology (Miltenyi) which does not permit for selection of particular populations. Thus, all cells that bind pMHC tetramer will also bind the magnetic beads, and cells of high and low affinities will be sorted. Overall, IL-21 conditioned media established highly peptide-responsive 5T4p17-specific CD8 T cells, which have shown a better cytotoxic potential (defined by CD107a expression) as compared to their counterparts generated in IL-2 & IL-15 conditioned media (**Figure 4.15**), while memory differentiation phenotype was favourable (**Figure 4.14**). Many tumour-specific antigens provoke only weak immune responses that are generally incapable of eliminating all tumour cells. Predicting the potency of activation and T cell response is desirable since it will assist in the rational design of anti-tumour vaccines. Although there are many molecules on the surface of T cells that interact with ligands on other cells, the TCR/pMHC is the only receptor-ligand pair that offers antigen specificity and dictates the functional response of the T cell. Any given TCR can bind to a large number of distinct pMHCs, leading to various functional outcomes. Depending on the engaged pMHC, T cells can be activated (stronger or weaker agonist) or inactivated (antagonist), or pMHC binding can have no effect (null peptide). Accordingly, it is generally believed that the outcome of a T cell response is determined by the biochemical parameters of the TCR-pMHC interactions, whereby the potency of the response depends on the "strength" of a pMHC binding to the TCR. However, there is controversy over which chemical parameters governing binding are the primary determinants of successful activation. To obtain a more precise correlation of T cell response to TCR affinity, more detailed pMHC titrations to determine the potency (EC50) of the T response can be examined by one of many kinetics or affinity models (Lever *et al.*, 2014). However, one must be cautious when explaining T cell avidity using data obtained by current model systems since the generally positive correlation between TCR affinity and an immune response is questionable. Despite extensive experimental work and many mathematical models framed, the relationship between the TCR–pMHC binding parameters and T cell avidity remains elusive for the available data are far from conclusive and even controversial, especially for the low-affinity TCRs (Stone, Chervin and Kranz, 2009; Lever *et al.*, 2014). In addition to the strength of the TCR/pMHC

interaction, other cellular parameters along with the environment in which this interaction takes place are fundamental to how the T cell will respond. The relationship between ligand recognition and T cell avidity is complex and relies on the influence of co-receptor, costimulatory/inhibitory receptors, cytokines, or the differentiation state of a T cell. The CD8 co-receptor has received the most attention so far, as it is directly involved in T cell activation and can significantly enhance or reduce its activity (Stone *et al.*, 2011). The engagement of CD8 with pMHC I-complexes at the cell surface enhances the association rate of pMHC I-complex with the TCRs and increases the half-life of cognate TCR/pMHC I interaction by stabilising this interaction (Laugel *et al.*, 2011). The main contribution of CD8 to T cell activation may be from the enhancement of signalling by recruiting lck tyrosine kinase to the TCR signalling complex and second messenger pathways downstream of TCR triggering (Spear *et al.*, 2019). However, to add additional complexity to predicting T cell activation/response, it was shown that T cells could modulate their antigen sensitivity and response by tuning their CD8 co-receptor expression level via epigenetic reprogramming (Harland *et al.*, 2014).

There is an assumption that T cells with high-affinity TCRs possess a competitive advantage over the low-affinity TCR cells during the immune response because they would receive stronger and more prolonged activation signals (Stone, Harris and Kranz, 2015; Hoffmann and Slansky, 2020). How TCRs recognise pMHC complexes is important, yet the relationship between TCR structure, signalling strength, antigen affinity, and T cell response are not fully defined. A higher affinity TCR engagement can lead to better antitumour response only to a certain extent. For example, studies in mice have demonstrated that administration of high concentration of peptide antigen to high-affinity CTLs can cause proliferative inhibition and activation-induced death, partially induced by TNFα release (Martha A. Alexander-Miller *et al.*, 1996). Moreover, higher affinity T cells lose the ability to modulate specificity or cross-reactivity rationally. Thus, giving blinded importance to a single parameter - reinforcing interactions with the MHC at the expense of the peptide - more likely worsens off-target toxicity.

On the other hand, under suitable conditions, low-affinity CD8 T cells can control tumour growth equally well as high-affinity TCR cells (Hoffmann and Slansky, 2020), and in that, CD4 T cells help enhances their avidity against tumour cells *in vivo* (Bos *et al.*, 2012). In contrast, co-inhibitory molecules can regulate t-cell activation and response. Receptors such as LAG-3, PD-1 and TIM3 have been shown to negatively control activation responses of tumour infiltrating CD8 T cells (Anderson, Joller and Kuchroo, 2016).

Last but not least, here I have employed molecular advances to strengthen identification, isolation and expansion of rare and difficult tumour peptide-specific CD8 T cells from PBMC of healthy donors and cancer patients. The use of protein kinase inhibitors prevents TCR internalisation, and antibodies stabilise/secure pMHC-tetramer binding (Tungatt *et al.*, 2015); thus, enhanced staining and visualisation are achieved that conversely improves isolation of peptide-specific T cells. Moreover, the addition of IL-21 increased a final number of 5T4- peptide-specific T cells and seemed to improve their phenotype and reactivity to their cognate peptide. Despite that, the overall avidity of generated tumour peptide-specific T cells was still low compared to FluM1-specific CD8 T cells. At least 1000 fold more peptide was required to achieve half-maximal response in tumour peptide-specific T cells compared to FluM1 CD8 T cells (**Figure 4.7 and 5.8**). It is well known that in contrast to the TCRs recognising self-

antigens, the TCRs recognising pathogenic antigens are generally of higher affinities/avidities for their cognate peptides (Laugel, van den Berg, *et al.*, 2007; Stone, Harris and Kranz, 2015); thus, the generation of highly responsive anti-microbial CD8 T cells can be relatively fast and efficient. While the affinity issues from the TCR perspective on the respective natural CD8 T cell is fixed, one way to can tackle such issues could be by the use of modified synthetic peptides with increased antigenicity - created by substituting amino acid residues that do not impair TCR recognition (Laugel, van den Berg, *et al.*, 2007; Chen *et al.*, 2014; Galloway *et al.*, 2019).

Collectively, the results of this study demonstrate that rare tumour-antigen specific T cells can be successfully isolated from the peripheral blood of cancer patients and healthy donors and expanded to large numbers sufficient for extensive research demands. The pMHC-enriched T cell library approach together with non-specific stimulation and IL-2 and IL-15 cytokines in the presence of allogeneic feeder cells described here successfully generates a population of tumour antigen-responsive CD8 T cell cells from effector/memory repertoires; presumably, it reflects the active, circulating anti-tumour population. Conversely, expansion of CD8 T cells in the presence of IL-21 improves the expansion rate while sustaining a more desirable phenotype and functionality of generated cells, and thus is a method of choice.

In summary, the *ex vivo* strategy for generation of tumour peptide-specific CD8 T cells from pMHC-enriched T cell libraries and non-specific stimulation described here provides a relatively rapid, powerful and effective method for deriving multiple T cells clones and lines of various TCR specificities ready for research demanding large cell quantities.

Chapter 5 CROSS-PRESENTATION of TUMOUR ANTIGENS BY Vγ9/Vδ2 T cells

5.1 Introduction

As the prototype pro-APCs, DCs were long considered the optimal cell type for cell-based immunotherapies of various diseases, including cancer (Janikashvili, Larmonier and Katsanis, 2010). However, several limitations have become apparent, which impede the use of DC-based cellular vaccines in the clinic. One of the greatest issues is that not all types of DCs possess cross-presenting abilities. With mo-DCs and pDC1 being the only subtypes with antigen-presenting properties, this process has been shown efficient only for antigens of microbial origin rather than tumour origin. Another main issue is obtaining large numbers of antigen-loaded DCs with ease (Landmeier *et al.*, 2009) due to monocytes' inability to proliferate while most die during their differentiation into moDCs *in vitro* (Sallusto and Lanzavecchia, 1994). Furthermore, only a minimal number of studies show tumour antigen-cross presentation and TAA-specific T cell activation by DCs (Salimu *et al.*, 2015; Sánchez-Paulete *et al.*, 2017; Fu and Jiang, 2018; Ho *et al.*, 2018). Numerous studies have evidenced that DCs are relatively inefficient in taking up and processing exogenous TAAs for cross-presentation and thus cannot induce robust responses in TAA-specific CD8 T cells (Fu and Jiang, 2018).

In contrast, human Vγ9/Vδ2 T cells appear to possess favourable functional plasticity and can be easily expanded *ex-vivo* (Vantourout and Hayday, 2013; Lafont *et al.*, 2014; Fowler and Bodman-Smith, 2015; Tyler *et al.*, 2015). Moreover, with the ease of their manipulation, they show great potential for immunotherapy as a complementary or integrative strategy. On the one hand, human Vγ9/Vδ2 T cells possess cytotoxic effector functions against infected and cancerous cells in an MHC-independent manner and both TCR-dependent and -independent manner. For example, extensive investigations of human blood Vγ9/Vδ2 T cell effector functions against HMBPP producing bacteria, cancer cell lines, and tumour stem-like cells (Himoudi *et al* 2012)(Chen *et al.*, 2017) has highlighted their functional robustness. Other investigations have highlighted that Vγ9/Vδ2 T cells can also use a direct cytolytic function against cancer cells expressing stress-inducible ligands like MICA, MICB and ULBPs via NKG2D (Bauer, 1999; Wrobel *et al.*, 2007). At the same time, specific TLR ligands have been shown to directly or indirectly enhance and modulate Vγ9/Vδ2 T cell functions (Oberg *et al.*, 2011; Wesch *et al.*, 2011; Reynolds and Dong, 2013). On the other hand, they can also function as pro-APCs upon stimulation with phosphoantigens.

The discovery of APC characteristics and function in human blood Vγ9/Vδ2 T cells was pioneered in our laboratory (Brandes *et al.*, 2003) (Brandes, Willimann and Moser, 2005), was and later confirmed by others (Landmeier *et al.*, 2009; Wu *et al.*, 2009; Himoudi *et al.*, 2012; Howard *et al.*, 2017; Junqueira *et al.*, 2021). The processes involved *in vitro* cross-presentation of exogenous antigens of microbial origin to CD8 T cells by both short-term activated primary Vγ9/Vδ2 T cells and expanded Vγ9/Vδ2 T cells have been well characterised, and cross-presentation of antigens of various microbial sources has been demonstrated (Brandes *et al.*, 2009; Meuter *et al.*, 2010). To date, one study (Himoudi *et al.*, 2012)

established a co-culture system with Vγ9/Vδ2 T cells as APCs, cancer cells as a source of antigen, and antigen-specific CD8 T cells as responders. The authors of that study suggested that the opsonisation of targets with humanised antibodies may be required to "license" antigen cross-presentation by Vγ9/Vδ2 T cells to antigen-specific CD8 T cells. In that, they argue that a secondary signal via CD16 is required for phagocytosis of foreign antigens as well as it is an essential factor for efficient cross-presentation by Vγ9/Vδ2 T cells (Wu *et al.*, 2009; Himoudi *et al.*, 2012). In contrast, reports from our laboratory show considerable donor to donor variation in expression of CD16 in expanded Vγ9/Vδ2 T cells (Chen *et al* 2015), while the cross-presentation of microbial antigens is still intact, suggesting CD16 is not needed for APC function.

In this chapter, initial experiments were focused on optimising the culture conditions that generate γδ T-APCs with an optimal phenotype necessary for efficient tumour antigen-specific CD8 T cell stimulation. By optimising these protocols, a useful reference point would be generated for the comprehensive study of uptake, processing and cross-presentation of different kinds of self-antigens by γδ T-APCs as well as their ability to stimulate different T cell types under different environmental conditions (e.g. such as those of TME). This chapter describes a time point at which stimulated Vγ9/Vδ2 T cells to acquire the optimal professional APC phenotype and are then-after called γδ T-APCs. These *in vitro* generated γδ T-APCs were then used to study antigen-cross presentation of soluble tumour antigens and antigens expressed in cancer cells. Finally, the ability of Vγ9/Vδ2 T cells to cross-present soluble tumour antigens and antigens naturally expressed by cancer cells is demonstrated.

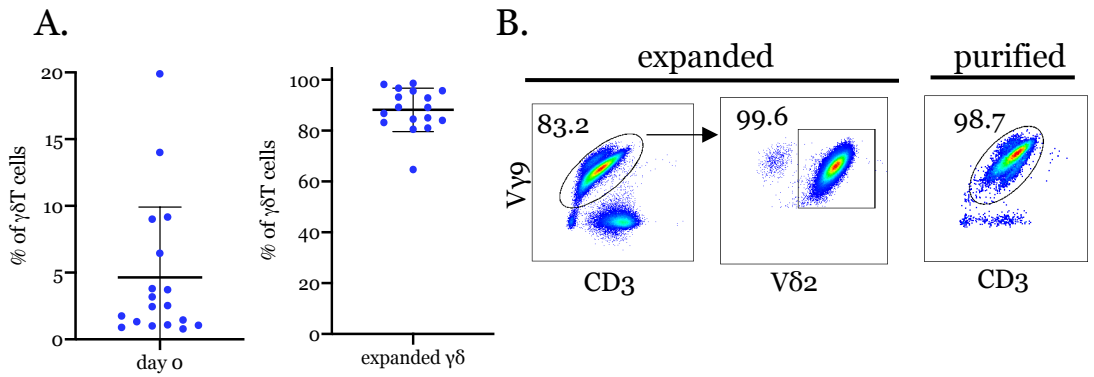
Aims

- *Ex vivo* expansion and characterisation of Vγ9/Vδ2 T cells
- Generation and optimisation of γδ T-APC protocols.
- Comparison of APC function in t *ex vivo* expanded Vγ9/Vδ2 T cells, and short term stimulated Vγ9/Vδ2 T cells (γδ T-APC)
- Analysis of acquisition of APC phenotype in Vγ9/Vδ2 T cells by stimuli from cancer cells
- Comparison of expanded Vγ9/Vδ2 T cells' and γδ T-APCs' ability to internalise cancer cell material
- Examination of cross-presentation efficiency of tumour antigens obtained from cancer cell lines by Vγ9/Vδ2 T cells

5.2 *Ex vivo* expansion of Vγ9/Vδ2 T cells and generation of γδ T-APCs

5.2.1 Selective expansion and phenotype characterisation of Vγ9/Vδ2 T cells

A series of experiments were sought to determine the expression level of markers associated with pro-APC phenotype throughout the Vγ9/Vδ2 T cell expansion period. For the selective expansion of Vγ9/Vδ2 T cells, PBMCs derived from healthy donors (n =17) were stimulated with zoledronate (ZOL) and cultured for two weeks in the presence of IL-2 and IL-15 starting from day five onwards. Although general protocols for expansion of Vγ9/Vδ2 T cells usually only include IL-2, the rationale behind the use of IL-15 is that previous studies from our lab demonstrated that the combination of IL-2 and IL-15 is approximately 10-fold better than IL-2 alone in terms of cell survival, γδ T cell count, and purity (Khan *et al.*, 2014). More recent extensive experimental work (Tyler *et al.*, 2017) has also demonstrated that IL-15 equally influences the "pro-APC maturation" of Vγ9/Vδ2 T cells, rendering them with fully functional APC abilities; that is, promoting their proliferation, APC phenotype, cytokine production, and increased ability to take up soluble antigen. For Vγ9/Vδ2 T cell phenotype examination, cells were stained using an eight-colour panel of fluorochrome-conjugated antibodies and analysed by flow cytometry. Lymphocytes were gated based on FSC vs SSC characteristics, and Vγ9/Vδ2 T cells were gated based on the expression of Vγ9 TCR and CD3. Initially, the Vγ9/Vδ2 T cell frequency within the CD3 population in freshly isolated PBMC was 4.64±5.28%, and at two weeks of expansion reached 86.1±9.58% (**Figure 5.1 A**). In agreement with previous work from our lab and others (Kondo *et al.*, 2011; Khan *et al.*, 2014; Xiao *et al.*, 2018), >99% of zoledronate expanded Vγ9 expressing γδ T cells co-expressed the Vδ2 TCR chain at the end of expansion period (**Figure 5.1B**).



***In vitro* expansion of Vγ9/Vδ2 T cells from PBMC**	Mean (± SD)
Input of Vγ9+ T cells (x 10^6/10^6 PBMC)*	0.0618 ± 0.056
Live CD3+ cells at day 14-15 (%)**	94.7 ± 8.56
Vγ9+ T cells at day 14-15 (%)**	90.2 ± 5.56
Yield of Vγ9+ T cells (x 10^6 /10^6 PBMC)***	6.89 ± 4.39
Expansion fold +	1740 ± 964
* Initiation of cultures at 2 x 10^6 PBMC per well (24-well plate). ** Percentage of total live (Aqua) cells. *** Number of Vγ9+ T cells per 10^6 of input PBMC after 14 days of culture. + Total yield of Vγ9+ T cells divided by input Vγ9+ T cells.	

Figure 5.1. Selective Vγ9/Vδ2 T cell expansion.

Freshly isolated PBMC from healthy donors were stimulated with zoledronate (ZOL) to facilitate the selective expansion of Vγ9/Vδ2 T cells and cultured in the presence of IL-2 and IL-15 from day five onwards. (**A**) Frequency of Vγ9/Vδ2 T cells (among the CD3 population) in freshly isolated PBMC (left) and two weeks expanded Vγ9/Vδ2 T cells (right). Each data point represents individual donors from individual experiments. The horizontal line and error bars display the mean of samples ±SD. Gated on live, single, CD3+, Vγ9+ cells. n = 17. (**B**) Example plot taken from one representative donor at the end of the expansion period demonstrates the selective expansion of Vγ9/Vδ2 cells at day 14. Expanded cells were enriched (purified) to ≥95% Vγ9/Vδ2 T cell purity using custom made γδ negative selection kit (see methods) before being used in experiments. The table below shows Vγ9/Vδ2 T cell expansion details.

To evaluate the potential of ZOL-expanded Vγ9/Vδ2 T cells serving as pro-APCs, the expression levels of MHC class I and II molecules and immune-co-stimulatory molecules - hereafter collectively called pro-APC markers - were examined (**Figure 5.2**). This can be used as a phenotypical readout for pro-APC function. In rested cells, relatively low expression of CD86 (10.38 ± 17.87 %), HLA-DR (4.1 ± 4.89 %) CD40 (1.05 ± 0.86 %), CD80 (0.5 ± 0.24 %) were observed, while CD83 and CCR7 presented at low-intermediate level (20-30 %). CD70 was variable, with most of the donors presenting intermediate-high expression levels (50-70 %) and two donors showing low expression levels (0-10 %) (**Figure 5.2**). On the other hand, expression of HLA-DR was highly variable among donors by the end of the expansion period (58.50 ±28.46 %; range 23 - 98 %) (**Figure 5.2**). In contrast, expression of CD80 was absent in resting cells and remained high (99 ± 1.68 %) at the end of the expansion period. Likewise, CD86 expression varied greatly by the end of the expansion period (58.50 ±28.46 %; range 15.8-99.5 %)

(**Figure 5.2**). Expression of CD40 was not detectable in resting cells, and at the end of the expansion, the expression level was low or absent (**Figure 5.2**). Similar findings have been observed in previous studies (Khan *et al.*, 2014). The variation in the pro-APC markers expression profiles in expanded Vγ9/Vδ2 T cells among donors suggested potential inconsistency in downstream antigen-presenting experiments. It was hypothesised Vγ9/Vδ2 T cells with low expression level of pro-APC markers would have a reduced ability to cross-present and/or to induce potent CD8 T repsonses; thus, optimisation of Vγ9/Vδ2 T cell stimulation was required.

.

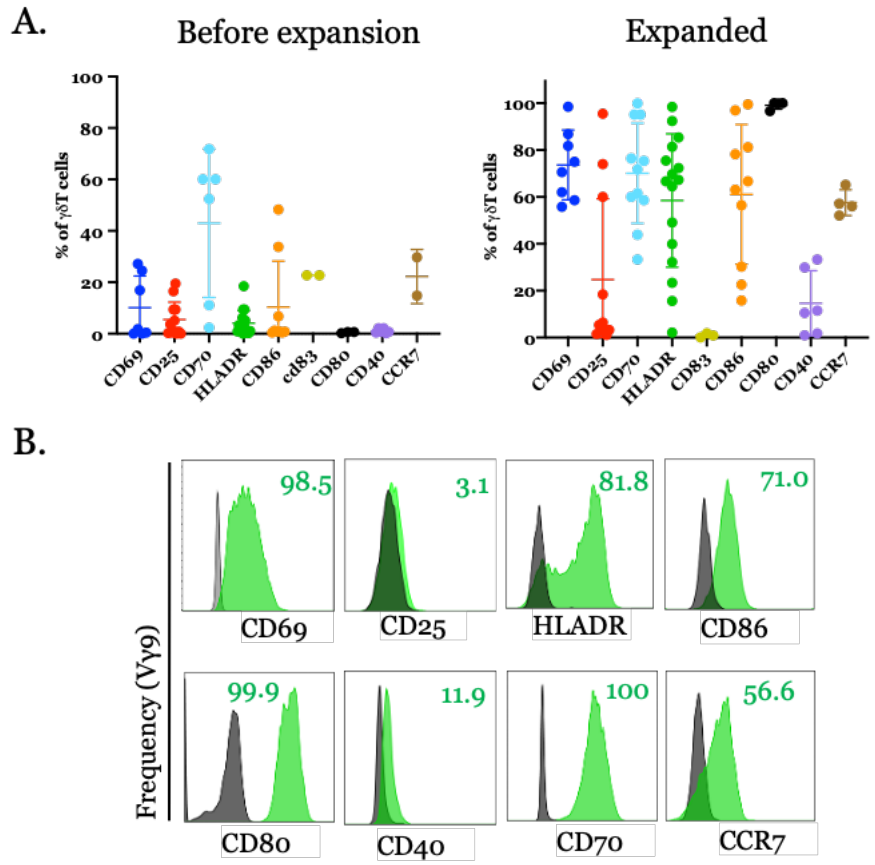


Figure 5.2. Phenotype characterisation of fresh and expanded Vγ9/Vδ2 T cells.
Freshly isolated PBMC from healthy donors were stimulated with zoledronate and cultured for two weeks, as described in **Figure 5.1**. Cells were stained with fluorescently labelled antibodies and analysed by flow cytometry. (**A**) Shown are the expression of activation markers (CD25, CD69) and APC molecules HLA-DR, CD40, CD80, CD83, CD86, CD70, and lymph node homing receptor CCR7 at day 0 (fresh) and the end of two weeks expansion period (expanded). Each data point represents an individual donor; horizontal lines represent the mean of all data from individual experiments. Error bars display ±SD. (**B**) Representative plots from one donor showing surface marker expression in expanded Vγ9/Vδ2 T cells. Isotype controls are shown in grey, and the stained markers are shown in green.

To understand how the behaviour of pro-APC markers during the Vγ9/Vδ2 T cell expansion, first MHC II and activation markers were simultaneously monitored over time. The expression of activation markers was relatively consistent on fresh Vγ9/Vδ2 cells among donors; both CD69 and CD25 were expressed at very low levels. However, their expression was rapidly upregulated upon zoledronate

stimulation, and after that, behaved differently among different donors (**Figure 5.3**). CD25 reached peak levels between day 5 and day 8 and was gradually downregulated in the second half of the two-week expansion period, approaching the expression level of resting cells by the time cells stop proliferating (**Figure 5.3 A**). In agreement with other studies (Lafont *et al.*, 2001), gradual downregulation of CD25 made Vγ9/Vδ2 cells less responsive to cytokines and slowed down proliferation; hence the cells finally stopped proliferating as the CD25 approaches the expression level of resting cells. In three donors, the expression of CD25 remained at an intermediate/high level (60-95 %) at day 14 after stimulation, which indicated these Vγ9/Vδ2T cells were still active and had the potential to expand further. In contrast, the expression of the early activation marker CD69 was rapidly increased and reached its maximum in the second half of the expansion period and remained at intermediate expression level when CD25 had (almost) disappeared (**Figure 5.3 A**). Similarly, the expression of antigen-presenting molecule MHC II was at low levels in fresh Vγ9/Vδ2 T cells, and it was gradually upregulated, reaching peak levels between days 8 and 11; however, the expression level was in donors among donors throughout the expansion (**Figure 5.3**).

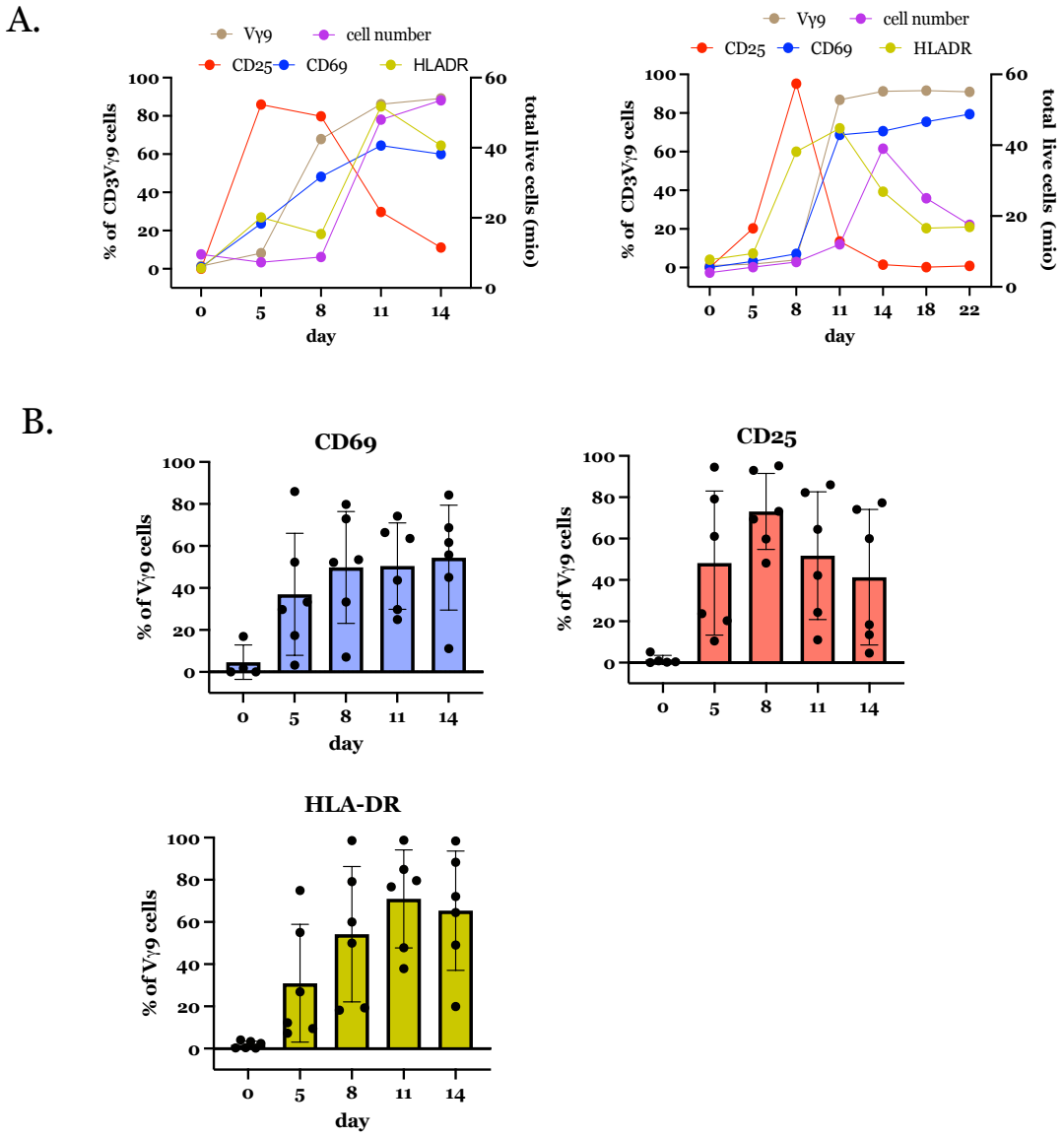


Figure 5.3. Expression of activation makers and MHC II during expansion of Vγ9/Vδ2 T cells. Vγ9/Vδ2 T cell expansion was monitored over time in relation to early activation (CD69), late activation marker (CD25) and antigen-presenting molecule (HLA-DR) by flow cytometry. (**A**) Shown are representative plots from two healthy donors. Differential expression of CD25, CD69 and percentage of total γδ T (Vγ9) cells in cultures are depicted on the left y-axis, and the total number of viable cells is shown on the right y-axis. (**B**) CD25, CD69 and HLA-DR expression over time in expanding Vγ9/Vδ2 T cells from eight healthy donors. Each data point represents individual donors; columns represent the mean of all data from individual experiments; error bars display ±SD.

To understand the difference in the potency of the stimulus on pro-APC marker levels, ZOL was compared with HMBPP stimulation of PBMC over two weeks expansion (**Figure 5.4**). Overall, ZOL and HMBPP induced similar expression of CD70, CD80, CD86, and HLA-DR and HLA-A2, while the expression levels were slightly higher in HMBPP stimulated cells. Upon Vγ9/Vδ2 T cell stimulation, all APC molecules got rapidly upregulated and reached their maximum between day 3-9 after stimulation (**Figure 5.4 A**). Upon stimulation with either ZOL or HMBPP, MHC I and MHC II expression reached the peak level at day 6, but the expression level was higher in HMBPP stimulated cells (**Figure 5.4 B**).

Similarly other pro-APC markers showed higher expression levels in HMBPP stimulated cells, but the peak levels were reached at earlier timepoints than in ZOL stimulated cells (**Figure 5.4).**

The memory status of Vγ9/Vδ2 T cells was examined to check the potential persistency and efficacy after adoptive transfer. As shown in **Figure 5.4 C**, the majority of both ZOL (50%) and HMBPP (70%) stimulated cells showed a CD45RA−/CD27− effector memory phenotype. Cells stimulated with ZOL contained a small proportion (~10%) of residual TN (CD45RA+/CD27+) cells, while HMBPP stimulated cells did not. The expansion with either stimulus produced a similar proportion (~30%) of TCM (CD45RA−/CD27+) cells. This is in contrast to the previous study where day 14 expanded Vγ9/Vδ2 T cells showed mainly TEM phenotype (Khan *et al.* 2014), or the study where cultures were supplemented with IL-2 (Dieli *et al.*, 2003). Further experiments are required to determine the potential diversity in memory phenotype of Vγ9/Vδ2 T cells among different donors of different ages and cancer patients.

Taken together, these data showed that stimulation of Vγ9/Vδ2 T cells with ZOL or HMBPP resulted in the acquisition of pro-APC phenotype as noted by a sharp upregulation of APC markers in the first 9 days, but thereafter, the APC status declined and was partially lost as the cells approached their steady-state. Moreover, these experiments are suggestive that the expression level of all APC markers is higher (optimal) upon stimulation with HMBPP. Further re-stimulation experiments were sought to determine an exact time window when Vγ9/Vδ2 T cells re-acquire optimal pro-APC phenotype.

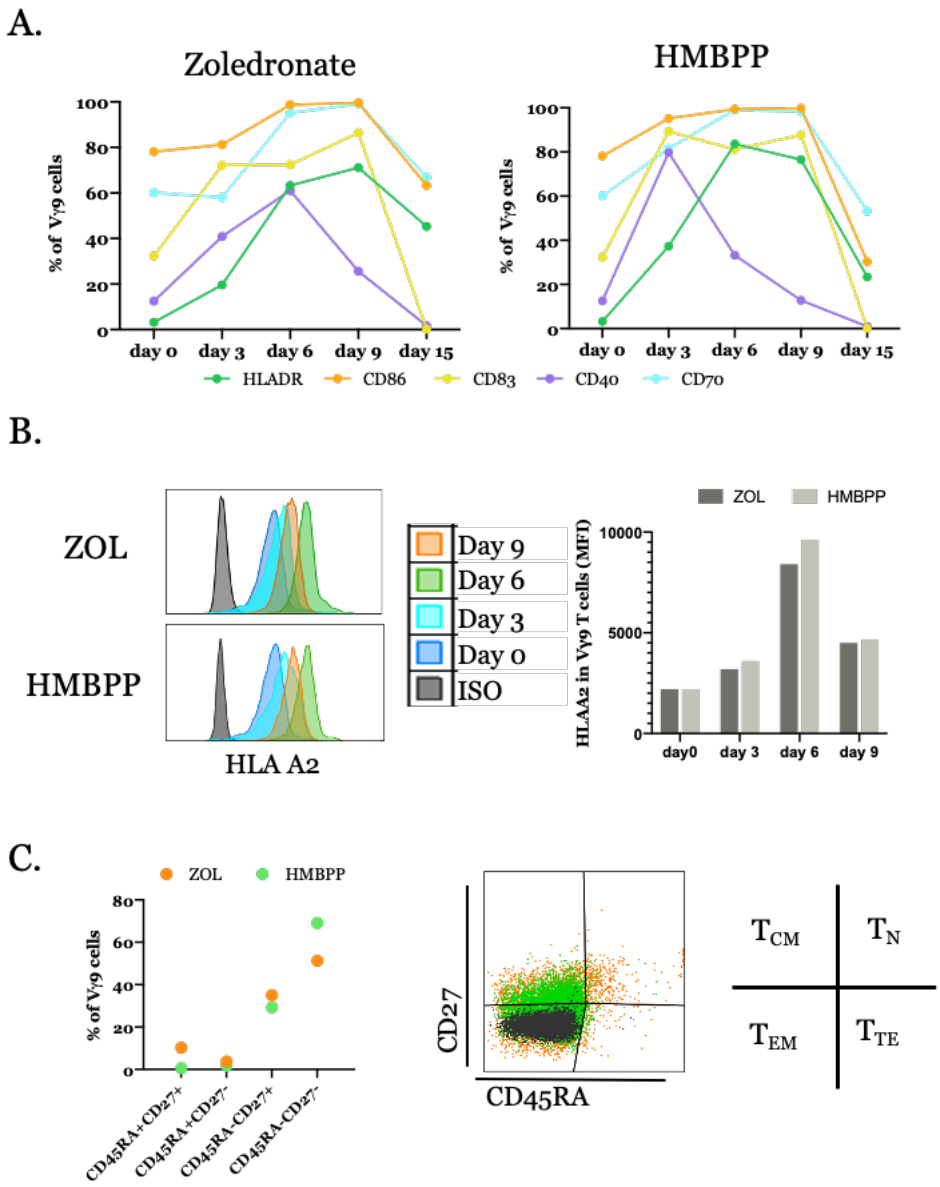


Figure 5.4. Comparison of APC phenotype in Vγ9/Vδ2 T cells over time after stimulation with zoledronate or HMBPP.

Freshly isolated PBMC from a healthy donor were stimulated using either zoledronate (ZOL) or HMBPP, and cells were cultured with IL-2 and IL-15. (**A**) Cells were monitored for the expression of APC molecules HLA-DR, CD83, CD86, CD70, and CD40. (**B**) Expression of MHC class, I (HLA-ABC, HLA-A2) in the first 9 days is shown as FACS plots (left) and as MFI values (right). Gated on single, live, CD3+, Vδ9+ cells. (**C**) Memory status of Vγ9/Vδ2T cells after expansion. The proportion of $CD45RA^{+}/CD27^{+}$ (T_N), $CD45RA^{-}/CD27^{+}$ (T_{CM}), $CD45RA^{-}/CD27^{-}$ (T_{EM}), and $CD45RA^{+}/CD27–$ (T_{TE}) cells was examined by flow cytometry. Stained on single, live, CD3+, Vγ9+ cells. A representative dot plot is shown on the right, with zoledronate expanded cells shown in orange, HMBPP expanded cells shown in green and isotype control shown in grey. The data shown is derived from one experiment.

5.2.2 Optimisation of culture conditions to generate optimal γδ T-APCs

Expanded Vγ9/Vδ2 T cells showed somewhat a semi-resting status at around 14 days in culture, judged by their relatively low expression level of some pro-APC markers. Thus, in the following set of experiments, I aimed to optimise culture conditions for short-term re-stimulation of expanded Vγ9/Vδ2 T cells that would result in enhanced-optimal pro-APC phenotype using HMBPP, and to establish the optimal time window when this would be achieved. Considering initial stimulation experiments, it was hypothesised that HMBPP restimulation of expanded Vγ9/Vδ2 T cells would acquire optimal pro-APC phenotype between day 3 and day 6.

In the ideal experimental model for studying the APC function in Vγ9/Vδ2 T cells, the culture system would include Vγ9/Vδ2T cells and CD8 responder T cells only. Previous reports suggested that accessory cells are required for optimal stimulation and expansion of Vγ9/Vδ2 T cells with ZOL and HMBPP (Roelofs *et al.*, 2009) (Nerdal *et al.*, 2016). Nevertheless, given that all immune cells express BTNs (Arnett and Viney, 2014), γδ T cells could potentially present HMBPP to each other and get activated under appropriate culture conditions. Thus, the first aim was to determine whether HMBPP re-stimulation would boost the APC phenotype of expanded Vγ9/Vδ2 T cells in the absence of accessory cells. To this end, initially, three concentrations of HMBPP (2 nM, 5, nM, 10 nM) were tested to determine the optimal concentration that induces the highest expression of APC molecules but results in low level of activation-induced cell death. All accessory cells were subjected to high γ radiation to ensure they only serve for stimulation of Vγ9/Vδ2T cells and are not kept in culture. The accessory cells tested were from HLA-A2 negative donors at a 1:10 ratio (monocyte: γδ) and 1:5 ratio (PBMC: γδ). The presence of accessory cells markedly influenced the activation status of expanded Vγ9/Vδ2T cells, as noted by the downregulation of TCR in an HMBPP dose-dependent manner (**Figure 5.5**). As expected, the cell viability was best at 2 nM HMBPP, followed by 5 nM HMBPP, and the least viable cells were detected in cultures stimulated with 10 nM HMBPP (not shown). The least viable cells were detected in cultures without accessory cells, while similar cell viability was detected among cultures with either type of accessory cells (not shown). Re -stimulation with 10 nM HMBPP resulted in the highest expression levels of all pro-APC markers (**Figure 5.5**). Re-stimulation in the presence of accessory cells resulted in a higher percentage of Vγ9/Vδ2T cell expressing all co-stimulatory molecules and lymph node homing receptor at all HMBPP concentrations tested compared to cultures without accessory cells (**Figure 5.5**). Surprisingly, the of HLA-A2 and HLA-DR was reduced on the surface of Vγ9/Vδ2T cells stimulated in the presence of accessory cells (**Figure 5.5**). This observation appeared to correlate with stimulation-induced TCR internalisation/downregulation (**Figure 5.6**). MHC molecules can get internalised due to the invagination of the plasma membrane during the formation of phagosomes and/or are recycled to be used for loading and subsequent cross-presentation of exogenously derived peptides (Colbert, Cruz and Rock, 2020). Such internalisation of both MCH I and MHC II is also noted in DCs (van Niel *et al.*, 2006; Platt *et al.*, 2010; Reinicke *et al.*, 2019). Future examination of intracellular MHC molecules together with the use of deubiquitinase UCH-L1 will confirm the intrenalisation (Reinicke *et al.*, 2019).

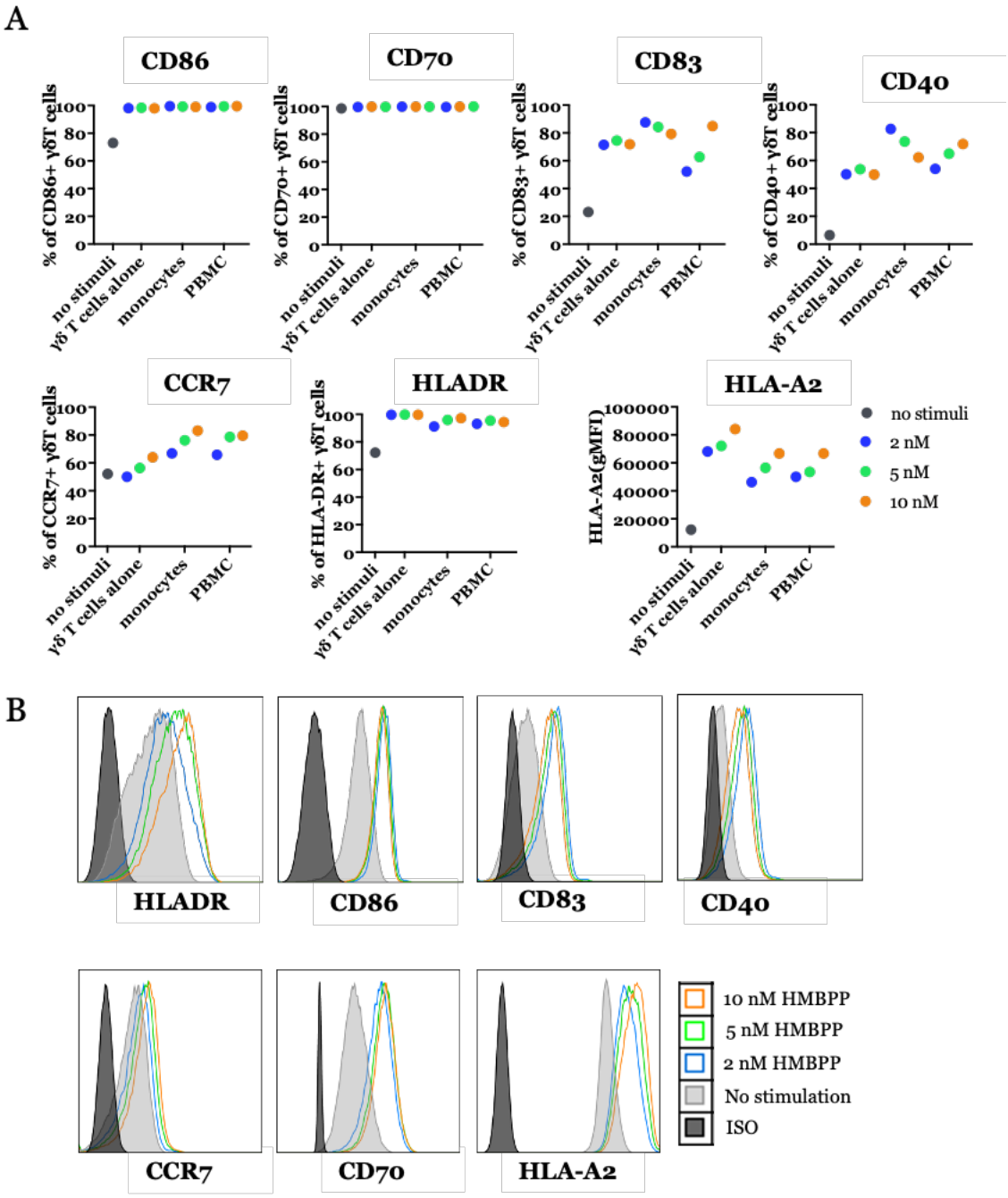


Figure 5.5. Re-stimulation of expanded Vγ9/Vδ2 T cells: analysis of APC molecules expression.

Expanded Vγ9/Vδ2 T cells were re-stimulated or not with HMBPP and IL-2 and IL-15 in the presence or absence of irradiated accessory cells (allogenic HLA-A2 negative monocytes or PBMC). On day 3, cells were stained with fluorescently conjugated antibodies and analysed for expression of APC molecules by flow cytometry. (**A**) Expression of costimulatory molecules (CD86, CD70, CD83, CD40), lymph node homing receptor (CCR7) and MHC molecules (HLA-DR, HLA-A2) are shown as a percentage of Vγ9+ cells. Data points represent one donor from a single experiment. (**B**) Representative histogram plots of these markers are shown for Vγ9/Vδ2T cells re-stimulated in the presence of monocytes. Gated on live single CD3+ Vγ9+ cells.

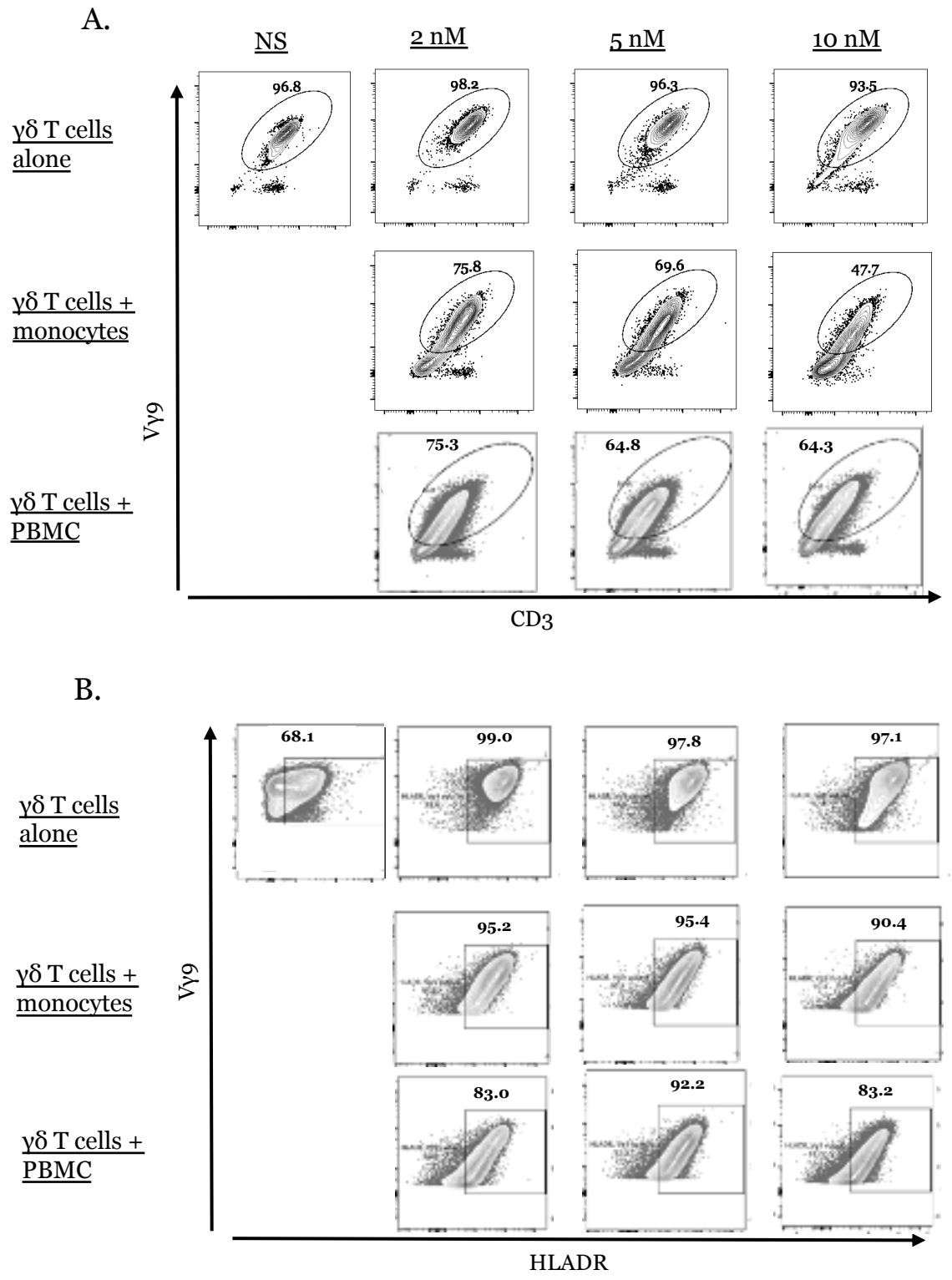


Figure 5.6. HMBPP re-stimulation induces internalisation of TCR and MHC molecules in Vγ9/Vδ2 T cells.

Expanded Vγ9/Vδ2T cells were re-stimulated with HMBPP at indicated concentrations in the presence or absence of allogenic irradiated accessory cells as described in **Figure 5.4**. **(A)** Shows expression of Vγ9 TCR and **(B)** shows the expression of HLA-DR. Gated on live, single, CD3 positive cells.

The final aim was to determine the optimal duration of re-stimulation that would result in the highest expression level of pro-APC markers in Vγ9/Vδ2T cells (Figure 5.7.) Expanded Vγ9/Vδ2T cells from three donors were stimulated with 5 nM HMBPP and cytokines in the presence of monocytes, and expression of MHC class I and II, CD86, CD83, CD40, CD70 and CCR7 was measured three and five days later. Surprisingly, no difference in the expression level of HLA-A2 at either time point after re-stimulation was detected. Compared to unstimulated Vγ9/Vδ2T cells, a sharp upregulation of all pro-APC molecules was reached on day 3, however by day 5, expression of CD40 and CD83 diminished to intermediate and low levels, respectively (Figure 5.7). Nevertheless, due to the low sample number, the statistical analysis did not show the significance for expression of HLA-DR, CD86, and CD70, even though marked differences were noted. In conclusion, 3-day re-stimulation was chosen as the optimal length.

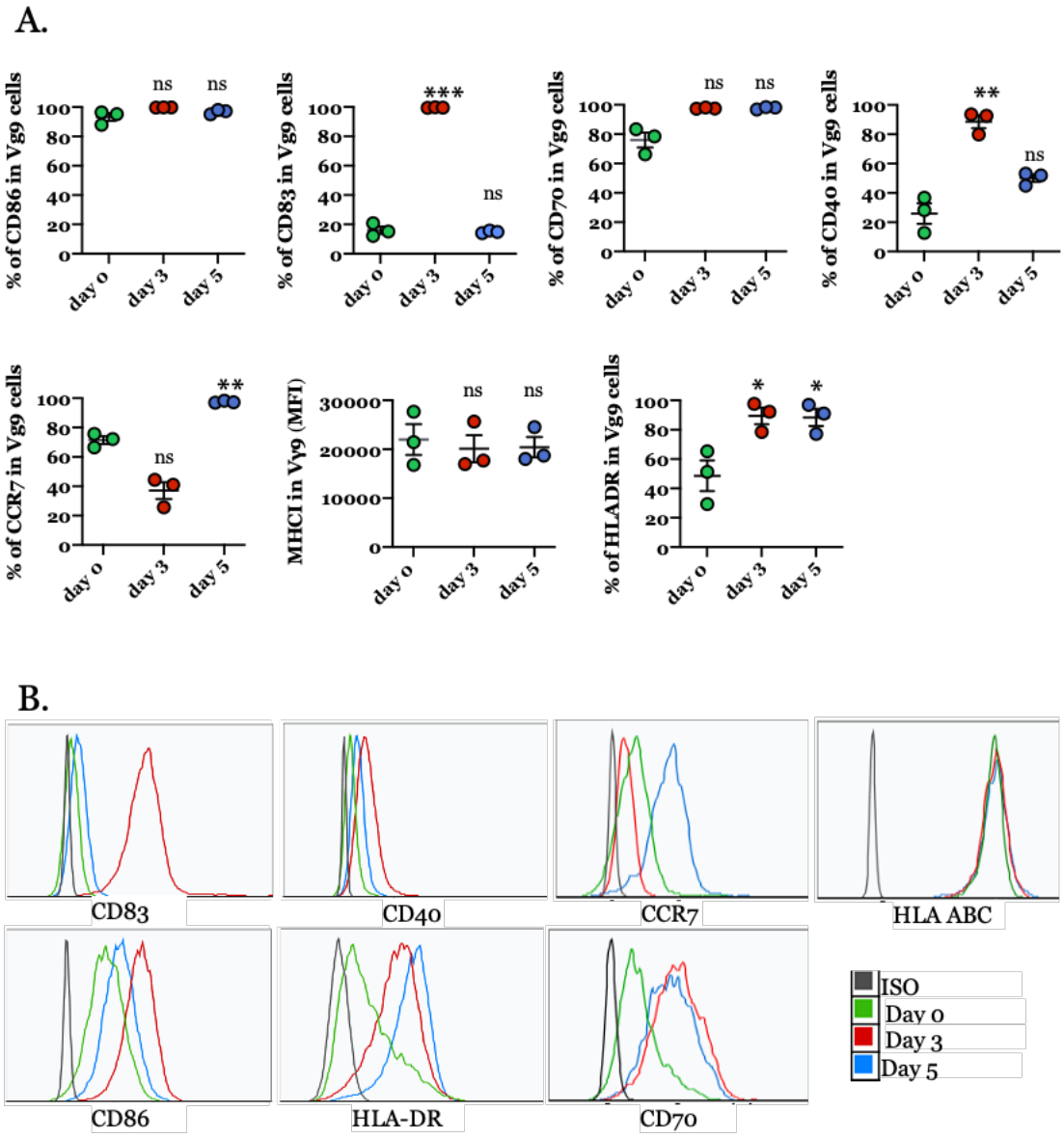


Figure 5.7. Expression of APC molecules in HMBPP re-stimulated Vγ9/Vδ2 T cells at different timepoints.

Zoledronate expanded Vγ9/Vδ2 T cells were re-stimulated or not with 5 nM HMBPP in the presence of accessory cells and cultured in the presence of cytokines for 5 days. Cells were stained with fluorescently labelled antibodies targeting surface markers and analysed by flow cytometry at 3 time points. **(A)** Comparison of expression levels of antigen-presenting molecules (HLA-DR, HLA-A2), homing receptor (CCR7) and costimulatory molecules (CD86, CD83, CD40) by Vγ9/Vδ2 T-cells on day 3 and day 5 after re-stimulation. Data points represent three donors from a single experiment. Horizontal lines display the means of samples, and error bars represent SD. Statistical significance was determined by two-way ANOVA, followed by Dunn's multiple comparison test, *=p<0.05, ns=not significant. Significance displayed in comparison to unstimulated control (day 0). **(B)** Representative histogram plots of cell surface markers expressed by re-stimulated γδ T-APC over time. Gated on live, single, CD3+, TCR Vγ9+ cells.

Taken together, re-stimulation of expanded Vγ9/Vδ2 T cells without accessory cells turned out to be suboptimal. Although stimulation with 10 nM HMBPP induced the expression levels of pro-APC markers, 5 nM HMBPP was chosen as optimal concentration for Vγ9/Vδ2 T cell re-stimulation since the activation-induced cell death was lower than in cultures stimulated with 10 nM HMBPP. Monocytes were chosen as the optimal cells as irradiated monocytes offer several benefits over another accessory cells examined here. They do not produce IFN-γ and hence would not interfere with functional APC assays. Moreover, since monocytes present HMBPP, they are susceptible to Vγ9/Vδ2 T cell killing while their apoptosis is also assured by high dose irradiation, and hence, further purification steps before the APC assays with responder CD8 T cells would not be necessary. A duration of 3 days was chosen to be optimal for use in future APC assay experiments.Vγ9/Vδ2 T cells restimulated under these conditions will be referred to as γδ T-APCs.

5.3 Cross-presentation of soluble antigens by Vγ9/Vδ2 T cells

5.3.1 Cross -presentation of soluble FluM1 protein by expanded Vγ9/Vδ2 T cells and γδ T -APCs

Before evaluating the ability of Vγ9/Vδ2 T cell to cross-present tumour antigens, it was logical to test the antigen cross-presentation within a known system with an antigen of microbial origin. I turned to an experimental model frequently used in our lab to evaluate antigen presentation capacity of APCs using the well-defined influenza virus-encoded matrix protein M1 (FluM1) that induces strong responses in peptide-specific CD8 T cells of HLAA*0201 (HLA-A2)-positive individuals (M. Brandes et al., 2009). This was achieved by using CD8 T-cells specific for the M1p58-66 peptide immunodominant peptide generated in Chapter 4. Expanded Vγ9/Vδ2 T cells or γδ T-APCs were incubated with decreasing concentrations of FluM1 protein, and following overnight incubation, γδ T cells were washed and incubated with FluM1 responder T cells. Induction of peptide-specific CD8 T cell responses requires a protein to be taken up and processed by proteasomal degradation pathway, which results in 8-9 aa long peptides (in this case M1p58-66) being loaded onto intracellular MHC class I (HLA-A2) molecules and transported to the cell surface. Successful antigen cross-presentation was determined by intracellular IFN-γ production measured in responder T cells by flow cytometry after 4 h of co-culture with cross-presenting Vγ9/Vδ2 T cells in the presence of BRE A (**Figure 5.9**). Since uptake and intracellular processing is not required for the presentation of exogenously added peptides by HLA-A2, γδ T and CD8 T cells pulsed with an M1p58–66 peptide served as positive controls for maximal responses to peptide stimulation. Likewise, cells stimulated non-specifically with a mitogen (PMA/ionomycin) served as a positive control for maximal response. Processing and presentation of fragments of 1 and 0.1 μM of FluM1 protein by γδ T-APC resulted in robust IFNγ responses in CD8 T cells (Figure 5.9 A), similar to the previously published findings (Brandes, Willimann and Moser, 2005; M. Brandes et al., 2009; Khan et al., 2014). However, the ability of expanded Vγ9/Vδ2 T cells to induce robust M1p58-specific CD8 T cells varied among donors. In two donors, a weak FluM1-CD8 T cell response was noted when expanded Vγ9/Vδ2 T were loaded with a lower concentration of FluM1

protein. In contrast, expanded Vγ9/Vδ2 T cells loaded with 1 μM FluM1 protein-induced responses in ~ 70% FluM1-CD8 T cells (**Figure 5.9**).

Taken together, these experiments support previous findings that expanded Vγ9/Vδ2 T cells can function as pro-APCs. Furthermore, seeing that pro-APC phenotype varied in expanded Vγ9/Vδ2 T cells among different donor and γδ T-APCs, these results suggest that the expression level of pro-APC markers plays a vital role in the induction of robust CD8 T cell responses by cross-presenting Vγ9/Vδ2 T cells.

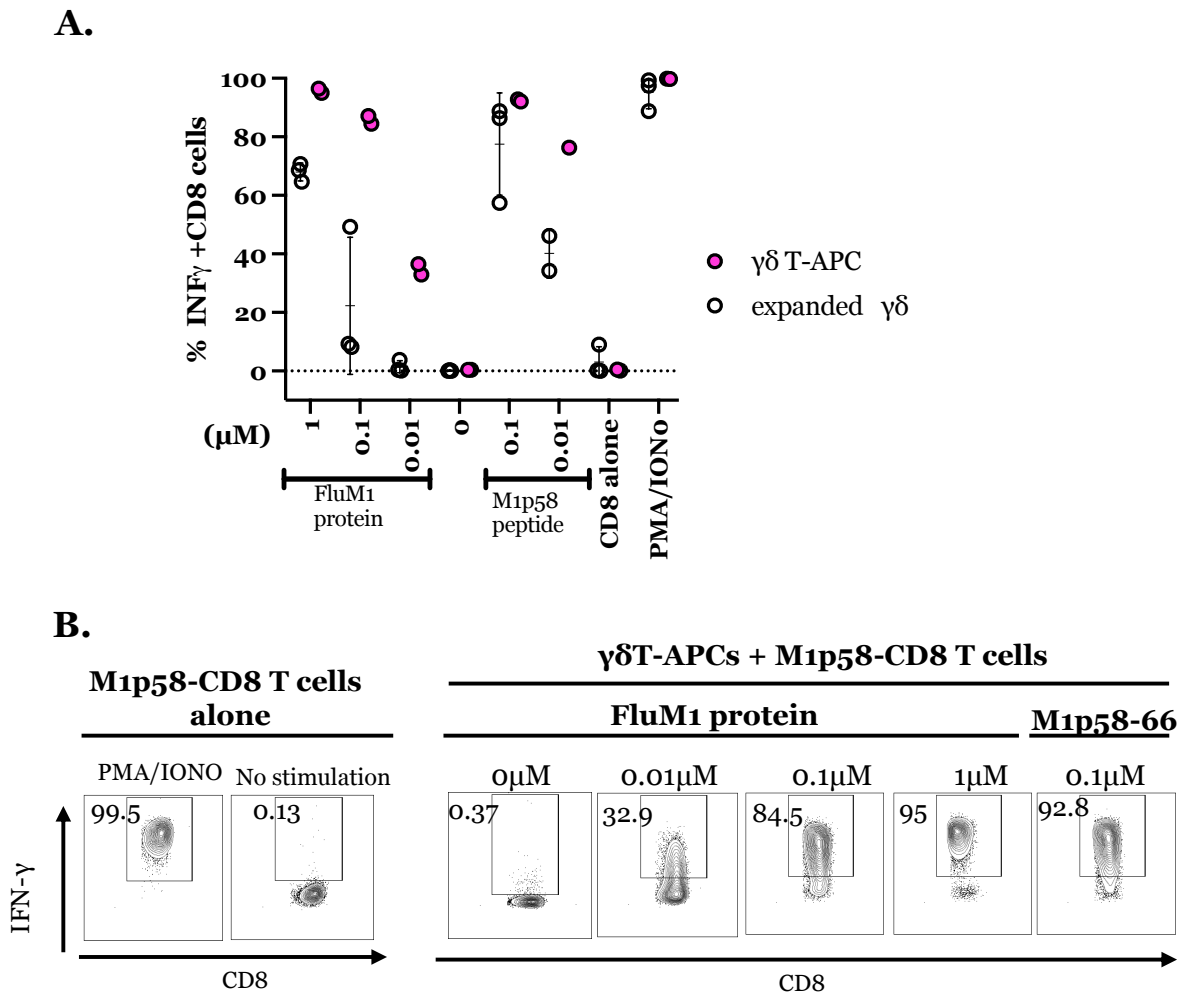


Figure 5.8. Vγ9/Vδ2 T cells cross-present recombinant influenza M1 protein.

(A) In separate experiments, expanded Vγ9/Vδ2 T cells (black, n =3) and γδ T-APCs (pink, n= 2) from healthy individuals were incubated overnight in the presence of recombinant influenza M1 protein (FluM1 protein) at indicated concentrations. After extensive washes, M1p58-specific CD8 responders T cells were added to the cell culture in the presence of BRE A, and cross-presentation was assessed employing intracellular IFN-γ staining in CD8 T cells and flow cytometry 4 h later. CD8 T cells stimulated with PMA/ionomycin and γδ T cells pulsed with M1p58 peptide (GILGFVFTL) served as positive controls for maximal responses and maximal responses peptide, respectively. CD8 T cells alone and CD8 T cells plus γδ T cells cultures served as negative controls. Each data point represents one donor from individual experiments; horizontal lines demonstrate the means of data sets; error bars represent ± SD. **(B)** Representative plots from one experiment with γδ T-APCs. Gated on single, live, CD3+, Vγ9-, CD8+ cells.

5.3.2 Cross-presentation of soluble tumour antigens by expanded Vγ9/Vδ2 T cells

A few studies have examined antigen presentation in Vγ9/Vδ2 T cells, but only one study (Himoudi *et al.*, 2012) investigated the ability of Vγ9/Vδ2 T cells to cross-present tumour antigens. Himoudi and collegues used cell proliferation of cognate tumour peptide-specific CD8 T responder cells within complicated mixed-lymphocyte and EBV population experiments as a readout for successful antigen cross-presentation by Vγ9/Vδ2 T cells. However, so far, no reports exist using more detailed examination, measuring early activation and cytokine production in cognate tumour-specific CD8 T cells induced in response to cross-presenting Vγ9/Vδ2 T cells.

Since different response rates were observed in FluM1-CD8 T cells in co-cultures with cross-presenting γδ T-APCs vs expanded Vγ9/Vδ2 T cells (section 5.3.1), thus first the cross-presentation function of tumour antigens was examined in expanded Vγ9/Vδ2 T cells. The APCA experimental setting was identical to the one described for FluM1 protein, and patient-derived NY-ESO-1 specific CD8 T cells and tESO-1 CD8 T (described in Chapter 4) were used as responder cells to measure successful NY-ESO-1 antigen cross-presentation. For experiments with 5T4 protein, the polyclonal 5T4p17 cell line (p17.wz.3B-LN1 cells) and "monoclonal" CD8 T cells (p17.BM.A11 cells,) were employed, which are described in Chapter 4.

Knowing that the activation threshold of CD8 T cells reactive to self-antigens is higher than for FluM1-reactive CD8 T cells, higher concentrations of the recombinant NY-ESO-1 protein were supplied (5 μM and 10 μM) to γδ T cells. In addition to γδ T cells pulsed with cognate peptides, T2 cells served as a positive control. T2 cells are defective in the peptide supply and have impaired ability to process and present self-antigens via both classes- I and class-II pathways (Salter and Cresswell, 1986) while retaining a high expression of co-stimulatory molecules (Steinle and Schendel, 1994; Bossi *et al.*, 2013). Thus, in this study, T2 cells were used as a positive control for assessing the strength of peptide-specific responses in cognate CD8 T cells, as well as a suitable negative control for studying antigen processing and presentation. Mitogen stimulation of CD8 responder T cells with PMA/IONO served as a general positive control. Co-culture of γδ T cells and CD8 responder T cells without stimulants served as a negative control. Additional negative controls included: i) unstimulated APCs alone, and ii) peptide-pulsed APCs in cultures without CD8 T cells.

A low IFNγ production was detected in ESO157 peptide-specific CD8 responder T cells incubated with expanded Vγ9/Vδ2 T cells pulsed with NY-ESO-1 protein (**Figure 5.9 A**). However, due to background IFNγ (13%) produced by CD8 responders in co-culture with expanded Vγ9/Vδ2 T cells alone, it was not possible to determine whether the cross-presentation of NY-ESO-1 occurred or not. Such nonspecific CD8 T cell response could occur because allogeneic cells were used rather than autologous. However, it cannot be excluded that the stimulation arose due to the FSC in the assay medium. Likewise, the IFNγ production by the ESO157 peptide-specific CD8 T cells was similar in co-cultures with ESOp157 peptide-

pulsed expanded Vγ9/Vδ2 T cells. In contrast, T2 cells pulsed with ESO157 peptide could induce a high IFNγ response in the same CD8 responder T cells (**Figure 5.9A**).

Tumour antigen cross-presentation was also examined by IFNγ ELISpot assay. High background (e.g., spots in the absence of specific antigen stimulation) has been a general problem with the ELISpot. It is usually encountered due to complicating factors, such as aberrantly active cells, low-intensity responses, and high background associated with strongly responding lines. Separating relevant responses from the background, i.e. the signal to noise ratio, may be challenging, especially when working with polyclonal lines. Usually, this is dealt with by subtracting background noise from reactivity observed after specific stimulation. In this essay, however, the background was extremely low, and no subtraction was required. Pulsing of expanded Vγ9/Vδ2 T cells with 5 μM and 10 μM NY-ESO-1 protein did not elicit any IFN-γ response, but a moderate IFN-γ response at the same peptide concentrations was detected (**Figure 5.9 B**). In comparison, T2 cells stimulated cognate CD8 T cells and elicited high responses at peptide concentration as low as 5 μM, while at 10 μM concentrations, the response was as high as with mitogen stimulation (**Figure 5.9 B**). To show the specificity of CD8 responder cells for their cognate peptide, pulsing antigen-presenting cells with FluM1 peptide did not elicit a response in ESOp157 specific responders.

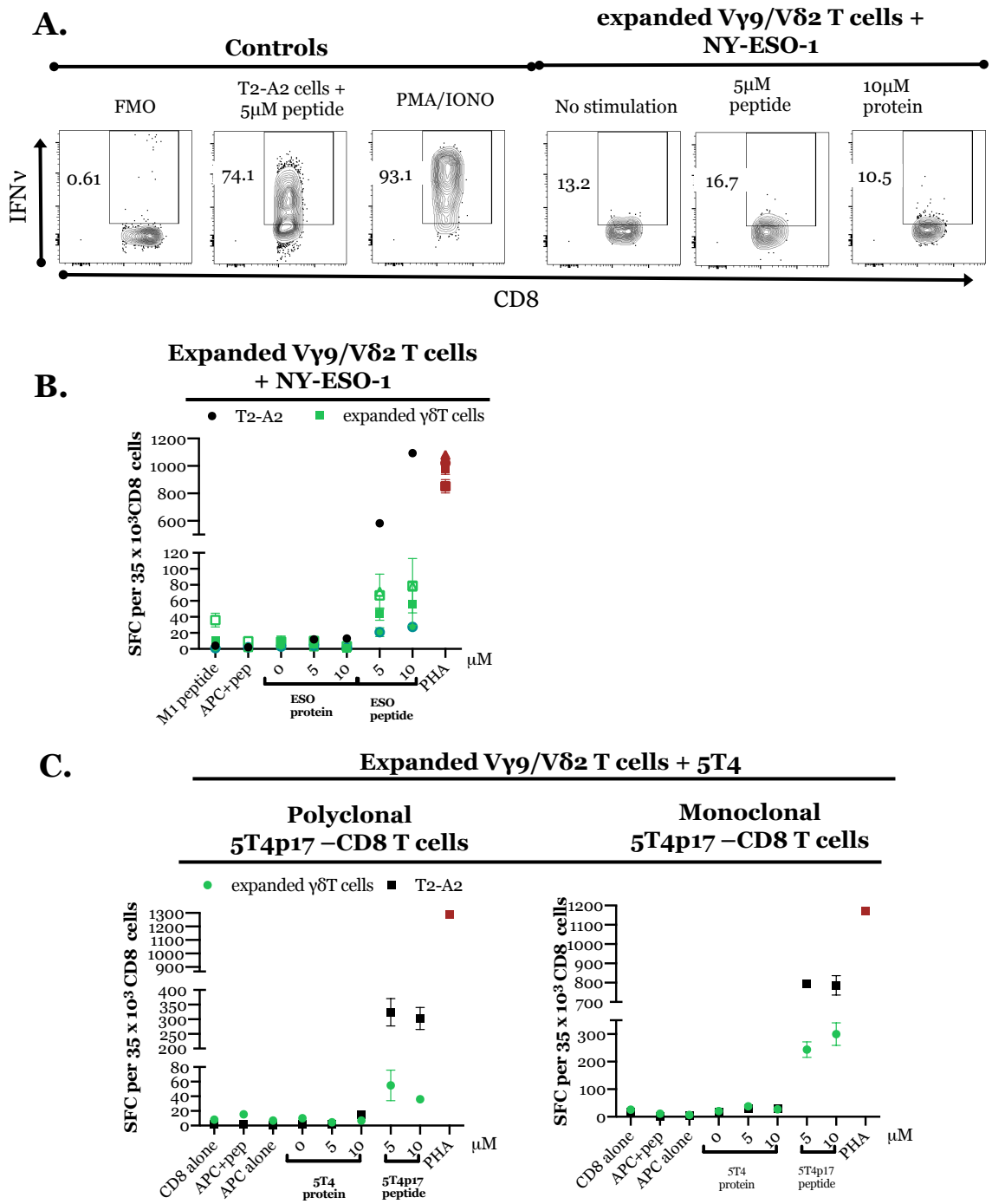


Figure 5.9. Expanded Vγ9/Vδ2 T cells do not cross-present tumour antigens to peptide specific CD8 T cells.

Vγ9/Vδ2 T cells expanded from PBMCs were cultured for 24 h in the presence of recombinant 5T4 or NY-ESO-1 (ESO) protein, or ESOp157-165 peptide (SLLMWITQC) or 5T4p17-p25 (RLARLALVL) at various concentrations and then used as APCs for cross-presentation to ESO or 5T4 specific CD8 T cell responders. The T2-A2 cell line was used as a positive control for responses to peptide presentation. PMA/IONO or PHA stimulated cells served as a general positive control; co-culture of γδ T cells with CD8 responders without stimulants as a negative control. Additional negative controls included CD8 T cells in cultures without APCs (NCTRL), and APCs (APC alone) and peptide-pulsed APCs (APC+pep) in cultures without CD8 T cells. **(A)** Representative plots showing activation of CD8 T cell responders in co-cultures with γδ

T-APCs measured by intracellular staining of IFN-γ and analysed by flow cytometry. (B) Cross-presentation of NY-ESO-1 antigen was examined by IFNγ –ELISpot assay. Briefly, 35,000 CD8 responder cells were plated onto 100,000 antigen-pulsed Vγ9/Vδ2 T cells or 50,000 T2 cells at two different concentrations. Each data point represents the mean ± SD of the IFNγ-spot forming cell (SFC) number of duplicated samples from three healthy donors. Negative controls: responder cells stimulated with an irrelevant peptide (FluM1), APC alone stimulated with cognate peptide. Responder CD8 T cells stimulated with PHA served as a control for maximal response. (C) Presentation of 5T4 antigen and p17-25 peptide (RLARLALVL) by expanded Vγ9/Vδ2 T cells examined by IFNγ –ELISpot; the assay performed as described for NY-ESO-1 in **(B)**. Data points represent IFNγ producing responses in cognate-5T4 specific CD8 T cell clone (left) and cell line (right). Each data point represents the mean ± SD of the SFC number of duplicated samples. SFC, spot forming cell.

Similar results were obtained when the cross-presentation of the 5T4 tumour antigen was examined. ELISpot APCA setting was identical to experiment with NY-ESO-1 protein. The pulsing of expanded Vγ9/Vδ2 T cells with 5 μM and 10 μM 5T4 protein did not elicit an IFN-γ response in cognate CD8 T cells, but responses were detected in polyclonal and monoclonal 5T4p17 specific CD8 responder cells when incubated with 5T4p17 peptide pulsed expanded Vγ9/Vδ2 T cells, or T2 cells (**Figure 5.9 C**).

Taken together, these assays demonstrated that expanded Vγ9/Vδ2 T-cells pulsed with NY-ESO-1 or 5T4 proteins are unable to induce responses in cognate CD8 T cells. These results indicated that either: 1) stimulation via costimulatory signals provided by expanded Vγ9/Vδ2 T cells was not strong enough for induction of tumour-antigen specific CD8 T cell responses; or 2) the supply (overall concentration) of processed tumour peptides on the surface of expanded Vγ9/Vδ2 T cells was insufficient to provide a threshold for triggering a strong response, or 3) the expanded Vγ9/Vδ2 T cells were not able to process NY-ESO-1 and 5T4 proteins.

5.3.3 γδ T-APCs cross-present soluble recombinant 5T4 protein to cognate CD8 T cells

5.3.3.1 5T4 antigen cross-presenting γδ T-APCs induce low IFNγ responses in cognate polyclonal CD8 T cells

First, I tested the ability of 5T4-protein pulsed γδ T-APC to induce IFNγ response in cognate effector/memory CD8 responder T cells, established as a polyclonal cell line (p17.wz.3B-LN1 cell) in Chapter 4. In contrast to the use of ELISpot in the evaluation of APC function in DCs (Schnurr *et al.*, 2005) or expanded Vγ9/Vδ2 T cells, attempts to optimise ELISpot assay for evaluation of APC function of γδ T-APCs failed. This is because stimulated Vγ9/Vδ2 T cells (γδ T-APCs) produce too much IFNγ themselves, while the tumour-specific CD8 responder cells are weaker IFNγ producers (not shown). Thus, the implication of ELISpot assays for evaluating tumour antigen cross-presentation in γδ T-APCs turned out to be challenging and unsuitable.

With the IFNγ-ICS assay optimised to detect tumour antigen-specific CD8 T cells responses, the experimental setting was identical to that described in Chapter 4. To allow for antigen processing, γδ T

cells were incubated with a protein for the final 24 h during re-stimulation, before being combined with 5T4p17 specific CD8 T cells, and the IFNγ read-out was taken 18 h later.

As expected, the potency of IFNγ response in cognate 5T4p17 CD8 responder cells correlated with the duration of γδ T cell re-stimulation. 5T4 protein pulsed γδ T-APCs generated in 48 h induced a very low IFNγ response in cognate CD8 T cell responders (**Figure 5.10**).

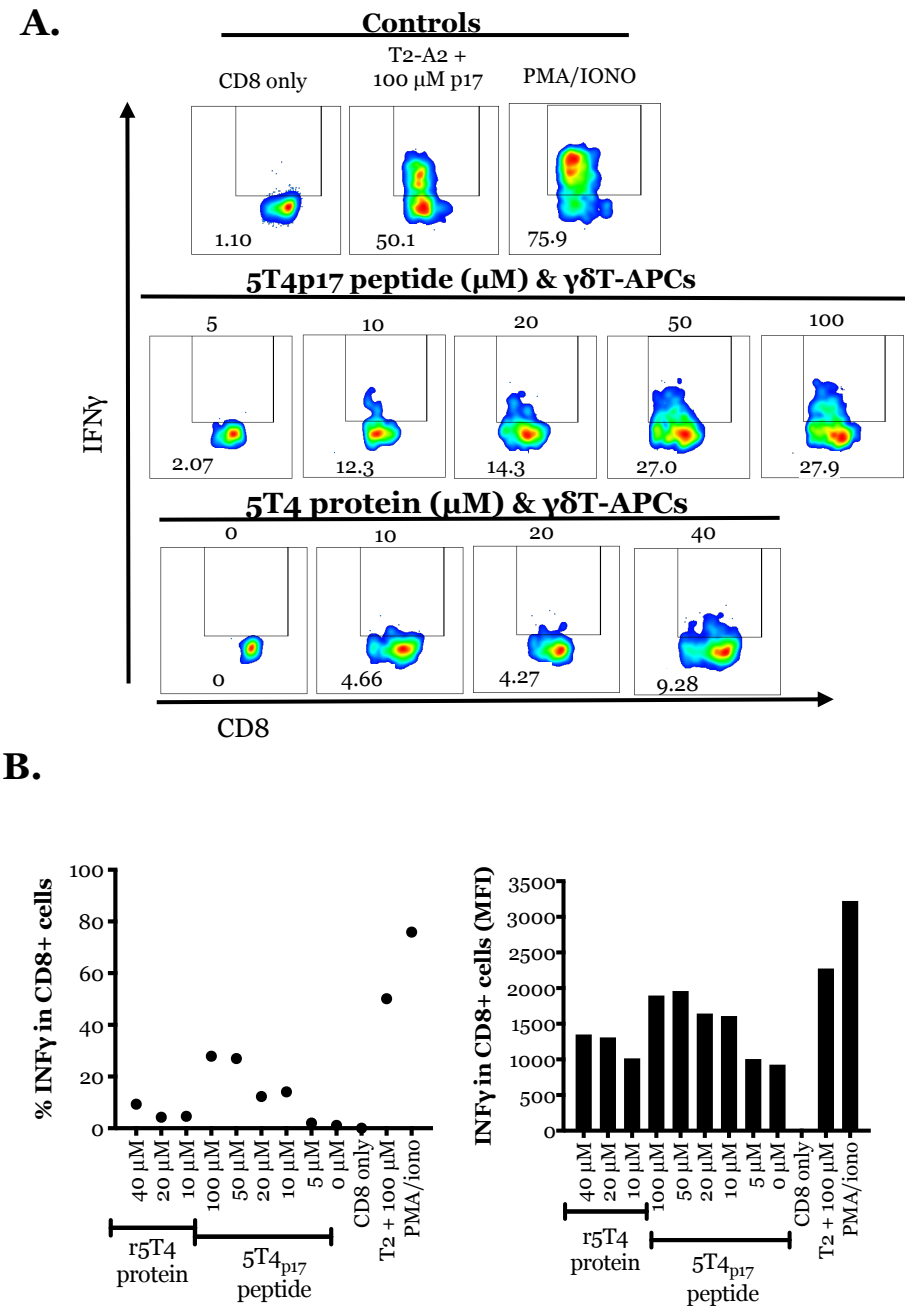


Figure 5.10. 5T4-Cross-presenting γδ T-APCs generated in 48 h induce low response in polyclonal peptide-specific CD8 T cells.

(**A**) Expanded γδ T cells were restimulated in the presence of irCD14 for 24 h. Recombinant 5T4 and proteins were added at indicated concentrations, and cells were incubated for another 24 h before 5T4p17 specific CD8 T cells were added to the cell culture. Cross-presentation by γδ T-APC was measured by ICS of IFNγ and flow cytometry. T2-A2 cell line was used as a positive control for responses to MHC-peptide stimulation. PMA/IONO stimulated responder cells served as a general positive control; coculture of γδ T

cells and CD8 responders without stimulants and CD8 responder cells alone served as a negative control. **(B**) Graphical Presentation of the percentage of IFNγ produced by CD8 responders and their corresponding MFI values in (**A)**.

Cross-presenting γδ T-APCs generated throughout 72 h, induced proportionally higher IFNγ response in cognate CD8 T cells. A specific IFNγ response to the cross-presentation of 40 μM 5T4 protein was detected in cognate CD8 T cells, roughly equivalent to the 30% response detected in the 100μM peptide-loaded T2 cell condition (**Figure 5.11)**. However, no responses were detected in cognate CD8 T responder cells stimulated with γδ T-APCs pulsed with lower 5T4 protein concentrations. Surprisingly, 5T4p17 peptide-loaded γδ T-APCs generated in 72 h could not induce higher IFNγ responses compared to γδ T-APCs generated in a shorter time (**Figure 5.11**), suggesting the fitness of polyclonal CD8 T cells was poor. In conclusion, these data support the notion that the antigen-presenting ability and induction of cognate CD8 T cell responses by γδ T-APCs is superior at least 3-days after stimulation with HMBPP.

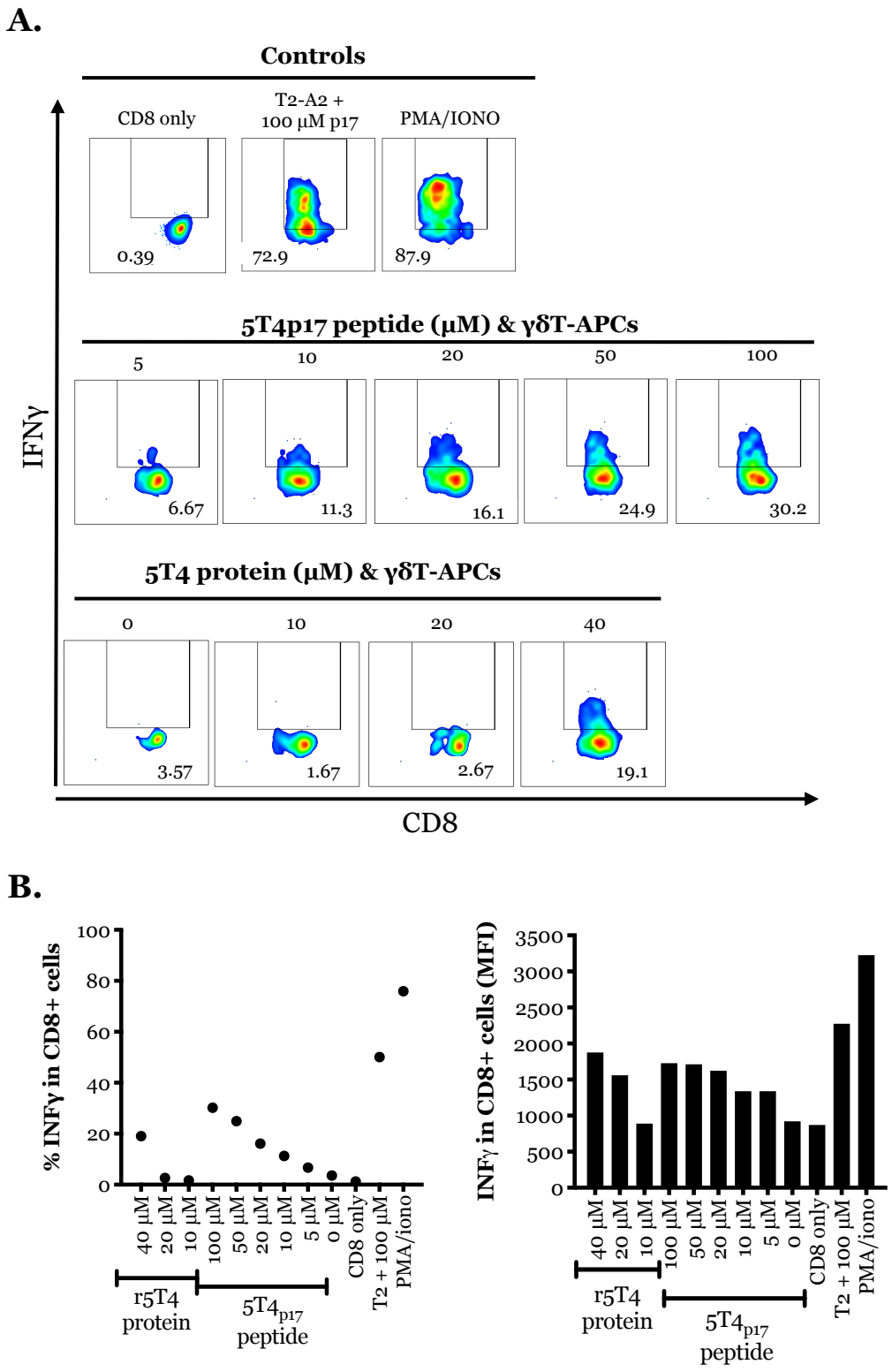


Figure 5.11. Cross-presentation of 5T4 tumour antigen to polyclonal peptide specific CD8 T cells by γδ T-APCs generated in 72 h.

(A) Vγ9/Vδ2 T cells were re-stimulated with 5 µM HMBPP/IL-2+IL-15 cytokine cocktail and cultured for 72 h in the presence of irradiated monocytes from a HLA-A2 negative donor. Recombinant 5T4 and peptides were added to cultures at indicated concentrations for the final 24 h, before 5T4p17 specific CD8 T cells were added to the culture. Successful antigen cross-presentation by γδ T-APC was determined by measuring intracellular IFNγ produced in CD8 responder cells, using flow cytometry. T2-A2 cell line pulsed with p17

peptide was served as a positive control for responses to peptide presentation. PMA/IONO stimulated responder cells served as a general positive control; coculture of γδ T cells and CD8 responders without stimulants and CD8 responder cells alone served as a negative control. Additional negative controls included, T2 cells pulsed with 5T4 protein. Data shown from one experiment. (**B**) Plots showing percentage of IFNγ (left) produced in 5T4p17 specific CD8 T cell line.

5.3.3.2 Cross-presenting γδ T-APCs induce intermediate responses in cognate monoclonal CD8 T cells

In the following experiments, the ability of γδ T-APCs to cross-present 5T4 antigen was investigated using cognate CD8 T responder cells (p17.BM.A11) that were generated by single-cell cloning in Chapter 4.3.2.2. These CD8 responder cells contained high-affinity TCR, judged by pMHC-TET staining intensity (**Figure 4.4 C).** γδ T-APCs generated in 72 h were pulsed with protein for the final 24 h, and antigen cross-presentation assay was carried out as described in the previous section. Specific CD8 T cell responses were observed for all protein concentrations tested. As expected, γδ T-APCs pulsed with the highest protein concertation induced the highest CD8 T cell response, which was roughly a half-maximal response detected in peptide-pulsed APCs (**Figure 5.12**). When pulsing γδ T-APCs with lower 5T4 protein concentration, proportionally lower responses were detected.

In conclusion, these preliminary data support the notion that γδ T-APCs (i.e. short term re-stimulated Vγ9/Vδ2 T cells) can cross-present 5T4 tumour antigen and induce responses in 5T4 specific CD8 T cells. However, future studies are required to confirm and expand these findings to other types of tumour antigens.

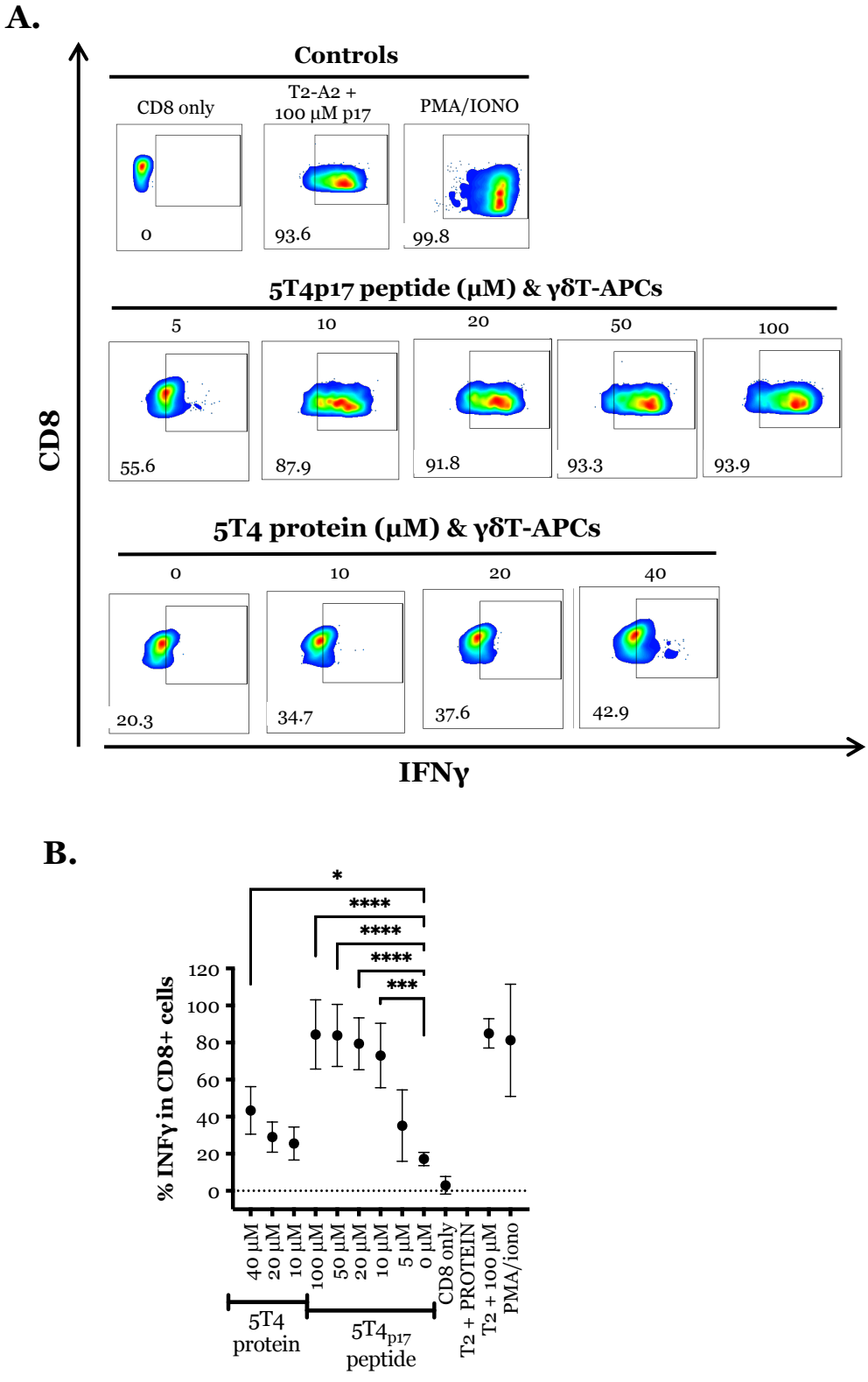


Figure 5.12. γδ T-APC generated in 72 h cross-present 5T4 tumour antigen to cognate monoclonal CD8 T cells.

Vγ9/Vδ2 T cells were restimulated with an HMBPP/cytokine cocktail and cultured for 48 h in the presence of irradiated monocytes from an HLA-A2 negative donor. 5T4 protein and peptides were added at indicated concentrations, and cells were incubated for another 24 h before 5T4p17 specific CD8 responder cells were added to the culture for a further 18 h. **(A)** Representative plots from one experiment. **(B)** Combined data

from three individual experiments, error bars represent ±SD. The significance of differences was calculated by one-way ANOVA followed by Fisher's LSD multiple comparison test. *=p<0.05. Significance displayed in comparison to unstimulated γδ T-APC: CD8 T cell coculture (0 μM).

5.4 Uptake and cross-presentation of antigens expressed in cancer cells by Vγ9/Vδ2 T cells

5.4.1 5T4 protein expression in cancer cell lines

Before assessing the ability of Vγ9/Vδ2 T cells to cross-present the naturally occurring tumour antigens obtained from cancer cells, I first determined the expression of 5T4 antigen and HLA type in cancer cells. Three human cancer cell lines of different tissue origin, cancer grade and HLA type were used as a cancer model in all experiments: 1) MCF7 breast adenocarcinoma, derived from invasive ductal carcinoma at a metastatic site; 2) HT1080 fibrosarcoma, and 3) PC3 prostate carcinoma, derived from the bone metastasis. High doses of γ-irradiation have been shown to significantly increase the surface expression of all antigens present in the cells (Santin *et al.*, 1996). Thus, here in this study, cancer cell lines were exposed to 30 Gγ and were compared to untreated, and zoledronate treated cancer cell lines. The choice of MCF cell line was due to their expression of HLA-A2 and 5T4 antigen (Boegel *et al.*, 2014; Scholtalbers *et al.*, 2015; http://celllines.tron-mainz.de/) **(Figure 5.13),** and thus serving as a positive control. As expected, a high-intensity band of ~65 kDa, corresponding to 5T4 protein, was observed in irradiated MCF7 cells, whereas untreated and zoledronate treated MCF7 cells showed moderate-intensity protein bands (**Figure 5.13**). The choice of the PC3 cell line was for the lack of expression of HLA-A2 (**Figure 5.13**) (Boegel *et al.*, 2014; Scholtalbers *et al.*, 2015), high expression of 5T4 antigen (Al-Taei *et al.*, 2012), and a great record of published data available for PC3 cell line, including experiments examining cross-presentation of 5T4 antigen by DCs (Salimu *et al.*, 2015). Hence, in theory, PC3 cells would not induce responses in HLA-A2 restricted, 5T4-antigen-specific CD8 T cells in antigen presentation assays described in later sections of this subchapter. Western blot analysis of PC3 cell lysates of irradiated, zoledronate treated, and untreated cells revealed different intensities of 5T4 bands (**Figure 5.13**). As expected and following previously reported data (Salimu *et al.*, 2015), the most intense 5T4 band was detected in irradiated PC3 cells (**Figure 5.13**). According to the literature, the choice of HT1080 was for the lack of HLA-A2 and 5T4 antigen expression (to my knowledge, no data is available that shows 5T4 expression in the HT1080 line)- serving as a negative control in ACPAs. However, to a surprise, Western blot analysis of HT1080 whole cell lysate revealed a faint to moderate intensity bands of ~65 kDa, corresponding to 5T4 antigen in all conditions tested, but it was not as intense as seen in MCF7 and PC3 cell lysates (**Figure 5.13**).

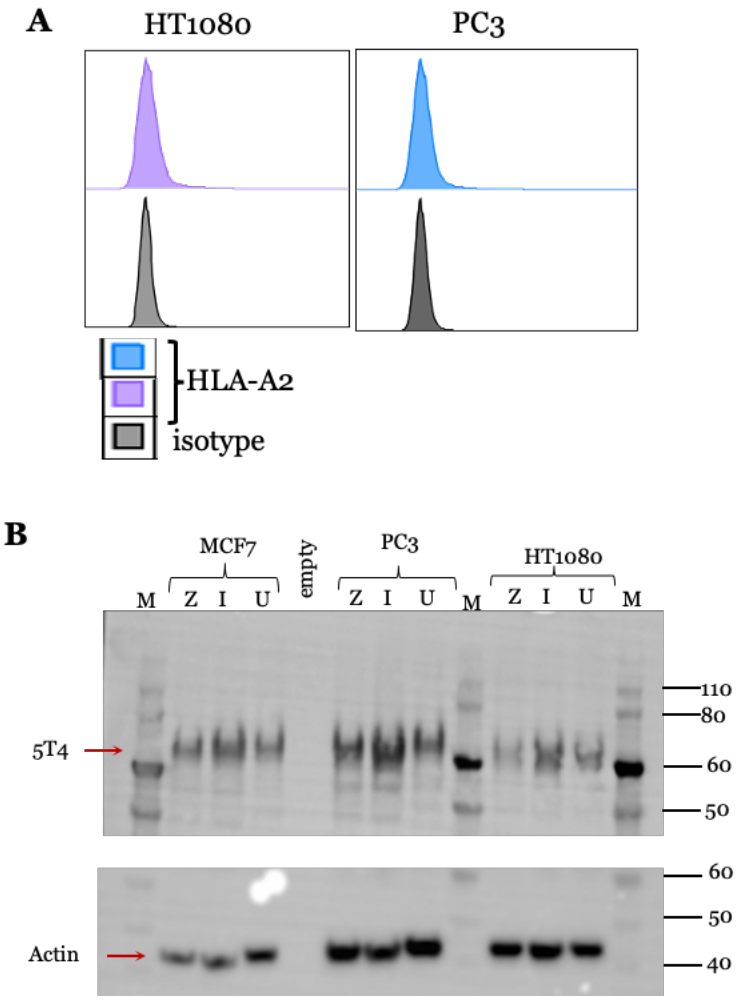


Figure 5.13. Expression of HLA-A2 and 5T4 protein in PC3, MCF7 and HT1080 cell lines. (**A**) HLA type was determined by fluorescently labelled HLA-A2 antibody and flow cytometry. Grey histograms show isotype controls, purple and blue histograms show expression of HLA-A2 in HT1080 and PC3 cell lines, respectively. (**B**) Top panel: Western blotting of 5T4 antigen in zoledronate treated (Z), irradiated (I), and untreated (U) PC3, MCF7 and HT1080 cell lines. Bottom panel: the membrane was extensively washed and re-blotted with an actin antibody. M, protein marker (Novex Sharp Pre-stained protein ladder).

5.4.2 Co-culture with cancer cell lines boosts APC phenotype in expanded Vγ9/Vδ2 T cells

Cancer cells show various levels of dysfunctional cellular functions depending on the cancer cell type, differentiation status, and mutational burden. Thus, they will produce/express various ligands that can be recognised by Vγ9/Vδ2 T cells via their TCR or other receptors, including NKG2D or TLRs. Upon zoledronate treatment, different types of cancer cells will produce different amounts of IPP and ApppI, which positively correlates with the efficacy of Vγ9/Vδ2 T cell-mediated killing of cancer cells (Mönkkönen *et al.*, 2008; Benzaïd *et al.*, 2011). On the other hand, only limited evidence shows that upon cancer cell contact/lysing, Vγ9/Vδ2 T cells acquire APC phenotype, take-up cancer cell material, and cross-present antigens to CD8 T cells (Himoudi *et al.*, 2012). Thus, the first set of experiments was

conducted to confirm whether expanded Vγ9/Vδ2 T cells get activated and acquire a professional APC phenotype in co-culture with cancer cells.

To this end, PC3, MCF7 and HT1080 cells were either treated with γ-radiation, zoledronate or left untreated prior to co-culture with expanded Vγ9/Vδ2 T cells. High doses of gamma irradiation have been shown to significantly increase protein expression (Santin *et al.*, 1996), while during the cells death process, they release many ligands, including TLR-ligands, which can stimulate Vγ9/Vδ2 T cells. In contrast, zoledronate imposes cell by inhibiting isoprenoid biosynthesis by blocking the FPPS enzyme, leading to a significant accumulation of various pyrophosphate molecules including IPP, DMAPP and 'by products' (Oldfield 2010, Roelofs *et al.* 2009) (Vantourout *et al.*, 2009; Moulin *et al.*, 2017) that are recognised by Vγ9/Vδ2 TCR. Thus, cancer cells were treated 48 h before ("pre-treated CRCs") or were treated at the time of co-culture ("same-day CRCs") with Vγ9/Vδ2 T cells in the presence of IL-2 and IL-15. The co-cultures were then incubated for 48 - 72 h, and expression of cell activation markers CD25, and CD69, APC molecules HLA-DR, CD86/CD80, CD70 was analysed in Vγ9/Vδ2 T cells by flow cytometry. Stimulation of expanded Vγ9/Vδ2 T cells with the HMBPP plus cytokines alone served as a positive control, while unstimulated expanded Vγ9/Vδ2 T cells maintained in complete RPMI medium with IL-2 and IL-15 were used as a negative control.

The expression of early and late activation markers CD69 and CD25 in Vγ9/Vδ2 T cells varied considerably, depending on cancer cell type and treatment. Although all Vγ9/Vδ2 T cells re-expressed these markers in co-cultures with zoledronate or irradiation pre-treated PC3 cells, the expression level did not reach the level seen in the HMBPP re-stimulated Vγ9/Vδ2 T cells (**Figure 5.14**). Similar activation was noted after co-cultures with MCF7 and HT1080 cell lines (**Appendix 3**). This was expected as IPP is not as potent Vγ9/Vδ2 T cell stimulator as HMBPP. Yet, it would be interesting to examine the level of IPP and/or other phosphoantigens produced by these cancer cells for a comparison.

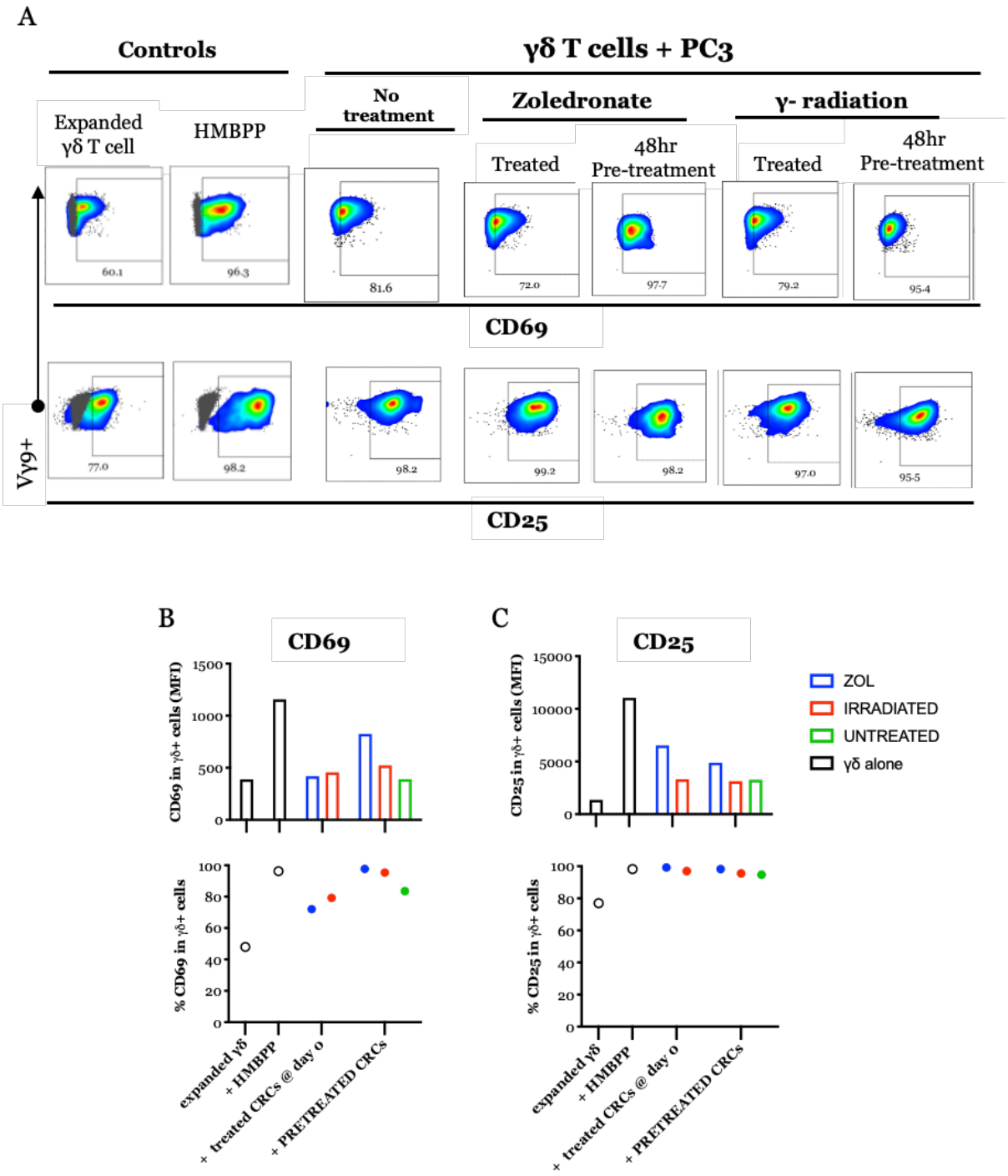


Figure 5.14. PC3 cells stimulate CD25 and CD69 expression in Vγ9/Vδ2 T cells.
(**A**) Cancer cells were treated or not with zoledronate (5 μM) or ionising radiation (30Gγ) 48h prior (pre-treated) or immediately before co-culture with expanded Vγ9/Vδ2 T cells (treated @ day 0). Vγ9/Vδ2 T cells were analysed by flow cytometry for expression of CD25 and CD69 72 hours after co-culture with cancer cells. Expanded Vγ9/Vδ2 T cells re-stimulated with HMBPP served as a positive control and expanded Vγ9/Vδ2 T cells maintained in complete RPMI with IL-2 and IL-15 served as a negative control. Cell populations depicted in grey represent isotype controls. Comparison of the expression level of CD69 (**B**) and CD25 (**C**) activation markers by Vγ9/Vδ2 T cells in response to co-culture with PC3 cells shown as percentages (bottom columns) and MFI values (top columns). Data represent one experiment.

A drastic increase in HLA-DR expression was observed when Vγ9/Vδ2 T cells were cultured with untreated or pretreated PC3 cancer cells for 72 h - even higher than in HMBPP re-stimulated Vγ9/Vδ2 T cells. (**Figure 5.15**). Similarly, the co-cultures with pretreated MCF7 and HT1080 cells resulted in a high percentage (~ 96%) HLA-DR expressing Vγ9/Vδ2 T cells (**Appendix 4**), which was higher than in HMBPP re-stimulated Vγ9/Vδ2 T cells. These results indicate that it is likely that molecules other than phosphoantigens also contribute to establishing pro-APC phenotype in Vγ9/Vδ2 T cells. Further investigation will reveal the type of ligands and receptors involved.

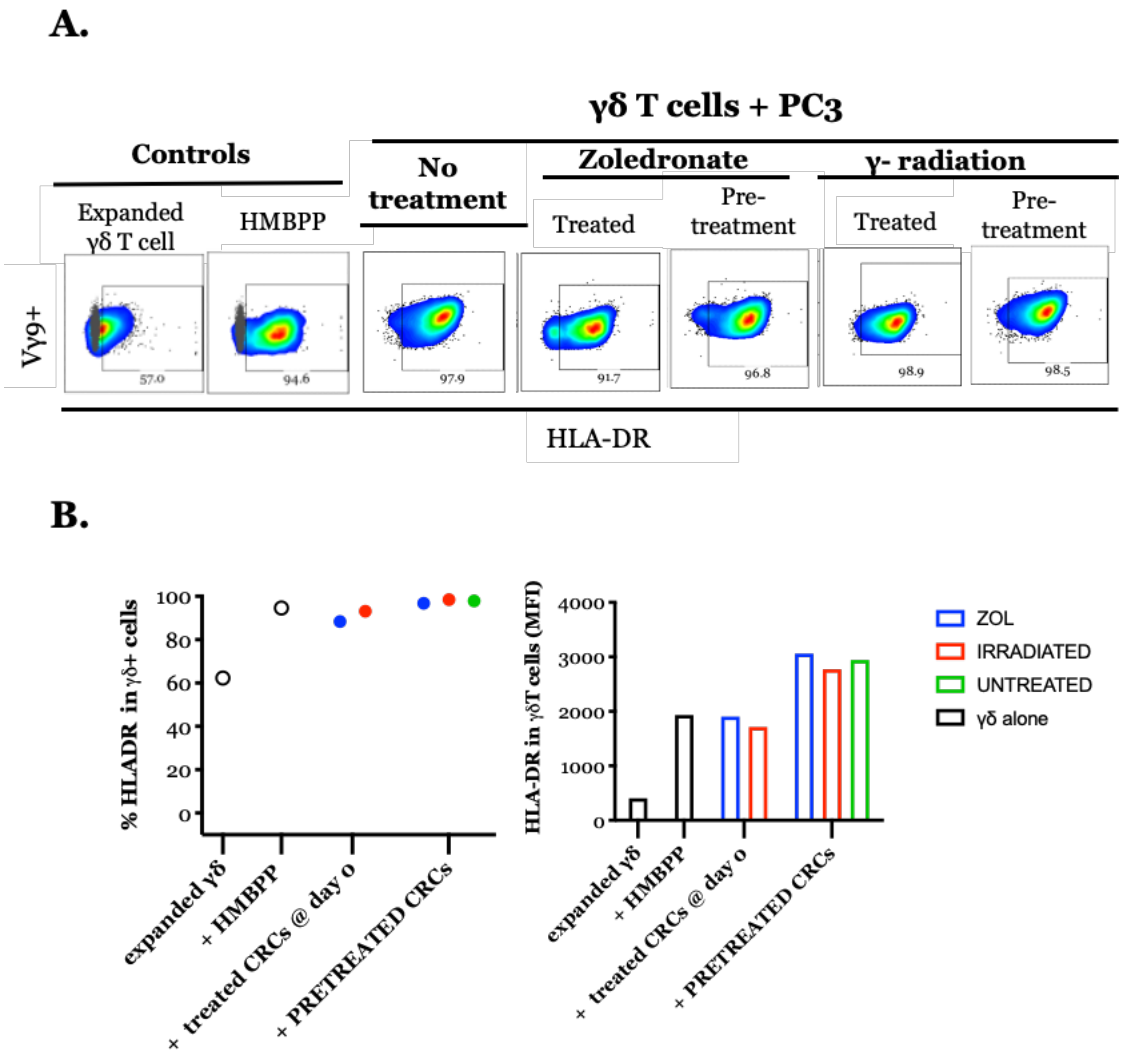


Figure 5.15. Vγ9/Vδ2 T cells upregulate MHC class II after co-culture with PC3 cells.
(**A**) Cancer cells were treated or not with zoledronate (5 μM) or ionising radiation (30Gγ) 48h prior (pre-treated) or immediately before co-culture with expanded Vγ9/Vδ2 T cells (treated @ day 0). HLA-DR expression was analysed by flow cytometry after cells were in co-culture for 72 h. Expanded Vγ9/Vδ2 T cells re-stimulated with HMBPP served as a positive control and expanded Vγ9/Vδ2 T cells maintained in complete RPMI with IL-2 and IL-15 served as a negative control. Cell populations depicted in grey represent isotype controls. (**B**) Comparison of the expression level of HLA-DR by Vγ9/Vδ2 T cells in response to co-culture with PC3 cells shown as percentages (left columns) and MFI values (right columns). The data shown represent one experiment.

.

Similar trends were observed in the expression level of costimulatory molecules CD80, CD86 and CD70 after co-cultures with all three cancer cell lines (**Figure 5.16**, **Appendix 5**). However, the expression level of CD80 and CD86 was best (2.5 fold higher than in HMBPP re-stimulated Vγ9/Vδ2 T cells) in co-culture with zoledronate pre-treated PC3 cancer cells. Likewise, zoledronate treated PC3 cells could induce a high expression level of CD70 in expanded Vγ9/Vδ2 T cells comparable to re-stimulation with HMBPP (**Figure 5.16)**.

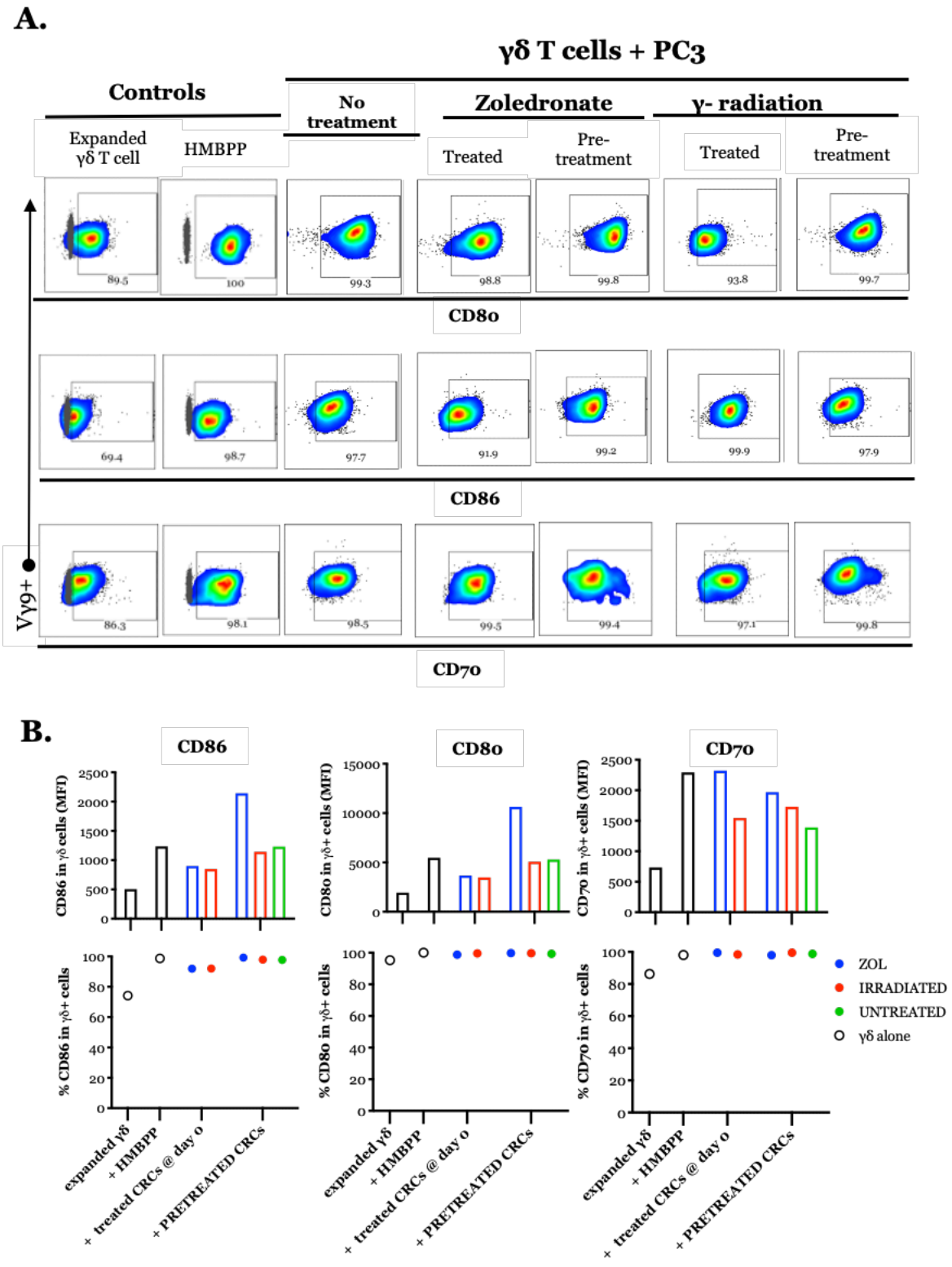


Figure 5.16. PC3 cancer cells induce upregulation of CD86, CD80 and CD70 in Vγ9/Vδ2 T cells

Cancer cells were treated or not with zoledronate (5 μM) or ionising radiation (30Gγ) 48h prior (pre-treated) or immediately before co-culture with expanded Vγ9/Vδ2 T cells (same-day treated). Expanded Vγ9/Vδ2 T cells re-stimulated with HMBPP served as a positive control; expanded Vγ9/Vδ2 T cells maintained in complete RPMI with IL-2 and IL-15 served as a negative control. (**A**) Expression of CD86, CD80 and CD70 was analysed in g9/d2 T cells by flow cytometry 72 hours after co-culture with cancer cells. Gated on single, live, CD3+, Vγ9+ cells. Overlayed cell populations in control samples depicted in grey represent isotype control. (**B**) Comparison of expression of CD80, CD86, and CD70 in Vγ9/Vδ2 T cells in response to co-culture with PC3 cells shown as MFI values (top columns) and as percentages (bottom columns). The data represent one experiment.

CD36 is a scavenger receptor widely expressed in antigen-presenting cells, including dendritic cells, monocytes and macrophages. It plays an important role in the endocytosis of apoptotic cell material by DCs (Albert *et al.*, 1998). A more recent study has shown that CD36 expressed on CD8a DCs, mediates acquisition and presentation of cell-surface antigens from mTECs for T cell receptor repertoire development and allo-tolerance during bone marrow transplantation (Perry *et al.*, 2018). In macrophages, it was shown that CD36 plays an important role in the phagocytic clearance of *Plasmodium falciparum*-infected erythrocytes in a nonopsonic manner (McGilvray *et al.*, 2000). In the field of γδ T cells, CD36 expression was previously demonstrated on bovine γδ T cells where its function was linked to the recognition of LTA molecules (Lubick and Jutila, 2006). In contrast, a recent report has documented CD36 expression on human γδ T cells in patients with the autoimmune skin disorder *Pemphigus vulgaris* (Das *et al.*, 2018). Thus, I was interested to see if CD36 would be expressed in Vγ9/Vδ2 T cells upon engagement with cancer cells and potentially be involved in their uptake/endocytosis and thus antigen presentation.

To examine CD36 expression, identical culture conditions were employed as described above. The highest percentage of CD36 expressing Vγ9/Vδ2 T cells (~72%) were noted in co-cultures with zoledronate pre-treated PC3 cells, while zoledronate pre-treated HT1080 or MCF7 cells induced CD36 expression in ~30-40% of Vγ9/Vδ2 T cells Likewise, the expression level of CD36 was highest under these conditions (**Figure 5.17**). In contrast, either type of cancer cells treated with radiation at day 0 did not induce CD36 expression in Vγ9/Vδ2 T cells. Likewise, HT1080 and MCF7 lines treated with zoledronate could not induce CD36 expression in Vγ9/Vδ2 T cells. Surprisingly, HMBPP re-stimulation of Vγ9/Vδ2 T cells did not induce a noticeable expression of CD36. It would be interesting to examine whether CD36 in Vγ9/Vδ2 T cells associates with TLR receptors or other types of receptors and to discriminate if it is involved in phagocytosis or antigen transfer of apoptotic bodies (Triantafilou *et al.*, 2006; Perry *et al.*, 2018).

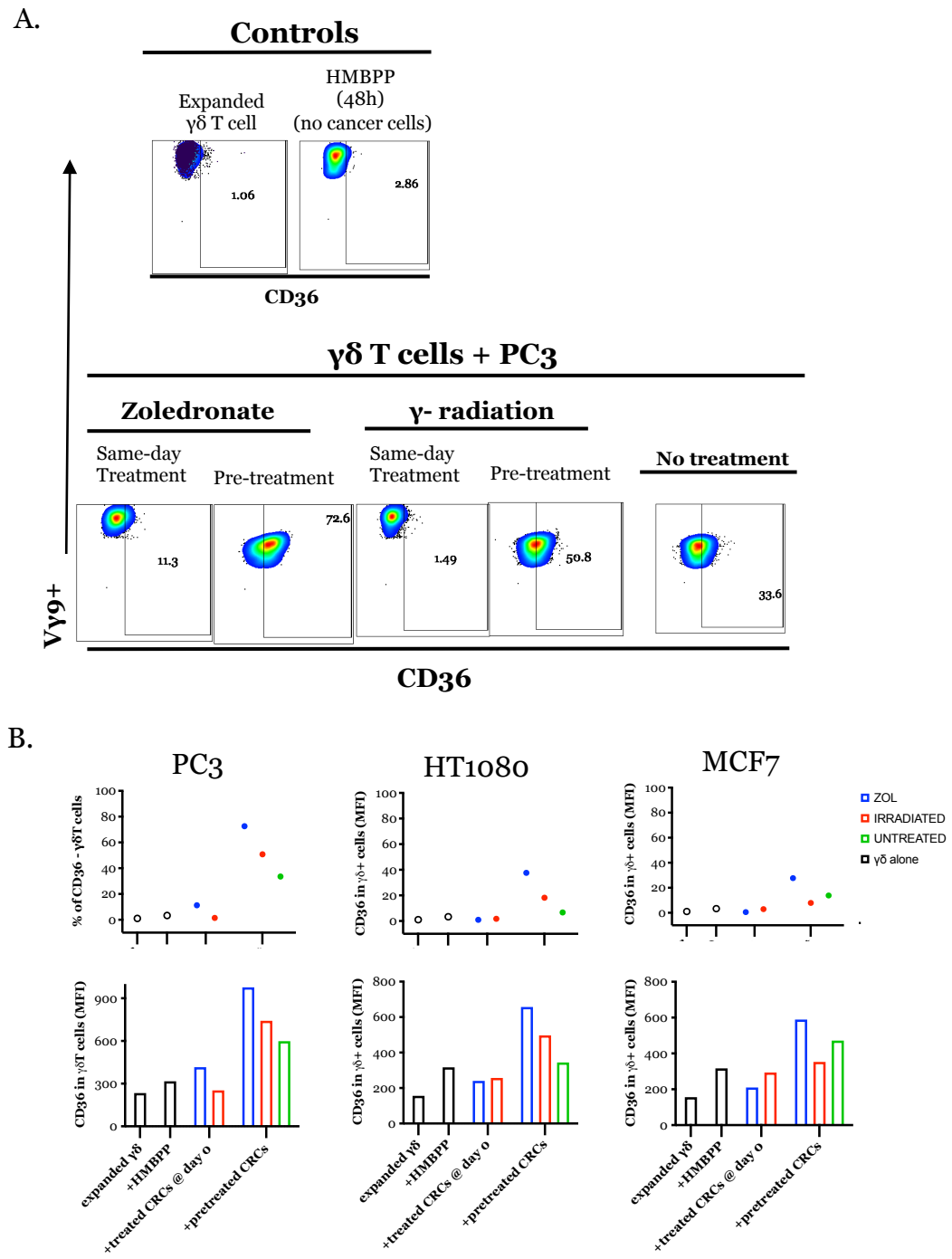


Figure 5.17. Cancer cells induce the expression of CD36 in Vγ9/Vδ2 T cells, but not HMBPP stimulation alone.

(**A**) Cancer cells were treated or not with zoledronate (5 μM) or ionising radiation (30Gγ) 48h prior (pre-treated) or immediately before co-culture with expanded Vγ9/Vδ2 T cells (treated @ day 0). Re-stimulation of expanded Vγ9/Vδ2 T cells with HMBPP served as a positive control, and expanded Vγ9/Vδ2 T cells maintained in complete RPMI with IL-2 and IL-15 served as a negative control. The expression of CD36 in Vγ9/Vδ2 T cells was studied by flow cytometry 72 hours after co-culture with cancer cells. Overlaid cell population depicted in grey represents isotype control. (**B**) Comparison of the expression level of CD36 in γδ T-APCs after co-culture with PC3, HT1080 and MCF7 cells shown as MFI values (top panel) and percentages (bottom panel). Data shown represent one experiment.

Taken together, these preliminary data demonstrated that the pro-APC characteristics in expanded Vγ9/Vδ2 T cells could be greatly escalated upon contact with sensitised or unsensitised cancer cells. Furthermore, the preliminary data suggest that molecular signals received from sensitised cancer cells can also induce the expression of CD36. It can only be speculated that CD36 is involved in the phagocytosis of dying cells, and further analysis is required to confirm this. Lastly, these data suggest that stimulation and APC characteristics may be induced in a TCR independent or synergistic manner; further analysis is required to determine the receptors and ligands involved.

5.4.3 Uptake of cancer cell material by Vγ9/Vδ2 T cells

Nonspecific and receptor-mediated phagocytosis of live and dead material from the environment (from proteins to whole infected cells and apoptotic cells) by DCs have been well established within the past twenty years (Albert *et al.*, 1998; Jenne *et al.*, 2000; Platt *et al.*, 2010). In 2005, Poupot *et al.* (Poupot, Pont and Fournié, 2005) described for the first time that blood Vγ9/Vδ2 T cells of healthy donors take up fragments of many different cancer cell lines. They suggested this happens by trogocytosis. A few years later, reports from Gustafsson's laboratory demonstrated that activated blood Vγ9/Vδ2 T cells are capable of receptor-mediated phagocytosis of *E. coli*, 1 μm synthetic beads, tumour antigens, and cancer cells (Wu *et al.*, 2009; Himoudi *et al.*, 2012; Barisa *et al.*, 2017). In addition, the latest study demonstrated that Vγ9/Vδ2 T cells are excellent in phagocytosing red blood cells infected with *Plasmodium falciparum* (Junqueira *et al.*, 2021). These reports emphasised that opsonisation and CD16 receptor signalling (FcγRIII) is required for the phagocytotic function by Vγ9/Vδ2 T cells.

Nevertheless, based on previous observations from our lab that demonstrated Vγ9/Vδ2 T cells are capable of capturing soluble antigens (Meuter, Eberl and Moser, 2010), I hypothesised that γδ T-APCs would also endocytose/micropinocytose parts of cancer cells or dying cancer cells. Experiments in the Section 5.4.2 suggested that upon activation and contact with sensitised cancer cells, Vγ9/Vδ2 T cells upregulate scavenger receptor CD36, which can play an important role in phagocytosis of apoptotic cell material by DCs (Albert *et al.*, 1998). Here, I investigated the capacity of expanded Vγ9/Vδ2 T cells, and γδ T-APCs take up parts of cancer cells in an opsonisation independent manner (**Figure 5.18**). Generally speaking, mDCs show impaired uptake of exogenous material from the extracellular environment; thus, mature moDCs were used as a negative control, whereas it is known that iDCs are excellent in endocytosis, thus were used as a positive control. I adapted a simple experimental model (Salimu *et al.*, 2015). Briefly, cancer cells were labelled with CFSE 48 h after irradiation (30Gγ) or treatment with zoledronate, and APCs were added to cultures in a 1:1 ratio. The uptake of cancer cells was measured 24 hours later by determining the proportion of Vγ9+CFSE+ or CD209+CFSE+ cells by flow cytometry (**Figure 5.18**). Untreated cancer cells were also included in the experiment.

In contrast, the proportion of endocytic γδ T cells was significantly higher for all three different cancer cell lines (3-fold increase) than DCs. The treatment of cancer cells did not significantly influence the capacity of Vγ9/Vδ2 T cells to uptake exogenous material; it was equally efficient either with treated or untreated PC3 and Ht1080 cancer cells. However, the proportion of endocytic γδ T-APCs was higher as

compared to expanded Vγ9/Vδ2 T cells, especially when encountering MCF7 cells. The proportion of endocytic expanded Vγ9/Vδ2 T cells increased by about 1.5-fold when encountering zoledronate treated MCF7 cells, while irradiation seemed to have the opposite effect on endocytosis of MCF7 cells by expanded Vγ9/Vδ2 T cells. In contrast, the proportion of endocytic iDCs increased significantly upon encountering irradiated MFC7 and HT1080 cells, while no significant difference was observed with PC3 cells. As expected and following previous studies (Albert *et al.*, 1998), almost no endocytosis was observed in mDC encountering irradiated MCF7 or HT1080; however, a two-fold increase was observed when encountering PC3 cells (**Figure 5.18**).

Taken together, these data confirmed the ability of Vγ9/Vδ2 T cells to take up exogenous cancer cell material. These experiments demonstrated that Vγ9/Vδ2 T cells are notably more efficient in acquiring exogenous cancer cell material than DCs. These results confirm that sensitisation of cancer cells with irradiation can improve the endocytic capacity of DCs. In contrast, the treatment of cancer cells with either irradiation or zoledronate did not seem to have a substantial effect on Vγ9/Vδ2 T cells' ability to uptake cancer cell material - it was equally efficient in all conditions tested. Additional analysis is required to confirm statistical significance, while the use of antagonist antibodies and inhibitors specific for endocytosis/micropinocytosis will define which route of antigen uptake has taken place.

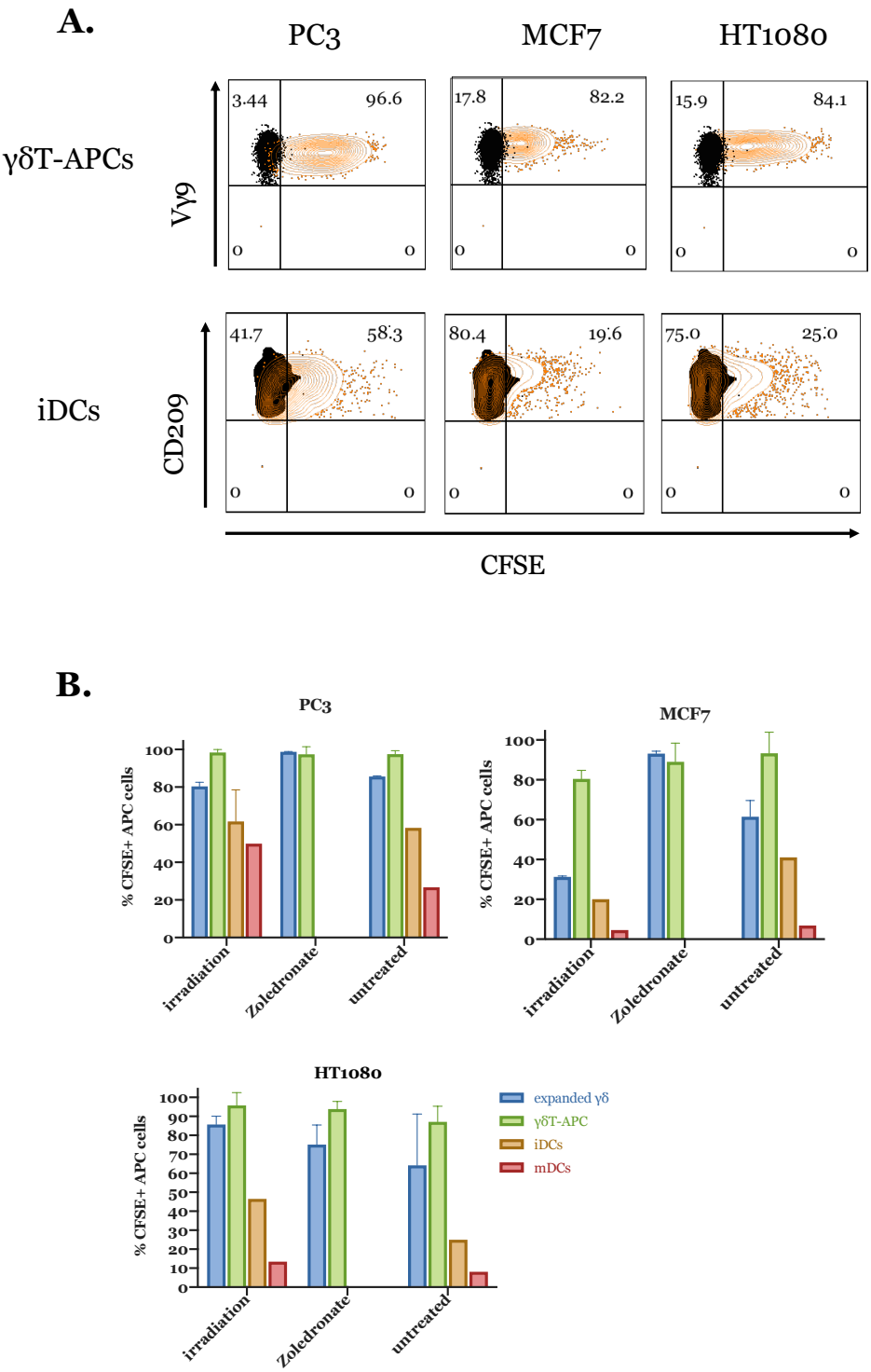


Figure 5.18. Uptake of cancer cell lines by Vγ9/Vδ2 T cells.
Monocyte-derived DC EXPRESS were either stimulated for 48 hours with LPS (mDCs) or not (iDC). Vγ9/Vδ2 T cells, or re-stimulated with HMB-PP-cocktail for 48 hours. PC3, MCF7 and HT1080 cancer cells were either irradiated (30Gy) or treated with zoledronate (5μM) or left untreated and incubated for 48 h. Cancer cells were labelled with CFSE before coculture with γδ T cells and DCs. Representative dot plots showing uptake of untreated CFSE-prelabeled PC3, MCF7, and HT1080 cells (x-axis) by re-stimulated Vγ9/Vδ2 T cells and iDCs (**A**). The numbers in the top right quadrants display percentages of endocytic APCs, determined by Vγ9/CFSE or CD209/CFSE double-positive cells as assessed by flow cytometry. Gated on live, single, Cd3+, Vγ9+ cells.; or live, single, CD209+ cells. Double positivity was established by gating

on Vγ9 alone and followed by CFSE labelled cancer cells alone. (**B**) Summary of results. Bar represents the mean values of data +_SD from two donors from two independent experiments. Gated on live, single, Cd3+, Vγ9+ cells; or live, single , CD209+ cells.

5.4.4 Cross-presentation of 5T4 antigen expressed in cancer cell lines by Vγ9/Vδ2 T cells

Having established that Vγ9/Vδ2 T cells can take up cancer cell material from the extracellular environment, I next investigated 5T4 antigen cross-presentation derived from PC3, HT1080 and MCF7 cancer cell lines. Cancer cells were either sensitised with low dose zoledronate, ionising radiation or left untreated. At the same time, γδT-APCs were generated 48 h before co-culture (pre-restimulated γδs) or at the time of co-culture (same-day stimulated γδs) with cancer cells or were used as expanded Vγ9/Vδ2 T cells. The cancer cell- γδ T cell co-cultures were then incubated for 48 h. Cross-presentation of cancer cell-derived 5T4 antigen was measured by assessing intracellular cytokine production of 5T4-peptide-specific T cells (Methods 2.3.6.3). Successful antigen presentation was determined if IFNγ production was higher than those obtained in control co-cultures, containing cancer cells and peptide-specific CD8 T cells alone (no γδ T cells).

Although some T cell response was triggered by cancer cells alone, significantly more IFNγ production was observed when 5T4 antigen was cross-presented by Vγ9/Vδ2 T cells from ZOL treated PC3 cells (**Figure 5.13**). However, no significant responses were triggered in specific CD8 T cell responses by cross-presenting pre-stimulated γδT cells. This may be because the expression of pro-APC markers by these pre-stimulated γδT-cells had already diminished by the time CD8 T cells were added to co-cultures.

Although the highest IFNγ production was observed when 5T4 antigen was provided by irradiated PC3 cells, the result was insignificant due to increased response detected in the control condition (**Figure 5.13)**. In contrast to PC3 cells that provided a good source of 5T4 antigen (**Figure 5.11**), HT1080 cells provided little 5T4 antigen for cross-presentation; thus, no significant responses were triggered in CD8 T cells (**Appendix 6**). On the other hand, cross-presentation of 5T4 antigen from HLA-A2+ MCF7 cells expectedly did not trigger significant responses in CD8 T cells (**Appendix 6**). To further confirm antigen cross-presentation at the molecular level, inhibitors such as brefeldin and ammonium chloride would demonstrate that the 5T4 antigen was processed intracellularly. Take together, these experiments demonstrate that Vγ9/Vδ2 T cells are able to present a naturally expressed tumour antigen to peptide-specific T cells and that this process is not significantly enhanced by tumour cell sensitisation. However, the induction of robust CD8 T response appears to depend on the antigen expression level, the cancer cell type, and the pro-APC status of Vγ9/Vδ2 T cells. Additional experimental repeats are required to increase statistical power. Further research is required to expand these findings to additional cancer cell lines.

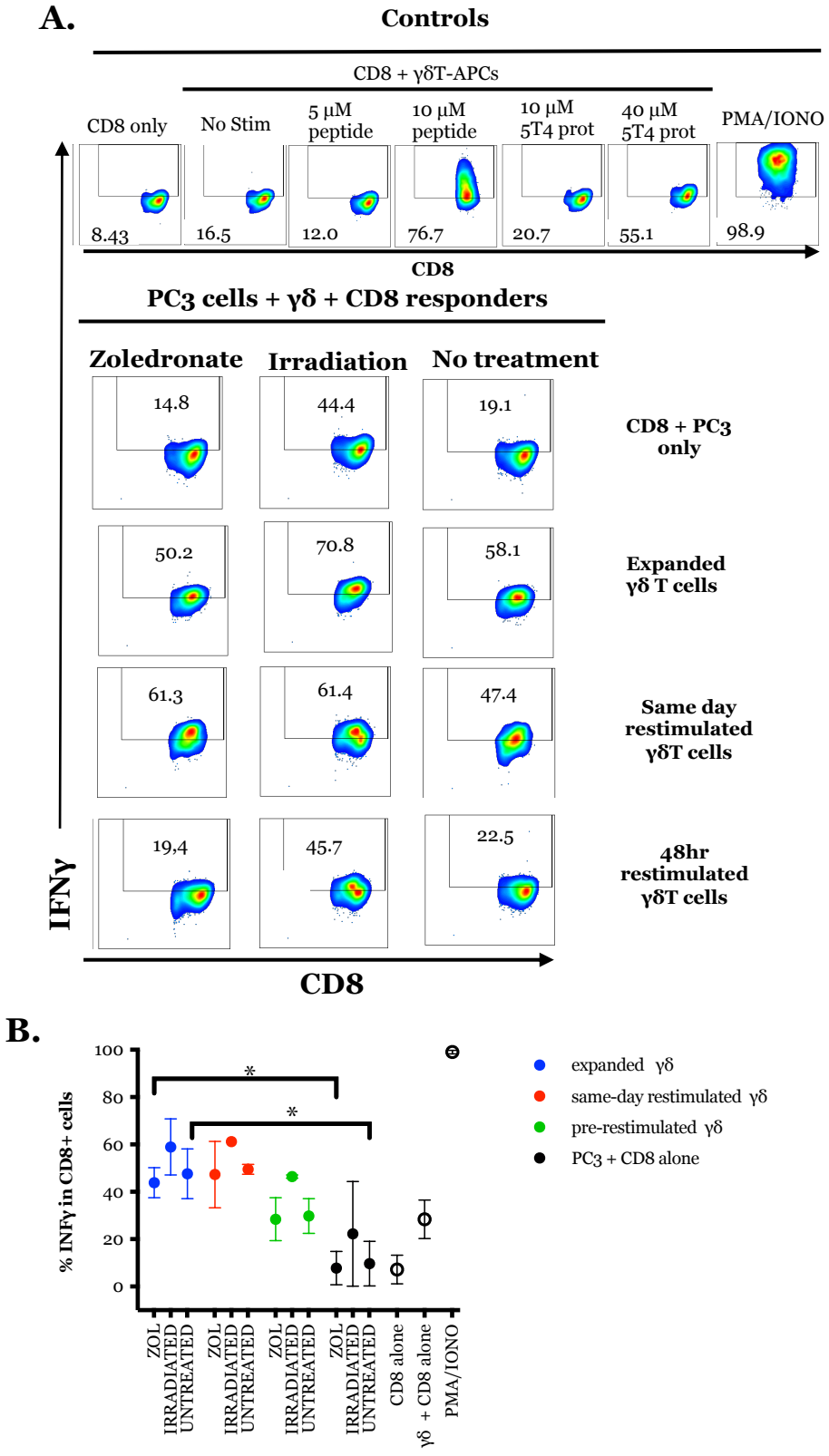


Figure 5.19. Cross-presentation of 5T4 antigen expressed in PC3 cancer cell line to cognate peptide-specific CD8 T cells by γδ-T-APCs.

(**A**) Expanded Vγ9/Vδ2 T cells were either re-stimulated with HMB-PP for 48 hours before (pre-restimulated γδ) or at the time of coculture (same-day re-stimulated γδs) with cancer cells or were left untreated (expanded γδ). Cancer cells were either left untreated or were treated with low dose zoledronate (10 μM) or irradiation (30Gγ) 48 h before coculture with γδ T cells at 1:1 ratio in the presence of IL-2 and

IL-15. The cancer cell - γδ T cell cocultures were then incubated for 48 h and the 5T4p17 - peptide specific CD8 T cells were added directly to cell cultures and were incubated overnight in the presence of BRE A. Negative controls included CD8 T cells alone, cocultures of cancer cells and CD8 T cells alone, and cocultures of γδ T cells with CD8 T cells alone. Positive controls included CD8 T cells stimulated with PMA/ionomycin, γδ T-APCs pulsed with 5T4 protein or 5T4p17 peptide at indicated concentrations. Successful antigen cross-presentation was measured by IFNγ production in CD8 T cells, determined by ICS and flow cytometry. Gated on single, live, Vγ9 negative cells, CD8 cells. (**B**) Combined data from 2 individual experiments shown as percentages of IFNγ positive CD8 T cells. The significance of differences was calculated by two-way ANOVA followed by Dunnett's multi comparison test. Significance displayed compared to cocultures with PC3 + CD8 T cell alone and CD8 T cell alone. *=p<0.05

5.5 Discussion

In summary, the first part of this Chapter confirmed that the selective expansion of highly pure Vγ9/Vδ2 T cells could be achieved with ease in two weeks using zoledronate. The presented data confirm that Vγ9/Vδ2 T cells acquire robust pro-APC phenotype and function. Furthermore, it is demonstrated that pro-APC phenotype can be rapidly re-established in expanded Vγ9/Vδ2 T cells in 3-5 days upon HMBPP re-stimulation, and therefore these cells are called γδ T-APCs. Furthermore, findings here demonstrate that co-culture with sensitised cancer cells can also generate γδ T-APCs. Thus, the preliminary findings presented here show for the first time that, unlike HBMPP stimulation, cancer cells can stimulate CD36 expression in Vγ9/Vδ2 T cells. However, further analysis is required to confirm this discovery statistically. The second part of the Chapter compares and discusses the antigen-presenting function in expanded Vγ9/Vδ2 T cells and γδ T-APCs; shown is the ability to (i) uptake of exogenous cancer cell material, (ii) and to cross-present soluble and cancer cell-derived 5T4 antigen. The data presented in this Chapter support the notion that Vγ9/Vδ2 T cells can cross-present 5T4 and induce intermediate responses in tumour peptide specific CD8 T cells. Further examination is required to expand these findings to other tumour antigens.

5.5.1 Vγ9/Vδ2 T cells with pro-APC phenotype

Previous Vγ9/Vδ2 T cell studies from our lab have shown that IL-2 and IL-15 both together and independently promote APC phenotype and effector function in Vγ9/Vδ2 T cells (Khan *et al.*, 2014; Chen *et al.*, 2017; Tyler *et al.*, 2017). In agreement with previous reports, data presented here show that expansion of Vγ9/Vδ2 T cells with zoledronate in the presence of both IL-2 and IL-15 selectively expanded Vγ9/Vδ2 T from PBMC and rendered their ability to cross-present exogenous FluM1 antigen and induce potent responses in M1p58 specific CD8 T cells (Brandes *et al.*, 2009; Meuter, Eberl and Moser, 2010). Likewise, in this study, upon phosphoantigen stimulation, Vγ9/Vδ2 T cells showed prominent upregulation of all pro-APC markers at the cell surface that reached their peak levels within the first 5-8 days (**Figure 5.3 – 5.4**). However, after that period, the staining for most of the APC markers returned to basal levels. A similar pro-APC phenotype in Vγ9/Vδ2 T cells was also reported by others (Landmeier *et al.*, 2009).

Previously, Gustafsson laboratory showed that APC molecules CD40, CD86, CD80, and HLA-DR are upregulated in freshly isolated Vγ9/Vδ2 T cells upon 5-day co-culture with cancer cells and EBV–transformed B cells (Himoudi *et al.*, 2012). In this chapter, I first demonstrated that expanded Vγ9/Vδ2 T cells re-acquired fully mature pro-APC phenotype within 5 days after re-stimulation with HMBPP (**Figure 5.5 – 5.6**) or co-culture with cancer cells (**Figure 5.15-17**, **Appendix 4-5**). These short-term restimulated Vγ9/Vδ2 T cells are thereafter called γδ T-APCs. In addition, Vγ9/Vδ2 T cells re-acquired the full pro-APC phenotype in co-cultures with sensitised cancer cells. Different types of cancer cells take up zoledronate with different efficiencies, and therefore the production of isopentenyl pyrophosphate and other metabolites of the mevalonate pathway varies among different cells, thus the strength of Vγ9/Vδ2 T cell stimulation. The expression of APC markers in Vγ9/Vδ2T cells was highest after co-cultures with zoledronate pre-treated PC3 cancer cells, suggesting TCR stimulation was vital for the re-establishment of full pro-APC phenotype. Interestingly, γ-radiated PC3 cells induced the expression of MHC molecules in Vγ9/Vδ2 T cells to the extent that exceeded the levels in HMBPP restimulated γδ T cells. Although, further experimental repeats are required to achieve statistical significance.

CD36 expression was previously described in bovine γδ T cells (Lubick and Jutila, 2006), and so far no expression of CD36 has been reported on human γδ T cells. In bovine γδ T cells, CD36 expression seems to be regulated by phorbol esters and facilitates recognition of LTA in conjunction with TLR2 (Lubick and Jutila, 2006). Data in this study demonstrated for the first time that intermediate-high CD36 expression could be induced in human γδ T cells upon engagement with stressed/dying cancer cells. However, unlike in conventional T cells (Tassone *et al.*, 1998), the CD36 expression on human γδ T cells in this study was not detected earlier than 48 h after re-stimulation. Thus, this suggests that the stimulation of human γδ T cells in this study was much weaker than the PHA induced CD36 in conventional T cells, and hence prolonged stimulation was required for induction of CD36 expression on γδ T cell surface. Zoledronate sensitised PC3 cells induced the highest CD36 expression in Vγ9/Vδ2 T cells. Seeing that the CD36 expression was induced only in co-cultures with cancer cells but not with HMBPP alone, these data indicate that an additional non-TCR signal was required to induce CD36 expression. Nevertheless, further experimental analysis is required to confirm these preliminary findings.

Although the function of CD36 is wildly divergent and cell type-specific (Silverstein and Febbraio, 2009), so far, CD36 expression and function in T cells have not been clearly established. In macrophages, monocytes and DCs, CD36 plays a prominent role in endocytosis and the internalisation of apoptotic cells, shed outer photoreceptor segments, oxidatively modified lipoproteins, glycated proteins, and amyloid-forming peptides (Silverstein and Febbraio, 2009)(Albert *et al.*, 1998). However, additional studies are required to determine the function of CD36 in Vγ9/Vδ2 T cells.

Finally, these preliminary data suggest that cancer cells produced a molecular signature that favourably induced APC maturation in expanded Vγ9/Vδ2 T cells. Future investigation should include additional cancer cell lines or primary cancer cells, and additional experimental repeats are needed to support these findings.

5.5.2 Cross-presentation of tumour antigens and induction of cognate CD8 T cell responses

So far, only one study has examined and reported the ability of Vγ9/Vδ2 T cells to cross-present tumour antigens (Himoudi *et al.*, 2012). While rather than day 14 expanded Vγ9/Vδ2 T cells, they utilised freshly isolated, and 5-day stimulated γδ T-APCs. Therefore, the expression of MHC molecules and co-stimulatory molecules examined in their study positively correlates with the phenotype of Vγ9/Vδ2 T cells in the early phase of stimulation in this study, but not with day 14 expanded Vγ9/Vδ2 T cells. Thus, successful induction of CD8 T cells responses by cross-presenting Vγ9/Vδ2 T cells seen in the study by Himoudi and colleagues (Himoudi *et al.*, 2012) appears to correlate with rapid induction of professional APC characteristics.

A study by Himoudi and colleagues demonstrated that activated Vγ9/Vδ2 T cells endocytosed Pax5 antigen from Daudi cells and cross-present it to antigen-specific CD8 T cells in a CD16 and opsonisation-dependent manner (Himoudi *et al.*, 2012). On the other hand, by confocal microscopy and molecular approaches, uptake of the soluble antigen and larger extracellular material by Vγ9/Vδ2 T cells was previously demonstrated in studies from our laboratory (Marlène Brandes *et al.*, 2009; Meuter, Eberl and Moser, 2010; Chen *et al.*, 2017). Furthermore, a more recent report demonstrated the ability of Vγ9/Vδ2 T cells to take up parts of zoledronate sensitised tdTomato- and FluM1-expressing breast cancer cells for antigen processing (Chen *et al.*, 2017), while work from other laboratories also reported uptake of large exogenous material such as whole bacteria, cancer cells and *Plasmodium falciparum*-infected red blood cells by γδ T-APCs in opsonisation and FCγ receptor-mediated fashion (Himoudi *et al.*, 2012; Barisa *et al.*, 2017; Junqueira *et al.*, 2021). On that note, it was shown that antigens associated with large (1 μm) structures could be processed and presented by Vγ9/Vδ2 T cells on MHC class II.

The data presented here demonstrate the ability of γδ T-APCs as well as expanded Vγ9/Vδ2 to take up parts of treated and untreated cancer cell lines of different origin (sarcoma, breast and prostate cancer), and it seems the uptake is more efficient than in iDCs (**Figure 5.18**).

We could speculate that the route of the antigen uptake may have been by receptor-mediated endocytosis, or it could have been non-specific, such as micro/macropinocytosis. However, many receptors are involved in endocytic pathways; thus, it is difficult to speculate which were involved here. Nevertheless, it is not always easy to draw clear distinctions among endocytic pathways and that some receptors, depending on the circumstances, are not necessarily restricted to a single endocytic pathway (Robinson, 1994; Sorkin, 2004; Benmerah and Lamaze, 2007). Targeting/blocking surface membrane water-channel proteins, Aquaporins (AQPs) and amiloride-sensitive Na+/H+ channels, which are known to play an important role in controlling the formation and concentration of macromolecules and efficient antigen presentation in immature DC (de Baey and Lanzavecchia, 2000) could have provided some evidence for macropinocytosis. In this study, the evaluation of the uptake of extracellular material by APCs was based on the flow cytometric analysis of double-positive events for γδ T cell or DC-specific antibodies and CFSE. While this method has been used before in studies evaluating endocytosis by DCs (Iyoda *et al.*, 2002; Salimu *et al.*, 2015), it cannot be excluded that the uptake of the cancer cell material

was *via* trogocytosis (Poupot, Pont and Fournié, 2005; Perry *et al.*, 2018). The future investigation will determine what route was utilised for the uptake of the extracellular material.

Regardless of the reduced expression level of pro-APC markers in expanded Vγ9/Vδ2 T cells, they were able to cross-present FluM1 protein and induce robust FluM1-specific CD8 T cell responses (**Figure 5.8**). However, these "semi-mature" 5T4-protein pulsed Vγ9/Vδ2 T cells could not induce responses in 5T4 specific CD8 T cells. This may be because FluM1 CD8 responders likely possess a high-affinity TCR, and thus their successful response did not require a high peptide concentration presented on MHC (Stone, Harris and Kranz, 2015), and the help from co-stimulatory molecules may not have been crucial either (Aleksic *et al.*, 2010). Notably, the data here support the notion that γδ T-APCs can cross-present soluble 5T4 tumour antigen as well as 5T4 derived from cancer cells; although, intermediate responses were induced in cognate CD8 T cells (**Figure 5.10 – 12**). This may likely be because, unlike with the exogenously added peptide, a variety of peptides generated through antigen-processing will occupy the MHC I molecules; thus, the actual concentration of the peptide of interest presented at the surface was likely at a much lower concentration. This is further supported by data using cancer cells as an antigen source - where the level of 5T4 expression in cancer cells positively correlated with the level of response induced in cognate CD8 T cells. On the other hand, through direct biophysical measurements, previous reports have shown that anti-cancer TCRs bind to their cognate peptide-HLA with affinities ~5-fold weaker than pathogen-specific TCR (Cole et al., 2007; Aleksic et al., 2012). TCR affinity is an important parameter in T-cell activation, and a 5-fold difference in TCR affinity can result in a marked difference in recognition and responses induced by tumour epitopes (Tan et al., 2015).

Since the expression of most of the co-stimulatory molecules in *expanded* Vγ9/Vδ2 T cells were at intermediate levels, and some were utterly absent at day 14, this suggests that the failure to induce responses in tumour-peptide specific CD8 T cells might also be due to insufficient co-stimulatory signalling. In contrast, a powerful co-stimulatory signal might come from the interaction of CD70 on γδ T-APC with CD27 on CD8 responder cells (**Figure 5.5; 5.7**). Furthermore, great responses were induced by tumour peptide-pulsed T2 cells that show a high expression level of all pro-APC markers (**Appendix 7**) but have defective peptide supply and impaired ability to process and present self-antigens via both class- I and class-II pathways (Salter, Howell and Cresswell, 1985; Salter and Cresswell, 1986; Steinle and Schendel, 1994). In addition, since T2-cells express "empty" MHC I molecules on their surface, the concentration of the tumour peptides presented by T2-A2 cells was likely much higher than by expanded Vγ9/Vδ2 T cells. This may also explain why responses induced in 5T4 tumour specific CD8 T cells by peptide-pulsed T2 cells were much more potent than by *expanded* Vγ9/Vδ2 T cells. Thus, the findings from this study suggest that the induction of potent responses in CD8 T cells with low-affinity TCRs may also rely on co-stimulatory signals provided by APCs (Rajasekaran et al., 2010). Further analysis using antagonistic antibodies will confirm this.

Unfortunately, there was not enough time to look closely into the intracellular mechanisms of antigen processing; proteasome blocking and measurement of pH would have confirmed the cross-presentation pathway. Moreover, using inhibitors lactacystin and brefeldin A in this investigation would reveal the routes of antigen processing leading to peptide loading onto MHC I and wheather the proteasome and

trans-Golgi network of classical MHC I pathway were involved. It would also be interesting to examine if the memory state influences the cross-presentation function in Vγ9/Vδ2 T cells.

Finally, using tumour peptide specific CD8 T cells as a tool for measuring successful antigen cross-presenting function proved to be challenging. Using molecular tools such as fluorophore coupled soluble TCRs, TCR-tetramers (Bossi et al., 2013) would be a great way to support these findings. To improve antigen cross-presentation, loading antigen onto γδ T-APCs in the form of mRNA or DNA encoding tumour antigen by electroporation (Anguille et al., 2009; Ang et al., 2020) or using adenovirus as a vehicle (Yoshio Ogawa, 2013) could improve the final epitope concentration presented on the surface of γδ T-APCs and thus improve responses in cognate CD8 T cells.

5.5.3 Concluding remarks

Data from this study provide evidence that responses in 5T4 tumour antigen-specific CD8 T cells can be induced by 5T4 antigen cross-presenting γδ T-APCs, but further analysis is required to expand this finding to additional antigens. Collectively, it is evident that the regulation of antigen uptake, processing and presentation by Vγ9/Vδ2 T cells likely differs compared to DCs in that immature DCs constantly take up and process antigen until maturation, upon which endocytic function is downregulated, and their function instead switches to inducing potent stimulation of adaptive immune cell responses. In contrast, Vγ9/Vδ2 T cells require a specific activation stimulus before uptake of extracellular material, and processing + presentation of antigen occurs. While the upregulation of co-stimulatory molecules for inducing robust T cell responses seem simultaneous in Vγ9/Vδ2 T cells, an additional secondary signal is probably required to induce their powerful opsonisation-independent endocytic feature. The data in this chapter thus demonstrate that opsonisation is not a requisite for antigen-cross presentation function in Vγ9/Vδ2 T cells and indicates that a secondary signal via Fcγ receptor may play a collaborative role for the uptake of tumour antigens and presentation as it is seen in mDCs (Platt et al., 2010; Himoudi et al., 2012), but is not required for the uptake, cross-presentation and induction of a potent tumour specific CD8 T responses by γδ T-APCs.

Lastly, the expert phagocytic properties and processing of particulate antigens are well known for moDCs. While many attempts have been made to use moDCs in the clinics, the uptake and cross-presentation of soluble tumour protein is relatively inefficient (Ho et al., 2018) and requires several adjustments and "artificial help". Help in the form of protein-conjugates, guiding DCs to detect and take up tumour antigen and directing the processing pathway towards proteasomal degradation and cross-presentation is usually required. In contrast, the ease of manipulating human γδ T-APCs and their ability to cross-present tumour protein in combination with providing strong T cell costimulatory signals could make them a promising new tool for cancer immunotherapy.

Chapter 6 GENERAL DISCUSSION

Summary

It has been 16 years since Vγ9/Vδ2 T cells were reported as a novel type of professional APC. This study aimed to investigate the ability of Vγ9/Vδ2 T cells to take up, process and present tumour antigens and induce robust responses in CD8 T cells. To do so, I first established protocols and generated recombinant tumour associated antigens 5T4 and NY-ESO-1 using bacterial systems as surrogates for economical production of large quantities of protein; and 5T4 specific CD8 T cell lines and clones with distinct phenotypical and functional characteristics, *ex vivo*. Both served as molecular and cellular tools to study and answer questions about antigen cross-presentation function in Vγ9/Vδ2 T cells. Previous studies established that direct or indirect TCR stimulation by HMB-PP, IPP or zoledronate in combination with the common γ-chain family cytokines IL-2 and IL-15 favourably induces the APC phenotype in Vγ9/Vδ2 T cells, while cells can be expanded to great numbers in ~2 weeks (Khan *et al.*, 2014; Van Acker, Anguille, Willemen, Van den Bergh, *et al.*, 2016; Tyler *et al.*, 2017). Notably, these findings showed upregulation of key APC molecules, cytokine production, and the ability to take up exogenous antigens. Although cytokine production was not studied in this thesis, other data presented here largely agrees with the previous studies. Upon stimulation, Vγ9/Vδ2 T cells showed excellent proliferation; however, expanded cells showed various degrees of APC marker expression that was donor-specific, probably due to the differences in their memory phenotype profiles. Expanding on previous studies into the generation of γδ T-APCs, I optimised and established an improved protocol for the generation of γδ T-APCs with uniformly high expression of APC markers. Likewise, data presented here show that zoledronate or γ-radiation sensitised cancer cells can enhance the APC phenotype in expanded Vγ9/Vδ2 T cells, while additionally inducing expression of the scavenger receptor CD36, which may add additional APC characteristics – potentially providing the ability to endocytose exogenous material as seen in DCs (Albert *et al.*, 1998; Perry *et al.*, 2018).

Regarding the pro-APC characteristics of γδ T-APCs cells, I have shown *in vitro* that γδ T-APCs but not expanded γδ T cells could cross-present soluble recombinant 5T4 tumour antigen and induce moderate responses in cognate CD8 T cells. Concerning the improvement of antigen cross-presentation, loading of antigen onto γδ T-APCs in the form of DNA/ mRNA encoding tumour antigen through transfection or transduction might result in an increased final concentration of presented peptides on the surface of γδ T-APCs, and thus more robust responses in cognate CD8 T cells. This observation contrasts with previous findings from our lab that demonstrated efficient cross-presentation of microbial antigens by expanded Vγ9/Vδ2 T cells and induction of robust cognate CD8 T cell responses (Marlène Brandes *et al.*, 2009; Khan *et al.*, 2014). In agreement with a previous study (Meuter, Eberl and Moser, 2010), data in this thesis demonstrate that γδ T-APCs as well as expanded γδ T cells readily take-up parts of cancer cells (sensitised or not) for processing, while they successfully cross-present 5T4 - tumour antigen obtained from these cancer cells and induce moderate responses in cognate CD8 T cells.

A few limitations and challenges have become evident during the study. Due to assay limitations, the readout for efficient tumour antigen cross-presentation was partially masked since it relied on the ability of tumour-antigen specific CD8 T cells to produce IFNγ. The TAA-specific CD8 T cells produced noticeable IFNγ in experimental controls, likely due to unknown factors in the FCS. Furthermore, as discussed in Chapter 4, these TAA-specific CD8 T cells were of central memory phenotype, and such cells are known to produce reduced levels of cytotoxic cytokines such as IFNγ as compared to cytotoxic effector CD8 T cells (*Section 1.3.3 The shades of mature CD8 T cell subsets*) (Gattinoni, Lugli, Ji, Pos, Chrystal M. Paulos, *et al.*, 2011). Combining a future γδ T-APC cell vaccine with less differentiated CD8 T cell subsets within the infusion product may ensure T cell expansion and potentially long term T cell persistence (Powell *et al.*, 2005; Xu *et al.*, 2014; Klebanoff *et al.*, 2016) . Importantly, a relatively recent study has shown that a remarkable efficacy in killing breast cancer stem-cell-like cells can be achieved with joined forces of Vγ9/Vδ2 T cells and CD8 cells (Chen *et al.*, 2017). Although previous reports demonstrated that γδ T-APCs of cancer patients are equally efficient in inducing responses in cognate CD8 T cells when cross-presenting FluM1 antigen (Khan *et al.*, 2014), it would be interesting to investigate if this also holds for TAAs. It is known that different antigens may trigger different routes of degradation due to their biochemical properties and may be rapidly degraded by immunoproteasomes. Since γδ T-APCs predominately possess the immunoproteasome (Marlène Brandes *et al.*, 2009), cross-presentation of some tumour associated peptides may not occur due to their rapid degradation, such as is seen for MELAN-A peptides (Marlène Brandes *et al.*, 2009). Significant variability in Vγ9/Vδ2 T cell functional potential has been found between individuals (Ryan *et al.*, 2016), manifested by differential mechanistic capacities to kill tumour targets. In addition, the cellular composition of PBMC from cancer patients differs substantially from healthy individuals, possibly reflecting the history of cancer treatment regimens and potential immunosuppressive conditions (Zou, 2005).

Moreover, while no differences were noted in antigen cross-presentation abilities of Vγ9 /Vδ2 T cells in healthy donors vs patients for microbial antigens (Khan *et al.*, 2014), it may be of value to investigate cross-presenting functionality in terms of tumour antigens. For example, monocytes isolated from cervical cancer patients are impaired and do not differentiate into functional mDCs *in vitro* compared to healthy controls (Roy *et al.*, 2011). Therefore, it would be valuable to understand if the tumour antigen-cross presentation function in Vγ9/Vδ2 cells of cancer patients remains intact.

In summary, the data in this thesis confirm the proof-of-concept and demonstrate that γδ T-APCs are capable of efficient cross-presentation of 5T4 tumour antigen, however further studies are required to expand these findings to other types of tumour antigens and to *in vivo* setting.

Unanswered questions

Before using γδ T-APCs in the clinic, a few open questions remain to be addressed. The most important question is whether an efficient migration of antigen-presenting γδ T-APC will be achieved when injected into patients. Upon stimulation, Vγ9/Vδ2 T cells rapidly and transiently switch off the

expression of CCR5 and start to express CCR7 in conjunction with other homeostatic chemokine receptors such as CCR4 and CXCR4. It was shown that *in vitro* activated Vγ9/Vδ2 T cells migrate efficiently toward the corresponding homeostatic ligands CCL21/SLC, CCL22/MDC and CXCL12/SDF-1, respectively, and not toward the inflammatory CCR5 ligand CCL5 (Brandes *et al.*, 2003). This is remarkably similar to DCs, which rapidly up-regulate CCR7 in response to TLR ligands such as LPS. However, no studies have yet demonstrated that this happens *in vivo* due to apparent technical limitations. The whole concept of γδ T-APC as cellular vaccines relies on their ability to migrate into lymph nodes to present the antigen to induce priming and activation of cognate T cells, yet no studies have yet demonstrated this *in vivo*. Until recently, a suitable animal model to study Vγ9/Vδ2 T cell function was not known (Fichtner *et al.*, 2018, 2020), and all clinical studies so far have focused on direct tumour cell killing properties of Vγ9/Vδ2 T cells rather than their APC function.

The data presented in this study indicate that the preparation of γδ T-APCs for infusion into cancer patients would require two stimulations: 1) to expand Vγ9/Vδ2 T cells to a sufficient number; and 2) to induce optimal γδ T-APC phenotype before being infused into patients. However, some patient PBMCs may contain a low number of cells, while these cells are functionally impaired, thus would require repeated stimulations. Therefore, a high yield of expanded, autologous Vγ9/Vδ2 T cells is undoubtedly desirable for further use as a cellular vaccine. However, the quality of expanded Vγ9/Vδ2 T cells in terms of their state of differentiation and responsiveness to re-stimulation is an equally important parameter to be considered. In agreement with previous studies, expanded Vγ9/Vδ2 T cells predominantly express surface markers suggestive of a TEM phenotype and, to a lesser extent, TCM phenotype (Dieli *et al.*, 2007), although the validity of adopting this terminology for γδ T cells is questionable. For example, while γδ T cells have shown remarkable persistence upon repeated stimulations than CD8 T cells, further investigations with additional memory differentiation markers are required to understand how their status changes over time.

Furthermore, will repeated activation and (prolonged) expansion of Vγ9/Vδ2 T cells result in upregulation of inhibitory molecules such as PD1/PDL1, CTLA-4, TIM3, which would limit their APC functionality? Substantial progress has been made, providing new insights into Vγ9/Vδ2 repertoire focusing, functional diversity and plasticity. Progress has also been made in our understanding of the TME dynamics, but a more profound knowledge of the complex immune processes involved in immunosurveillance and tumour progression is still lacking. It remains to be answered whether the interaction of Vγ9/Vδ2 T cells with tumour cells *in vivo* will not only lead to tumour cell killing but also induction of APC phenotype, uptake of TAAs for processing and presentation to induce αβT cell responses locally (tumour tissue) or in lymph nodes. Will γδ T-APCs remain functionally intact when injected into the patient and able to induce robust anti-tumour responses in αβT cells? On a similar note, even if γδ T-APC may remain intact, but the patient possesses little or no responsive and functional αβT cells, how successful will a γδ T-APC vaccine be in eradicating the tumour?

Considerations for the use of γδ T-APCs vaccine for cancer therapy

γδ T cells play an important role in cancer immune surveillance (Hayday, 2009) as evidenced by; (a) an increased incidence of tumours in γδ T cell-deficient mice (Girardi, 2001), (b) the increased numbers of γδ T cells in reactive lymphatic regions associated with neoplasia, (c) their infiltration into solid tumours (Gentles *et al.*, 2015), (d) their potential to kill a variety of tumour cells (Lo Presti, Dieli and Meraviglia, 2014), and (e) their potential to act as professional APCs (Brandes, Willimann and Moser, 2005). There is an ever-growing body of evidence suggesting that Vγ9/Vδ2 T cells can be manipulated to target tumours more efficiently. Therefore, γδ T cells have received attention as an alternative source for cellular immunotherapies (Fisher *et al.*, 2014). *In vitro* data evidenced that a multitude of tumour cell types are susceptible to Vγ9/Vδ2 T cell-mediated cytotoxicity, and *in vivo* data show that Vγ9/Vδ2 T cell-based therapies can have favourable clinical outcomes in a range of cancers. Their non-MHC-restriction gives Vγ9/Vδ2 T cells a significant advantage since they are unlikely to cause graft-versus-host disease, allowing them to be generated from healthy donors and given in an allogeneic setting as an "off-the-shelf" therapeutic (Godder *et al.*, 2007). Both adoptive transfer and *in vivo* activation/expansion of γδ T cells have proven to be safe therapeutic modalities that can result in objective clinical responses in the treatment of cancer (Fisher *et al.*, 2014)(Buccheri *et al.*, 2014; Sebestyen *et al.*, 2020). The review of 12 clinical trials and 157 patients who had received γδ T cell-based immunotherapies surmised that γδ T cell-based immunotherapy is superior to current second-line therapies such as prednisolone + docetaxel and everolimus for advanced prostate cancer and renal cell carcinoma, respectively (Fisher *et al.*, 2014). Although Vγ9/Vδ2 T cells have shown promise in immunotherapies against a range of tumours, patient responses and clinical outcomes have been variable and unpredictable in most clinical trials. This may be due to variability in Vγ9/Vδ2 T cell functional potential between individuals in the general population, which is manifested by differential mechanistic capacities to kill tumour targets (Ryan *et al.*, 2016), but also due to a particular suppressive TME or "whole organismal environment" (WOE) of an individual patient (Laplane *et al.*, 2019). In their review, Laplane and colleagues have nicely illustrated that not only does a particular TME influence phenotype and functionality of immune cells, but also the WOE can pose changes on the immune cell function (Laplane *et al.*, 2019).

The general purpose of the research presented in this thesis is to improve the Vγ9/Vδ2 T cell immunotherapy for cancer. Together with previously reported findings from our laboratory and others, this study suggests that γδ T-APC represent a promising alternative to moDC. In support of the original hypothesis underlying this thesis, I have demonstrated that upon activation, Vγ9/Vδ2 T cells become γδ T-APCs capable of cross-presenting tumour antigens and inducing responses in cognate peptide-specific CD8 T cells. By using γδ T-APC-based vaccines similarly to DC-based therapies, the bottlenecks associated with the difficulty of obtaining large numbers of homogenous functional APCs could be overcome. Even though γδ T-APCs would be directed to lymph nodes for induction of anti-tumour adaptive immune responses, the success of the therapy would then also rely on the ability of αβT cells to tackle the tumour burden and its potentially suppressive microenvironment. In addition, due to

harsh first line chemo-treatments, many cancer patients have a low number of immune cells, which may also possess dysfunctional phenotypes. Thus, a successful vaccine approach should go beyond γδ T-APC cells and be carefully designed to overcome suppressive microenvironment and inhibitory signals. For example, accumulating evidence has illustrated that the recruitment of immune cells into TME and/or their (persistent) activation is directly or indirectly influenced by the chemokine milieu produced in the TME due to oncogenic (Spranger, Bao and Gajewski, 2015) and epigenetic pathways (Timp and Feinberg, 2013; Peng *et al.*, 2015; Nagarsheth *et al.*, 2016; Nagarsheth, Wicha and Zou, 2017). Active β-catenin signalling in cold tumours inhibits CC-chemokine ligand 4 (CCL4) expression and limits DC recruitment and CD8 T cell activation and expansion.

Furthermore, the expression of the genes encoding chemokines that attract type 1 effector T cells is repressed through EZH2 and DNMT (DNA methyltransferase) epigenetic silencing. Consequently, effector CD8 T cells, NK cells and γδ T1 cells poorly infiltrate the tumour, and the tumour is immunologically "cold". In an ovarian cancer model, these "cold" tumour and stromal cells produce CXCL12 (Scotton *et al.*, 2001; Kryczek *et al.*, 2005), which attracts pDCs into tumours (Zou *et al.*, 2001). Conversely, ICOS-L/ICOS interactions between pDCs and T cells stimulate Treg function to secrete factors that inhibit cytotoxic T cell function (Conrad *et al.*, 2012). Along these lines, the presence of pDCs has been correlated with shorter overall survival despite the presence of high numbers of intratumoral T cells in breast cancer (Treilleux *et al.*, 2004). Interestingly, the regulatory and pro-tumour activity of certain γδ T cell populations was reported majorly in breast cancer (Peng *et al.*, 2007; Peters *et al.*, 2014; Ye *et al.*, 2013). Vδ1 T cells within breast tumours inhibit the activation of both CD4 and CD8 T cells and hinder the maturation of DCs, possibly through a TLR8 signalling-dependent mechanism (Peng *et al.*, 2007). Keeping in mind that effector γδ T cells are not as short-lived as CD8 T cells, they may migrate into tumour tissue after completing the pro-APC function in lymph nodes. Thus, given the remarkable functional plasticity of Vγ9/Vδ2 T cells and distinct functionalities of different subsets, γδ T cells might become distinctly polarised by the tumour microenvironment depending on the type of cancer and the disease stage (Lo Presti *et al.*, 2014), and may switch their cytokine secretory mode to a tumour-promoting one and/or become immunosuppressive (Wu *et al.*, 2014; Fleming *et al.*, 2017; Schilbach *et al.*, 2020). The study by Wu and colleagues has clearly demonstrated that tumour infiltrating pDCs in colorectal cancer polarise γδ T cells into γδ T17 cells in IL-23 dependent manner (Wu *et al.*, 2014). In addition to high levels of IL-17 secretion, these γδ T17 cells also secrete other cytokines including IL-8, GM-CSF, and TNF-a, and chemoattract PMN-MDSCs. These MDSCs in turn further expand and provide survival advantage to γδ 17 cells to maintain immune suppressive activity (Wu *et al.*, 2014). While γδ1 T cells represent the majority of intraepithelial γδ lymphocytes (~70-90%), the authors did not determine the γδ T cell subtype they studied. To date, there is no clinical study reporting such observation, particularly for Vγ9/Vδ2 T cells, but such activity cannot be excluded

This emphasises the need for the development of improved personalised clinical approaches to identify patients with particular types of tumour (hot/cold) and to tailor patient-specific approaches that will, together with the γδ T-APC vaccine, remodel TME while promoting the recruitment of type 1 effector immune cells with anti-tumour functions. Perhaps combination therapies with γδ T- APC-based vaccines and immune checkpoint inhibitors of PD1/PD-L1, CTLA-4/B7, or TIM3 may result in

synergistic outcomes, i.e., blockade of inhibitory immune cells by immune checkpoint inhibitors may facilitate the stimulatory effect of γδ T-APC-based vaccines leading to enhanced tumour-specific effector T-cell responses and long-lived immuno- surveillance and favourable T-cell formation. Furthermore, checkpoint inhibition might help to prevent the exhaustion of cytotoxic T cell responses as well as Vγ9/Vδ2 T-APC cells to prime favourable new αβ T cell response in the lymph nodes. In addition, modulation of the tumour microenvironment by local administration of cytokines and/or modulation of TLR signalling may be useful for boosting anti-tumour activity and reversing the suppressive immune cell function.

Finally, Vγ9/Vδ2 T cells represent a promising anti-cancer vaccine that could establish a potent and persistent anti-cancer immunity via their many innate and adaptive-like features, including their professional antigen-presentation function and stimulation of other immune cell functions coupled with instant cytolytic abilities. In contrast to DC-based vaccines, γδ T-APCs offer favourable, rapid and substantial expansion in *ex vivo* preparations. Using modern genetic and immunotherapy techniques to increase survival and manipulate cellular behaviours remains an attractive option for future clinical treatment settings. CAR transduction of γδ T cells may enhance their cytotoxicity while retaining their APC function and not compromising their migratory capabilities, all of which can be beneficial in treating solid tumours. Thus, it is important to recognise that Vγ9/Vδ2 T cells may mediate systemic immunity and provide indirect anti-tumour effects in cancer patients. Using genetic approaches to engineer γδ T cells can strengthen and redirect their cell function in difficult tumours. Nevertheless, the limited *in vivo* persistence of adoptively transferred Vγ9/Vδ2 T cells can be a problem. However, adoptive transfer of the transiently modified cells may potentially minimise the risk of chronic toxicity associated with the prolonged presence of effector cells.

References

Abdelsamed, H. A. *et al.* (2017) 'Human memory CD8 T cell effector potential is epigenetically preserved during in vivo homeostasis', *Journal of Experimental Medicine*. The Rockefeller University Press, 214(6), pp. 1593–1606. doi: 10.1084/jem.20161760.

Van Acker, H. H., Anguille, S., Willemen, Y., Smits, E. L., *et al.* (2016) 'Bisphosphonates for cancer treatment: Mechanisms of action and lessons from clinical trials', *Pharmacology and Therapeutics*. Elsevier Inc., 158, pp. 24–40. doi: 10.1016/j.pharmthera.2015.11.008.

Van Acker, H. H., Anguille, S., Willemen, Y., Van den Bergh, J. M., *et al.* (2016) 'Interleukin-15 enhances the proliferation, stimulatory phenotype, and antitumor effector functions of human gamma delta T cells', *Journal of Hematology & Oncology*. BioMed Central, 9(1), p. 101. doi: 10.1186/s13045-016-0329-3.

Ackerman, A. L. *et al.* (2003) 'Early phagosomes in dendritic cells form a cellular compartment sufficient for cross presentation of exogenous antigens', *Proceedings of the National Academy of Sciences of the United States of America*, 100(22), pp. 12889–12894. doi: 10.1073/pnas.1735556100.

Akoury, E. *et al.* (2019) 'Low-dose zoledronate for the treatment of bone metastasis secondary to prostate cancer', *Cancer Cell International*. BioMed Central, 19(1), p. 28. doi: 10.1186/s12935-019-0745-x.

Aktas, E. *et al.* (2009) 'Relationship between CD107a expression and cytotoxic activity', *Cellular Immunology*. Elsevier BV, 254(2), pp. 149–154. doi: 10.1016/j.cellimm.2008.08.007.

Albert, M. L. *et al.* (1998) 'Immature dendritic cells phagocytose apoptotic cells via alphavbeta5 and CD36, and cross-present antigens to cytotoxic T lymphocytes.', *The Journal of experimental medicine*. The Rockefeller University Press, 188(7), pp. 1359–68. doi: 10.1084/jem.188.7.1359.

Aleksic, M. *et al.* (2010) 'Dependence of T Cell Antigen Recognition on T Cell Receptor-Peptide MHC Confinement Time', *Immunity*. Elsevier Ltd, 32(2), pp. 163–174. doi: 10.1016/j.immuni.2009.11.013.

Aleksic, M. *et al.* (2012) 'Different affinity windows for virus and cancer-specific T-cell receptors: Implications for therapeutic strategies', *European Journal of Immunology*. Wiley, 42(12), pp. 3174–3179. doi: 10.1002/eji.201242606.

Alexander-Miller, M A *et al.* (1996) 'Role of antigen, CD8, and cytotoxic T lymphocyte (CTL) avidity in high dose antigen induction of apoptosis of effector CTL.', *The Journal of experimental medicine*, 184(2), pp. 485–92. doi: 10.1084/jem.184.2.485.

Alexander-Miller, Martha A. *et al.* (1996) 'Role of antigen, CD8, and cytotoxic T lymphocyte (CTL) avidity in high dose antigen induction of apoptosis of effector CTL', *Journal of Experimental Medicine*, 184(2), pp. 485–492. doi: 10.1084/jem.184.2.485.

Alexander-Miller, M. A., Leggatt, G. R. and Berzofsky, J. A. (1996) 'Selective expansion of high- or low-avidity cytotoxic T lymphocytes and efficacy for adoptive immunotherapy', *Proceedings of the National Academy of Sciences of the United States of America*, 93(9), pp. 4102–4107. doi: 10.1073/pnas.93.9.4102.

Alexander, A. A. Z. Z. *et al.* (2008) 'Isopentenyl pyrophosphate-activated CD56+ {gamma}{delta} T lymphocytes display potent antitumor activity toward human squamous cell carcinoma', *Clinical cancer research : an official journal of the American Association for Cancer Research*. American Association for Cancer Research, 14(13), pp. 4232–4240. doi: 10.1158/1078-0432.CCR-07-4912.

Allegra, A. *et al.* (2016) 'Adoptive immunotherapy for hematological malignancies: Current status and new insights in chimeric antigen receptor T cells', *Blood Cells, Molecules, and Diseases*. Elsevier BV, 62, pp. 49–63. doi: 10.1016/j.bcmd.2016.11.001.

Allen, T. M. *et al.* (1980) 'Detergent removal during membrane reconstitution', *BBA - Biomembranes*. doi: 10.1016/0005-2736(80)90537-4.

Altvater, B. *et al.* (2011) 'Activated human γδ T cells induce peptide-specific CD8+ T-cell responses to tumor-associated self-antigens', *Cancer Immunology, Immunotherapy*. Springer Science and Business Media LLC, 61(3), pp. 385–396. doi:

10.1007/s00262-011-1111-6.

Anderson, A. C., Joller, N. and Kuchroo, V. K. (2016) 'Lag-3, Tim-3, and TIGIT: Co-inhibitory Receptors with Specialized Functions in Immune Regulation.', *Immunity*. Elsevier, 44(5), pp. 989–1004. doi: 10.1016/j.immuni.2016.05.001.

Ang, W. X. *et al.* (2020) 'Electroporation of NKG2D RNA CAR Improves Vγ9Vδ2 T Cell Responses against Human Solid Tumor Xenografts.', *Molecular therapy oncolytics*. Elsevier, 17, pp. 421–430. doi: 10.1016/j.omto.2020.04.013.

Angelini, D. F. *et al.* (2004) 'FcγRIII discriminates between 2 subsets of Vγ9Vδ2 effector cells with different responses and activation pathways', *Blood*. American Society of Hematology, 104(6), pp. 1801–1807. doi: 10.1182/blood-2004-01-0331.

Anguille, S. *et al.* (2009) 'Short-term cultured, interleukin-15 differentiated dendritic cells have potent immunostimulatory properties', *Journal of Translational Medicine*, 7. doi: 10.1186/1479-5876-7-109.

Anikeeva, N. *et al.* (2012) 'Evidence that the Density of Self Peptide-MHC Ligands Regulates T-Cell Receptor Signaling', *PLoS ONE*. Edited by J. Kanellopoulos. Public Library of Science, 7(8), p. e41466. doi: 10.1371/journal.pone.0041466.

Arakawa, T. and Timasheff, S. N. (1985) 'Theory of Protein Solubility', in *Methods in Enzymology* . 114th edn, pp. 49–77. Available at: https://pdf.sciencedirectassets.com/273025/1-s2.0-S007668790X02340/1-s2.0-007668798514005X/main.pdf?x-amz-security-token=AgoJb3JpZ2luX2VjEO%2F%2F%2F%2F%2F%2F%2F%2F%2F%2FwEaCXVzLWVhc3QtMSJGMEQCIEw9876OGXl3G%2F8CbFSvKUCd9PD69126e6vlswk9bvwIAiAdsBqPKA%2B (Accessed: 26 May 2019).

Attaf, M. *et al.* (2015) 'The T cell antigen receptor: the Swiss army knife of the immune system', *Clinical & Experimental Immunology*. Blackwell Publishing Ltd, 181(1), pp. 1–18. doi: 10.1111/cei.12622.

Bansal, R. R. *et al.* (2012) 'IL-21 enhances the potential of human γδ T cells to provide B-cell help', *European Journal of Immunology*. John Wiley & Sons, Ltd, 42(1), pp. 110–119. doi: 10.1002/eji.201142017.

Barisa, M. *et al.* (2017) ' *E. coli* promotes human Vγ9Vδ2 T cell transition from cytokine-producing bactericidal effectors to professional phagocytic killers in a TCR-dependent manner', *Scientific Reports*. Nature Publishing Group, 7(1), p. 2805. doi: 10.1038/s41598-017-02886-8.

Battistini, L. *et al.* (2005) 'Homing and memory patterns of human γδ T cells in physiopathological situations', *Microbes and Infection*, 7(3), pp. 510–517. doi: 10.1016/j.micinf.2004.12.008.

Bauer, S. (1999) 'Activation of NK Cells and T Cells by NKG2D, a Receptor for Stress-Inducible MICA', *Science*. American Association for the Advancement of Science (AAAS), 285(5428), pp. 727–729. doi: 10.1126/science.285.5428.727.

Bekiaris, V. *et al.* (2013) 'The Inhibitory Receptor BTLA Controls γδ T Cell Homeostasis and Inflammatory Responses', *Immunity*. Cell Press, 39(6), pp. 1082–1094. doi: 10.1016/j.immuni.2013.10.017.

Benevides, L. *et al.* (2015) 'IL17 Promotes Mammary Tumor Progression by Changing the Behavior of Tumor Cells and Eliciting Tumorigenic Neutrophils Recruitment', *Cancer Research*. American Association for Cancer Research (AACR), 75(18), pp. 3788–3799. doi: 10.1158/0008-5472.can-15-0054.

Benmerah, A. and Lamaze, C. (2007) 'Clathrin-Coated Pits: Vive La Différence?', *Traffic*. Wiley, 8(8), pp. 970–982. doi: 10.1111/j.1600-0854.2007.00585.x.

Bennouna, J. *et al.* (2008) 'Phase-I study of Innacell γδ™, an autologous cell-therapy product highly enriched in γ9δ2 T lymphocytes, in combination with IL-2, in patients with metastatic renal cell carcinoma', *Cancer Immunology, Immunotherapy*. Springer Science and Business Media LLC, 57(11), pp. 1599–1609. doi: 10.1007/s00262-008-0491-8.

Benzaïd, I. *et al.* (2011) 'High Phosphoantigen Levels in Bisphosphonate-Treated Human Breast Tumors Promote Vγ9Vδ2 T-Cell Chemotaxis and Cytotoxicity In Vivo', *Cancer Research*. American Association for Cancer Research (AACR), 71(13), pp. 4562–4572. doi: 10.1158/0008-5472.can-10-3862.

Berzofsky, J. A., Wood, L. V and Terabe, M. (2015) 'Strategies for Improving Vaccines to Elicit T Cells to Treat Cancer', *Cancer Drug Discovery and Development*. Springer International Publishing, pp. 29–52. doi: 10.1007/978-3-319-21167-1_2.

Biasco, L. *et al.* (2015) 'In vivo tracking of T cells in humans unveils decade-long survival and activity of genetically modified T

memory stem cells.', *Science translational medicine*. American Association for the Advancement of Science, 7(273), p. 273ra13. doi: 10.1126/scitranslmed.3010314.

Blanchard, T., Srivastava, P. K. and Duan, F. (2013) 'Vaccines against advanced melanoma', *Clinics in Dermatology*. Elsevier BV, 31(2), pp. 179–190. doi: 10.1016/j.clindermatol.2012.08.005.

Block, A. *et al.* (2008) 'Impact of cell culture media on the expansion efficiency and T-cell receptor Vbeta (TRBV) repertoire of in vitro expanded T cells using feeder cells', *Medical Science Monitor*, 14(5), pp. 88–95. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18443543 (Accessed: 5 April 2017).

Boegel, S. *et al.* (2014) 'A catalog of HLA type, HLA expression, and neo-epitope candidates in human cancer cell lines.', *Oncoimmunology*. Taylor & Francis, 3(8), p. e954893. doi: 10.4161/21624011.2014.954893.

Bol, K. F. *et al.* (2016) 'Dendritic Cell–Based Immunotherapy: State of the Art and Beyond', *Clinical Cancer Research*. American Association for Cancer Research (AACR), 22(8), pp. 1897–1906. doi: 10.1158/1078-0432.ccr-15-1399.

Bondos, S. E. and Bicknell, A. (2003) 'Detection and prevention of protein aggregation before, during, and after purification', *Analytical Biochemistry*, 316(2), pp. 223–231. doi: 10.1016/S0003-2697(03)00059-9.

Bonifaz, L. *et al.* (2002) 'Efficient targeting of protein antigen to the dendritic cell receptor DEC-205 in the steady state leads to antigen presentation on major histocompatibility complex class I products and peripheral CD8+ T cell tolerance', *The Journal of experimental medicine*. The Rockefeller University Press, 196(12), pp. 1627–1638. doi: 10.1084/jem.20021598.

Bonneville, M., O'Brien, R. L. and Born, W. K. (2010) 'γδ T cell effector functions: a blend of innate programming and acquired plasticity', *Nature Reviews Immunology*. Nature Publishing Group, 10(7), pp. 467–478. doi: 10.1038/nri2781.

Bontkes, H. J. *et al.* (2007) 'Dendritic cells transfected with interleukin-12 and tumor-associated antigen messenger RNA induce high avidity cytotoxic T cells', *Gene Therapy*, 14(4), pp. 366–375. doi: 10.1038/sj.gt.3302874.

Born, W. K., Kemal Aydintug, M. and O'Brien, R. L. (2013) 'Diversity of γδ T-cell antigens', *Cellular & Molecular Immunology*. Nature Publishing Group, 10(1), pp. 13–20. doi: 10.1038/cmi.2012.45.

Bos, R. *et al.* (2012) 'Functional differences between low- and high-affinity CD8 + T cells in the tumor environment', *OncoImmunology*. Taylor & Francis, 1(8), pp. 1239–1247. doi: 10.4161/onci.21285.

Bossi, G. *et al.* (2013) 'Examining the presentation of tumor-associated antigens on peptide-pulsed T2 cells', *OncoImmunology*, 2(11), p. e26840. doi: 10.4161/onci.26840.

Boulet, S. *et al.* (2019) 'The orphan nuclear receptor NR4A3 controls the differentiation of monocyte-derived dendritic cells following microbial stimulation', *Proceedings of the National Academy of Sciences of the United States of America*. 2019/07/08. National Academy of Sciences, 116(30), pp. 15150–15159. doi: 10.1073/pnas.1821296116.

Boyle, J. M. *et al.* (1990) *Trophoblast glycoprotein recognised by monoclonal antibody 5T4 maps to human chromosome 6q14-q15, Hum Genet*. Springer-Verlag. Available at: https://link.springer.com/content/pdf/10.1007%2FBF00195819.pdf (Accessed: 19 May 2019).

Brandes, M. *et al.* (2003) 'Flexible migration program regulates γδ T-cell involvement in humoral immunity', *Blood*. American Society of Hematology, 102(10), pp. 3693–3701. doi: 10.1182/blood-2003-04-1016.

Brandes, M. *et al.* (2009) 'Cross-presenting human T cells induce robust CD8+ T cell responses', *Proceedings of the National Academy of Sciences*, 106(7), pp. 2307–2312. doi: 10.1073/pnas.0810059106.

Brandes, Marlène *et al.* (2009) 'Cross-presenting human gammadelta T cells induce robust CD8+ alphabeta T cell responses.', *Proceedings of the National Academy of Sciences of the United States of America*. 2009/01/26. National Academy of Sciences, 106(7), pp. 2307–12. doi: 10.1073/pnas.0810059106.

Brandes, M., Willimann, K. and Moser, B. (2005) 'Professional antigen-presentation function by human gammadelta T Cells.', *Science (New York, N.Y.)*, 309(5732), pp. 264–8. doi: 10.1126/science.1110267.

Bridgeman, J. S. *et al.* (2011) 'Structural and biophysical determinants of αβ T-cell antigen recognition', *Immunology*. Wiley, 135(1), pp. 9–18. doi: 10.1111/j.1365-2567.2011.03515.x.

Briseño, C. G. *et al.* (2016) 'Distinct Transcriptional Programs Control Cross-Priming in Classical and Monocyte-Derived Dendritic Cells', *Cell reports*. 2016/06/02, 15(11), pp. 2462–2474. doi: 10.1016/j.celrep.2016.05.025.

Brodie, S. J. *et al.* (1999) 'In vivo migration and function of transferred HIV-1-specific cytotoxic T cells', *Nature Medicine*, 5(1), pp. 34–41. doi: 10.1038/4716.

van den Broek, T., Borghans, J. A. M. M. and van Wijk, F. (2018) 'The full spectrum of human naive T cells', *Nature Reviews Immunology*. Nature Publishing Group, 18(6), pp. 363–373. doi: 10.1038/s41577-018-0001-y.

Brudno, J. N. and Kochenderfer, J. N. (2019) 'Recent advances in CAR T-cell toxicity: Mechanisms, manifestations and management', *Blood reviews*. 2018/11/14, 34, pp. 45–55. doi: 10.1016/j.blre.2018.11.002.

Buccheri, S. *et al.* (2014) 'Efficacy and safety of γδT cell-based tumor immunotherapy: a meta-analysis.', *Journal of biological regulators and homeostatic agents*. Italy, 28(1), pp. 81–90.

Buchan, S. L. *et al.* (2018) 'PD-1 Blockade and CD27 Stimulation Activate Distinct Transcriptional Programs That Synergize for CD8+ T-Cell-Driven Antitumor Immunity.', *Clinical cancer research : an official journal of the American Association for Cancer Research*. Clin Cancer Res, 24(10), pp. 2383–2394. doi: 10.1158/1078-0432.CCR-17-3057.

Buchholz, V. R., Schumacher, T. N. M. and Busch, D. H. (2016) 'T Cell Fate at the Single-Cell Level', *Annual Review of Immunology*. Annual Reviews , 34(1), pp. 65–92. doi: 10.1146/annurev-immunol-032414-112014.

Buckowitz, A. *et al.* (2005) 'Microsatellite instability in colorectal cancer is associated with local lymphocyte infiltration and low frequency of distant metastases', *British journal of cancer*. Nature Publishing Group, 92(9), pp. 1746–1753. doi: 10.1038/sj.bjc.6602534.

Buell, J. F., Gross, T. G. and Woodle, E. S. (2005) 'Malignancy after Transplantation', *Transplantation*. Ovid Technologies (Wolters Kluwer Health), 80(Supplement), pp. S254–S264. doi: 10.1097/01.tp.0000186382.81130.ba.

Bukowski, J. F., Morita, C. T. and Brenner, M. B. (1999) 'Human gamma delta T cells recognize alkylamines derived from microbes, edible plants, and tea: implications for innate immunity.', *Immunity*. Elsevier, 11(1), pp. 57–65. doi: 10.1016/s1074-7613(00)80081-3.

Burch, P. A. *et al.* (2000) 'Priming tissue-specific cellular immunity in a phase I trial of autologous dendritic cells for prostate cancer.', *Clinical cancer research : an official journal of the American Association for Cancer Research*. American Association for Cancer Research, 6(6), pp. 2175–82. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10873066 (Accessed: 11 March 2021).

Caballero, O. L. and Chen, Y.-T. (2009) 'Cancer/testis (CT) antigens: Potential targets for immunotherapy', *Cancer Science*. John Wiley & Sons, Ltd (10.1111), 100(11), pp. 2014–2021. doi: 10.1111/j.1349-7006.2009.01303.x.

Caccamo, N. *et al.* (2006) 'Sex-specific phenotypical and functional differences in peripheral human Vgamma9/Vdelta2 T cells.', *Journal of leukocyte biology*. J Leukoc Biol, 79(4), pp. 663–6. doi: 10.1189/jlb.1105640.

Caccamo, N. *et al.* (2011) 'Differentiation, phenotype, and function of interleukin-17–producing human Vγ9Vδ2 T cells', *Blood*. American Society of Hematology, 118(1), pp. 129–138. doi: 10.1182/blood-2011-01-331298.

Cai, Y. *et al.* (2011) 'Pivotal role of dermal IL-17-producing γδ T cells in skin inflammation', *Immunity*. 2011/10/06, 35(4), pp. 596–610. doi: 10.1016/j.immuni.2011.08.001.

Cairns, B. *et al.* (2006) 'Increased Toll-Like Receptor 4 Expression on T Cells May Be a Mechanism for Enhanced T cell Response Late After Burn Injury', *The Journal of Trauma: Injury, Infection, and Critical Care*. Ovid Technologies (Wolters Kluwer Health), 61(2), pp. 293–299. doi: 10.1097/01.ta.0000228969.46633.bb.

Callender, L. A. *et al.* (2018) 'Human CD8 + EMRA T cells display a senescence-associated secretory phenotype regulated by p38 MAPK', *Aging Cell*, 17(1), pp. 1–9. doi: 10.1111/acel.12675.

Capsomidis, A. *et al.* (2018) 'Chimeric Antigen Receptor-Engineered Human Gamma Delta T Cells: Enhanced Cytotoxicity with Retention of Cross Presentation', *Molecular Therapy*. 2017/12/08. American Society of Gene & Cell Therapy, 26(2), pp. 354–365. doi: 10.1016/j.ymthe.2017.12.001.

Carreno, B. M. *et al.* (2013) 'IL-12p70-producing patient DC vaccine elicits Tc1-polarized immunity', *The Journal of clinical investigation*. 2013/07/11. American Society for Clinical Investigation, 123(8), pp. 3383–3394. doi: 10.1172/JCI68395.

Carrió, M. M., Corchero, J. L. and Villaverde, A. (1999) 'Proteolytic digestion of bacterial inclusion body proteins during dynamic transition between soluble and insoluble forms', *Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology*. Elsevier, 1434(1), pp. 170–176. doi: 10.1016/S0167-4838(99)00177-6.

Carsberg, C. J., Myers, K. A. and Stern, P. L. (1996) 'Metastasis-associated 5T4 antigen disrupts cell-cell contacts and induces cellular motility in epithelial cells', *International Journal of Cancer*. Wiley-Blackwell, 68(1), pp. 84–92. doi: 10.1002/(SICI)1097-0215(19960927)68:1<84::AID-IJC15>3.0.CO;2-6.

Casetti, R. *et al.* (2009) 'Cutting Edge: TGF-β1 and IL-15 Induce FOXP3+γδ Regulatory T Cells in the Presence of Antigen Stimulation', *The Journal of Immunology*. The American Association of Immunologists, 183(6), pp. 3574–3577. doi: 10.4049/jimmunol.0901334.

Casorati, G. *et al.* (1989) 'Molecular analysis of human gamma/delta+ clones from thymus and peripheral blood', *The Journal of experimental medicine*. The Rockefeller University Press, 170(5), pp. 1521–1535. doi: 10.1084/jem.170.5.1521.

Castella, B. *et al.* (2017) 'The ATP-binding cassette transporter A1 regulates phosphoantigen release and Vγ9Vδ2 T cell activation by dendritic cells', *Nature communications*. Nature Publishing Group, 8, p. 15663. doi: 10.1038/ncomms15663.

Champagne, E. (2011) 'γδ T cell Receptor Ligands and Modes of Antigen Recognition', *Archivum Immunologiae et Therapiae Experimentalis*. Inserm, 59(2), pp. 117–137. doi: 10.1007/s00005-011-0118-1.

Chattopadhyay, P. K. *et al.* (2009) 'The cytolytic enzymes granyzme A, granzyme B, and perforin: expression patterns, cell distribution, and their relationship to cell maturity and bright CD57 expression.', *Journal of leukocyte biology*. J Leukoc Biol, 85(1), pp. 88–97. doi: 10.1189/jlb.0208107.

Chavez, J. C., Bachmeier, C. and Kharfan-Dabaja, M. A. (2019) 'CAR T-cell therapy for B-cell lymphomas: clinical trial results of available products', *Therapeutic advances in hematology*. SAGE Publications, 10, pp. 2040620719841581–2040620719841581. doi: 10.1177/2040620719841581.

Chen, D. S. S. and Mellman, I. (2013) 'Oncology Meets Immunology: The Cancer-Immunity Cycle', *Immunity*. Elsevier, 39(1), pp. 1–10. doi: 10.1016/j.immuni.2013.07.012.

Chen, H.-C. *et al.* (2017) 'Synergistic targeting of breast cancer stem-like cells by human γδ T cells and CD8+ T cells', *Immunology and Cell Biology*. Nature Publishing Group. doi: 10.1038/icb.2017.21.

Chen, J.-L. L. *et al.* (2005) 'Structural and kinetic basis for heightened immunogenicity of T cell vaccines', *Journal of Experimental Medicine*. Rockefeller University Press, 201(8), pp. 1243–1255. doi: 10.1084/jem.20042323.

Chen, J.-L. L. *et al.* (2014) 'Identification of NY-ESO-1 Peptide Analogues Capable of Improved Stimulation of Tumor-Reactive CTL', *The Journal of Immunology*. American Association of Immunologists, 165(2), pp. 948–955. doi: 10.4049/jimmunol.165.2.948.

Chen, L. *et al.* (2007) 'Epigenetic and Transcriptional Programs Lead to Default IFN-γ Production by γδ T Cells', *The Journal of Immunology*. The American Association of Immunologists, 178(5), pp. 2730–2736. doi: 10.4049/jimmunol.178.5.2730.

Chen, L. *et al.* (2013) 'Therapeutic Use of Dendritic Cells to Promote the Extranodal Priming of Anti-Tumor Immunity', *Frontiers in Immunology*. Frontiers, 4, p. 388. doi: 10.3389/fimmu.2013.00388.

Chen, Y.-T. *et al.* (1997) 'A testicular antigen aberrantly expressed in human cancers detected by autologous antibody screening', *Proceedings of the National Academy of Sciences*. National Academy of Sciences, 94(5), pp. 1914–1918. doi: 10.1073/pnas.94.5.1914.

Chitadze, G. *et al.* (2013) 'Generation of Soluble NKG2D Ligands: Proteolytic Cleavage, Exosome Secretion and Functional Implications', *Scandinavian Journal of Immunology*. Wiley, 78(2), pp. 120–129. doi: 10.1111/sji.12072.

Cieri, N. *et al.* (2013) 'IL-7 and IL-15 instruct the generation of human memory stem T cells from naive precursors', *Blood*. American Society of Hematology, 121(4), pp. 573–584. doi: 10.1182/blood-2012-05-431718.

Cipriani, B. *et al.* (2000) 'Activation of C-C beta-chemokines in human peripheral blood gammadelta T cells by isopentenyl pyrophosphate and regulation by cytokines', *Blood*, 95(1), pp. 39–47. Available at: https://pubmed.ncbi.nlm.nih.gov/10607682/.

Coffelt, S. B. *et al.* (2015) 'IL-17-producing γδ T cells and neutrophils conspire to promote breast cancer metastasis', *Nature*. 2015/03/30, 522(7556), pp. 345–348. doi: 10.1038/nature14282.

Colbert, J. D., Cruz, F. M. and Rock, K. L. (2020) 'Cross-presentation of exogenous antigens on MHC I molecules', *Current Opinion in Immunology*. Elsevier Ltd, 64, pp. 1–8. doi: 10.1016/j.coi.2019.12.005.

Cole, D. K. *et al.* (2007) 'Human TCR-Binding Affinity is Governed by MHC Class Restriction', *The Journal of Immunology*. The American Association of Immunologists, 178(9), pp. 5727–5734. doi: 10.4049/jimmunol.178.9.5727.

Comen, E. A., Bowman, R. L. and Kleppe, M. (2018) 'Underlying Causes and Therapeutic Targeting of the Inflammatory Tumor Microenvironment', *Frontiers in Cell and Developmental Biology*. Frontiers, 6, p. 56. doi: 10.3389/fcell.2018.00056.

Conrad, C. *et al.* (2012) 'Plasmacytoid dendritic cells promote immunosuppression in ovarian cancer via ICOS costimulation of Foxp3(+) T-regulatory cells', *Cancer research*. 2012/07/31, 72(20), pp. 5240–5249. doi: 10.1158/0008-5472.CAN-12-2271.

Conroy, H., Marshall, N. A. and Mills, K. H. G. (2008) 'TLR ligand suppression or enhancement of Treg cells? A double-edged sword in immunity to tumours', *Oncogene*. Springer Science and Business Media LLC, 27(2), pp. 168–180. doi: 10.1038/sj.onc.1210910.

Consortium, S. G. *et al.* (2008) 'Protein production and purification', *Nature Methods*. Nature Publishing Group, 5(2), pp. 135–146. doi: 10.1038/nmeth.f.202.

Constant, P. *et al.* (1994) 'Stimulation of human γδ T cells by nonpeptidic mycobacterial ligands', *Science*, 264(5156), pp. 267–270. doi: 10.1126/science.8146660.

Corchero, J. L. *et al.* (1997) 'Limited in vivo proteolysis of aggregated proteins', *Biochemical and Biophysical Research Communications*. doi: 10.1006/bbrc.1997.7132.

Cormier, J. N. *et al.* (1997) 'Enhancement of cellular immunity in melanoma patients immunized with a peptide from MART-1/Melan A.', *The cancer journal from Scientific American*. NIH Public Access, 3(1), pp. 37–44. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9072306 (Accessed: 4 March 2021).

Corvaisier, M. *et al.* (2005) 'V gamma 9V delta 2 T cell response to colon carcinoma cells.', *Journal of immunology (Baltimore, Md. : 1950)*. American Association of Immunologists, 175(8), pp. 5481–8. doi: 10.4049/jimmunol.175.8.5481.

Coscia, M. *et al.* (2012) 'Dysfunctional Vγ9Vδ2 T cells are negative prognosticators and markers of dysregulated mevalonate pathway activity in chronic lymphocytic leukemia cells', *Blood*. Elsevier, 120(16), pp. 3271–3279. doi: 10.1182/BLOOD-2012-03-417519.

Costa del Amo, P. *et al.* (2018) 'Human TSCM cell dynamics in vivo are compatible with long-lived immunological memory and stemness', *PLOS Biology*. Edited by A. Bhandoola. Public Library of Science, 16(6), p. e2005523. doi: 10.1371/journal.pbio.2005523.

Coussens, L. M. and Werb, Z. (2002) 'Inflammation and cancer.', *Nature*, 420(6917), pp. 860–7. doi: 10.1038/nature01322.

Couturier, J. *et al.* (2019) 'Lymphocytes upregulate CD36 in adipose tissue and liver', *Adipocyte*, 8(1), pp. 154–163. doi: 10.1080/21623945.2019.1609202.

Cox, M. A., Harrington, L. E. and Zajac, A. J. (2011) 'Cytokines and the inception of CD8 T cell responses', *Trends in Immunology*. Elsevier Current Trends, 32(4), pp. 180–186. doi: 10.1016/J.IT.2011.01.004.

Cox, M. A., Kahan, S. M. and Zajac, A. J. (2013) 'Anti-viral CD8 T cells and the cytokines that they love', *Virology*. Elsevier BV, 435(1), pp. 157–169. doi: 10.1016/j.virol.2012.09.012.

Crawford, G. *et al.* (2018) 'Epithelial damage and tissue γδ T cells promote a unique tumor-protective IgE response.', *Nature immunology*. Europe PMC Funders, 19(8), pp. 859–870. doi: 10.1038/s41590-018-0161-8.

Croft, M. *et al.* (1994) 'Generation of polarized antigen-specific CD8 effector populations: reciprocal action of interleukin (IL)-4

and IL-12 in promoting type 2 versus type 1 cytokine profiles.', *The Journal of Experimental Medicine*, 180(5), pp. 1715–1728. doi: 10.1084/jem.180.5.1715.

Cui, W. and Kaech, S. M. (2010) 'Generation of effector CD8+ T cells and their conversion to memory T cells', *Immunological Reviews*, 236(1), pp. 151–166. doi: 10.1111/j.1600-065X.2010.00926.x.

Cui, Y. *et al.* (2009) 'Human gammadelta T cell recognition of lipid A is predominately presented by CD1b or CD1c on dendritic cells', *Biology direct*. BioMed Central, 4(1), p. 47. doi: 10.1186/1745-6150-4-47.

Cummings, R. D. and Kornfeld, S. (1982) 'Characterization of the structural determinants required for the high affinity interaction of asparagine-linked oligosaccharides with immobilized Phaseolus vulgaris leukoagglutinating and erythroagglutinating lectins.', *Journal of Biological Chemistry*, 257(19), pp. 11230–11234.

Curtsinger, J. M. *et al.* (2007) 'Signal 3 availability limits the CD8 T cell response to a solid tumor.', *Journal of immunology (Baltimore, Md. : 1950)*. American Association of Immunologists, 178(11), pp. 6752–60. doi: 10.4049/jimmunol.178.11.6752.

Curtsinger, J. M. *et al.* (2012) 'Autocrine IFN-γ promotes naive CD8 T cell differentiation and synergizes with IFN-α to stimulate strong function.', *Journal of immunology (Baltimore, Md. : 1950)*. American Association of Immunologists, 189(2), pp. 659–68. doi: 10.4049/jimmunol.1102727.

Curtsinger, J. M. and Mescher, M. F. (2010) 'Inflammatory cytokines as a third signal for T cell activation', *Current Opinion in Immunology*. Elsevier Ltd, 22(3), pp. 333–340. doi: 10.1016/j.coi.2010.02.013.

D'Cruz, A. A. *et al.* (2013) 'Structure and function of the SPRY/B30.2 domain proteins involved in innate immunity', *Protein Science*. John Wiley & Sons, Ltd, 22(1), pp. 1–10. doi: 10.1002/pro.2185.

Daley, D. *et al.* (2016) 'γδ T Cells Support Pancreatic Oncogenesis by Restraining αβ T Cell Activation.', *Cell*. 2016/08/25. Elsevier, 166(6), pp. 1485-1499.e15. doi: 10.1016/j.cell.2016.07.046.

Dar, A. A., Patil, R. S. and Chiplunkar, S. V. (2014) 'Insights into the Relationship between Toll Like Receptors and Gamma Delta T Cell Responses', *Frontiers in Immunology*, 5, p. 366. doi: 10.3389/fimmu.2014.00366.

Das, D. *et al.* (2018) 'T helper type 1 polarizing γδ T cells and Scavenger receptors contribute to the pathogenesis of Pemphigus vulgaris', *Immunology*. John Wiley & Sons, Ltd, 153(1), pp. 97–104. doi: 10.1111/imm.12814.

Das, H. *et al.* (2001) 'MICA Engagement by Human Vγ2Vδ2 T Cells Enhances Their Antigen-Dependent Effector Function', *Immunity*. Elsevier BV, 15(1), pp. 83–93. doi: 10.1016/s1074-7613(01)00168-6.

Dasari, V. *et al.* (2016) 'Autophagy and proteasome interconnect to coordinate cross-presentation through MHC class I pathway in B cells', *Immunology and Cell Biology*, 94(10), pp. 964–974. doi: 10.1038/icb.2016.59.

Davis, M. M. and Bjorkman, P. J. (1988) 'The T cell receptor genes and T cell recognition', *Nature*, 334, pp. 395–402.

deBarros, A. *et al.* (2010) 'CD70-CD27 interactions provide survival and proliferative signals that regulate T cell receptor-driven activation of human γδ peripheral blood lymphocytes', *European Journal of Immunology*. Wiley, 41(1), pp. 195–201. doi: 10.1002/eji.201040905.

Deetz, C. O. *et al.* (2006) 'Gamma Interferon Secretion by Human Vγ2Vδ2 T Cells after Stimulation with Antibody against the T-Cell Receptor plus the Toll-Like Receptor 2 Agonist Pam3Cys', *Infection and Immunity*. American Society for Microbiology (ASM), 74(8), pp. 4505–4511. doi: 10.1128/IAI.00088-06.

Devilder, M.-C. *et al.* (2006) 'Potentiation of Antigen-Stimulated Vγ9Vδ2 T Cell Cytokine Production by Immature Dendritic Cells (DC) and Reciprocal Effect on DC Maturation', *The Journal of Immunology*. The American Association of Immunologists, 176(3), pp. 1386–1393. doi: 10.4049/jimmunol.176.3.1386.

Dieli, F. *et al.* (2001) 'Granulysin-Dependent Killing of Intracellular and ExtracellularMycobacterium tuberculosisby Vγ9/Vδ2 T Lymphocytes', *The Journal of Infectious Diseases*. Oxford University Press (OUP), 184(8), pp. 1082–1085. doi: 10.1086/323600.

Dieli, F. *et al.* (2003) 'Differentiation of effector/memory Vdelta2 T cells and migratory routes in lymph nodes or inflammatory sites', *The Journal of experimental medicine*. The Rockefeller University Press, 198(3), pp. 391–397. doi:

10.1084/jem.20030235.

Dieli, F. *et al.* (2007) 'Targeting human {gamma}delta} T cells with zoledronate and interleukin-2 for immunotherapy of hormone-refractory prostate cancer.', *Cancer research*. Cancer Res, 67(15), pp. 7450–7. doi: 10.1158/0008-5472.CAN-07-0199.

Dimova, T. *et al.* (2015) 'supp material - Effector Vγ9Vδ2 T cells dominate the human fetal γδ T-cell repertoire', *Proceedings of the National Academy of Sciences of the United States of America*. 2015/01/23. National Academy of Sciences, 112(6), pp. E556–E565. doi: 10.1073/pnas.1412058112.

Dokouhaki, P. *et al.* (2013) 'NKG2D regulates production of soluble TRAIL by ex vivo expanded human γδ T cells', *European Journal of Immunology*. Wiley, 43(12), pp. 3175–3182. doi: 10.1002/eji.201243150.

Domae, E. *et al.* (2017) 'Cytokine-mediated activation of human ex vivo-expanded Vγ9Vδ2 T cells', *Oncotarget*. Impact Journals LLC, 8(28), pp. 45928–45942. doi: 10.18632/oncotarget.17498.

Donia, M. *et al.* (2014) 'Simplified protocol for clinical-grade tumor-infiltrating lymphocyte manufacturing with use of the Wave bioreactor', *Cytotherapy*. Elsevier BV, 16(8), pp. 1117–1120. doi: 10.1016/j.jcyt.2014.02.004.

Dooley, J., Linterman, M. A. and Liston, A. (2013) 'MicroRNA regulation of T-cell development', *Immunological Reviews*, 253(1), pp. 53–64. doi: 10.1111/imr.12049.

Dudley, M. E. *et al.* (2010) 'CD8+ Enriched "Young" Tumor Infiltrating Lymphocytes Can Mediate Regression of Metastatic Melanoma', *Clinical Cancer Research*. American Association for Cancer Research (AACR), 16(24), pp. 6122–6131. doi: 10.1158/1078-0432.ccr-10-1297.

Dunbar, P. R. *et al.* (1999) 'Cutting edge: Rapid cloning of tumor-specific CTL suitable for adoptive immunotherapy of melanoma', *Journal of Immunology*, 162(12), pp. 6959–6962. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10358133 (Accessed: 22 June 2017).

Dunn, G. P. *et al.* (2002) 'Cancer immunoediting: from immunosurveillance to tumor escape', *Nature Immunology*. Nature Publishing Group, 3(11), pp. 991–998. doi: 10.1038/ni1102-991.

Dunn, G. P., Koebel, C. M. and Schreiber, R. D. (2006) 'Interferons, immunity and cancer immunoediting', *Nature Reviews Immunology*. Springer Science and Business Media LLC, 6(11), pp. 836–848. doi: 10.1038/nri1961.

Dvorak, H. F. (1986) 'Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing.', *The New England journal of medicine*, 315(26), pp. 1650–9. doi: 10.1056/NEJM198612253152606.

Eberl, M. *et al.* (2003) 'Microbial isoprenoid biosynthesis and human gammadelta T cell activation.', *FEBS letters*, 544(1–3), pp. 4–10. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12782281 (Accessed: 17 June 2016).

Eberl, M. *et al.* (2009) 'A rapid crosstalk of human gammadelta T cells and monocytes drives the acute inflammation in bacterial infections', *PLoS pathogens*. 2009/02/20. Edited by R. R. Isberg. Public Library of Science, 5(2), pp. e1000308–e1000308. doi: 10.1371/journal.ppat.1000308.

Elinav, E. *et al.* (2013) 'Inflammation-induced cancer: crosstalk between tumours, immune cells and microorganisms', *Nature Reviews Cancer*. Springer Science and Business Media LLC, 13(11), pp. 759–771. doi: 10.1038/nrc3611.

Enamorado, M. *et al.* (2018) 'Genealogy, Dendritic Cell Priming, and Differentiation of Tissue-Resident Memory CD8+ T Cells', *Frontiers in Immunology*. Frontiers, 9, p. 1751. doi: 10.3389/fimmu.2018.01751.

Epstein, L. B., Cline, M. J. and Merigan, T. C. (1971) 'The interaction of human macrophages and lymphocytes in the phytohemagglutinin-stimulated production of interferon', *Journal of Clinical Investigation*. American Society for Clinical Investigation, 50(4), pp. 744–753. doi: 10.1172/JCI106545.

Eyquem, J. *et al.* (2017) 'Targeting a CAR to the TRAC locus with CRISPR/Cas9 enhances tumour rejection', *Nature*. Nature Research. doi: 10.1038/nature21405.

Fahl, S. P. *et al.* (2018) 'Role of a selecting ligand in shaping the murine γδ-TCR repertoire.', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 115(8), pp. 1889–1894. doi: 10.1073/pnas.1718328115.

Falo, L. D. *et al.* (1995) 'Targeting antigen into the phagocytic pathway in vivo induces protective tumour immunity', *Nature Medicine*. doi: 10.1038/nm0795-649.

Ferrarini, M. *et al.* (2008) 'NF-κB Modulates Sensitivity to Apoptosis, Proinflammatory and Migratory Potential in Short- versus Long-Term Cultured Human γδ Lymphocytes', *The Journal of Immunology*. The American Association of Immunologists, 181(9), pp. 5857–5864. doi: 10.4049/jimmunol.181.9.5857.

Fichtner, A. S. *et al.* (2018) 'The Armadillo (Dasypus novemcinctus): A Witness but Not a Functional Example for the Emergence of the Butyrophilin 3/Vγ9Vδ2 System in Placental Mammals.', *Frontiers in immunology*. Front Immunol, 9, p. 265. doi: 10.3389/fimmu.2018.00265.

Fichtner, A. S. *et al.* (2020) 'Alpaca (Vicugna pacos), the first nonprimate species with a phosphoantigen-reactive Vγ9Vδ2 T cell subset.', *Proceedings of the National Academy of Sciences of the United States of America*. Proc Natl Acad Sci U S A, 117(12), pp. 6697–6707. doi: 10.1073/pnas.1909474117.

Finn, O. J. (2012) 'Immuno-oncology: understanding the function and dysfunction of the immune system in cancer', *Annals of Oncology*. Elsevier, 23, pp. viii6–viii9. doi: 10.1093/annonc/mds256.

Fisher, J. P. P. H. *et al.* (2014) 'γδ T cells for cancer immunotherapy: A systematic review of clinical trials', *Oncoimmunology*. 2014/01/17. Landes Bioscience, 3(1), pp. e27572–e27572. doi: 10.4161/onci.27572.

Fleming, C. *et al.* (2017) 'γδ T Cells: Unexpected Regulators of Cancer Development and Progression', *Trends in Cancer*. Elsevier, 3(8), pp. 561–570. doi: 10.1016/j.trecan.2017.06.003.

Fonteneau, J.-F. *et al.* (2001) 'Generation of high quantities of viral and tumor-specific human CD4+ and CD8+ T-cell clones using peptide pulsed mature dendritic cells', *Journal of Immunological Methods*. Elsevier, 258(1–2), pp. 111–126. doi: 10.1016/S0022-1759(01)00477-X.

Fowler, D. W. *et al.* (2014) 'Zoledronic acid causes γδ T cells to target monocytes and down-modulate inflammatory homing', *Immunology*. BlackWell Publishing Ltd, 143(4), pp. 539–549. doi: 10.1111/imm.12331.

Fowler, D. W. and Bodman-Smith, M. D. (2015) 'Harnessing the power of Vδ2 cells in cancer immunotherapy', *Clinical and experimental immunology*. BlackWell Publishing Ltd, 180(1), pp. 1–10. doi: 10.1111/cei.12564.

Franke, W. W. *et al.* (1981) 'Antibodies to the major insoluble milk fat globule membrane-associated protein: specific location in apical regions of lactating epithelial cells', *The Journal of cell biology*. The Rockefeller University Press, 89(3), pp. 485–494. doi: 10.1083/jcb.89.3.485.

Fu, C. and Jiang, A. (2018) 'Dendritic Cells and CD8 T Cell Immunity in Tumor Microenvironment', *Frontiers in Immunology*. Frontiers, 9, p. 3059. doi: 10.3389/fimmu.2018.03059.

Galloway, S. A. E. *et al.* (2019) 'Peptide super-agonist enhances T-cell responses to melanoma', *Frontiers in Immunology*. Frontiers Media S.A., 10(MAR). doi: 10.3389/fimmu.2019.00319.

Gameiro, S. R. *et al.* (2014) 'Radiation-induced immunogenic modulation of tumor enhances antigen processing and calreticulin exposure, resulting in enhanced T-cell killign', *Oncotarget*, 5(2), pp. 403–416. doi: 10.18632/oncotarget.1719.

Garg, A. D. *et al.* (2017) 'Integrating Next-Generation Dendritic Cell Vaccines into the Current Cancer Immunotherapy Landscape', *Trends in Immunology*. Elsevier BV, 38(8), pp. 577–593. doi: 10.1016/j.it.2017.05.006.

Gattinoni, L. *et al.* (2009) 'Wnt signaling arrests effector T cell differentiation and generates CD8+ memory stem cells', *Nature Medicine*. Nature Publishing Group, 15(7), pp. 808–813. doi: 10.1038/nm.1982.

Gattinoni, L., Lugli, E., Ji, Y., Pos, Z., Paulos, Chrystal M., *et al.* (2011) 'A human memory T cell subset with stem cell-like properties', *Nature Medicine*, 17(10), pp. 1290–1297. doi: 10.1038/nm.2446.

Gattinoni, L., Lugli, E., Ji, Y., Pos, Z., Paulos, Chrystal M, *et al.* (2011) 'A human memory T cell subset with stem cell–like properties', *Nature Medicine*. Nature Publishing Group, 17(10), pp. 1290–1297. doi: 10.1038/nm.2446.

Gattinoni, L. and Restifo, N. P. (2013) 'Moving T memory stem cells to the clinic', *Blood*. American Society of Hematology, 121(4), pp. 567–568. doi: 10.1182/blood-2012-11-468660.

Geginat, J., Lanzavecchia, A. and Sallusto, F. (2003) 'Proliferation and differentiation potential of human CD8+ memory T-cell subsets in response to antigen or homeostatic cytokines', *Blood*. American Society of Hematology, 101(11), pp. 4260–4266. doi: 10.1182/blood-2002-11-3577.

Geginat, J., Sallusto, F. and Lanzavecchia, A. (2003) 'Cytokine-driven proliferation and differentiation of human naïve, central memory and effector memory CD4+ T cells', *Pathologie Biologie*, 51(2), pp. 64–66. doi: 10.1016/S0369-8114(03)00098-1.

Gentles, A. J. *et al.* (2015) 'The prognostic landscape of genes and infiltrating immune cells across human cancers', *Nature Medicine*. 2015/07/20. Nature Research, 21(8), pp. 938–945. doi: 10.1038/nm.3909.

Gérard, A. *et al.* (2013) 'Secondary T cell–T cell synaptic interactions drive the differentiation of protective CD8+ T cells', *Nature Immunology*. Nature Publishing Group, 14(4), pp. 356–363. doi: 10.1038/ni.2547.

Gertner-Dardenne, J. *et al.* (2013) 'The co-receptor BTLA negatively regulates human Vγ9Vδ2 T-cell proliferation: a potential way of immune escape for lymphoma cells', *Blood*. American Society of Hematology, 122(6), pp. 922–931. doi: 10.1182/blood-2012-11-464685.

Geukes Foppen, M. H. *et al.* (2015) 'Tumor-infiltrating lymphocytes for the treatment of metastatic cancer', *Molecular oncology*. 2015/10/30. John Wiley and Sons Inc., 9(10), pp. 1918–1935. doi: 10.1016/j.molonc.2015.10.018.

Gibbons, D. L. *et al.* (2009) 'Neonates harbour highly active γδ T cells with selective impairments in preterm infants', *European Journal of Immunology*. Wiley, 39(7), pp. 1794–1806. doi: 10.1002/eji.200939222.

Giodini, A., Rahner, C. and Cresswell, P. (2009) 'Receptor-mediated phagocytosis elicits cross-presentation in nonprofessional antigen-presenting cells', *Proceedings of the National Academy of Sciences*, 106(9), pp. 3324–3329. doi: 10.1073/pnas.0813305106.

Girardi, M. *et al.* (2001) 'Pillars Article: Regulation of Cutaneous Malignancy by γδ T Cells. Science. 2001. 294: 605-609.', *Science*, 294(9), pp. 605-609. doi: 10.4049/jimmunol.1800364.

Girardi, M. (2001) 'Regulation of Cutaneous Malignancy by gamma delta T Cells', *Science*. American Association for the Advancement of Science (AAAS), 294(5542), pp. 605–609. doi: 10.1126/science.1063916.

Gnjatic, S. *et al.* (2002) 'CD8(+) T cell responses against a dominant cryptic HLA-A2 epitope after NY-ESO-1 peptide immunization of cancer patients.', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 99(18), pp. 11813–8. doi: 10.1073/pnas.142417699.

Gnjatic, S. *et al.* (2006) 'NY-ESO-1: Review of an Immunogenic Tumor Antigen', *Advances in Cancer Research*, 95(May 2016), pp. 1–30. doi: 10.1016/S0065-230X(06)95001-5.

Godder, K. T. *et al.* (2007) 'Long term disease-free survival in acute leukemia patients recovering with increased gammadelta T cells after partially mismatched related donor bone marrow transplantation.', *Bone marrow transplantation*. Bone Marrow Transplant, 39(12), pp. 751–7. doi: 10.1038/sj.bmt.1705650.

Gomes, A. Q. *et al.* (2010) 'Identification of a panel of ten cell surface protein antigens associated with immunotargeting of leukemias and lymphomas by peripheral blood gammadelta T cells.', *Haematologica*. Haematologica, 95(8), pp. 1397–404. doi: 10.3324/haematol.2009.020602.

Good, N. E. *et al.* (1966) 'Hydrogen ion buffers for biological research.', *Biochemistry*, 5(2), pp. 467–77. Available at: http://www.ncbi.nlm.nih.gov/pubmed/5942950 (Accessed: 30 May 2019).

Gorochov, G. *et al.* (1997) 'Oligoclonal expansion of CD8+ CD57+ T cells with restricted T-cell receptor beta chain variability after bone marrow transplantation', *Blood*. American Society of Hematology, 83(2), pp. 587–595. Available at: http://www.bloodjournal.org/content/90/9/3789?sso-checked=true (Accessed: 29 January 2019).

Gräslund, S. *et al.* (2008) 'No Title'. Nature Publishing Group, 5(2). doi: 10.1038/nmeth.f.202.

Griffiths, R. W. *et al.* (2005) 'Expression of the 5T4 oncofoetal antigen in renal cell carcinoma: a potential target for T-cell-based immunotherapy', *British Journal of Cancer*. Nature Publishing Group, 93(6), pp. 670–677. doi: 10.1038/sj.bjc.6602776.

Grivennikov, S. I., Greten, F. R. and Karin, M. (2010) 'Immunity, inflammation, and cancer', *Cell*, 140(6), pp. 883–899. doi:

10.1016/j.cell.2010.01.025.

Groh, V. *et al.* (1999) 'Broad tumor-associated expression and recognition by tumor-derived T cells of MICA and MICB', *Proceedings of the National Academy of Sciences*. National Academy of Sciences, 96(12), pp. 6879–6884. doi: 10.1073/pnas.96.12.6879.

Grotzke, J. E. *et al.* (2017) 'The ongoing saga of the mechanism(s) of MHC class I-restricted cross-presentation', *Current Opinion in Immunology*. Elsevier Ltd, 46(Figure 2), pp. 89–96. doi: 10.1016/j.coi.2017.03.015.

Gu, S. *et al.* (2015) 'Human gamma delta T cells: Evolution and ligand recognition', *Cellular Immunology*. NIH Public Access, 296(1), pp. 31–40. doi: 10.1016/j.cellimm.2015.04.008.

Gu, S. *et al.* (2018) 'Butyrophilin3A proteins and Vγ9Vδ2 T cell activation', *Seminars in Cell & Developmental Biology*. Academic Press, 84, pp. 65–74. doi: 10.1016/J.SEMCDB.2018.02.007.

Guarda, G. *et al.* (2007) 'L-selectin-negative CCR7– effector and memory CD8+ T cells enter reactive lymph nodes and kill dendritic cells', *Nature Immunology*. Nature Publishing Group, 8(7), pp. 743–752. doi: 10.1038/ni1469.

Guermonprez, P. *et al.* (2003) 'ER–phagosome fusion defines an MHC class I cross-presentation compartment in dendritic cells', *Nature*. Nature Publishing Group, 425(6956), pp. 397–402. doi: 10.1038/nature01911.

Guidoboni, M. *et al.* (2001) 'Microsatellite instability and high content of activated cytotoxic lymphocytes identify colon cancer patients with a favorable prognosis', *The American journal of pathology*. American Society for Investigative Pathology, 159(1), pp. 297–304. doi: 10.1016/S0002-9440(10)61695-1.

Guillaudeux, T. *et al.* (1996) 'Expression of HLA Class I Genes in Meiotic and Post-Meiotic Human Spermatogenic Cells1', *Biology of Reproduction*. Narnia, 55(1), pp. 99–110. doi: 10.1095/biolreprod55.1.99.

Guillaume, B. *et al.* (2010) 'Two abundant proteasome subtypes that uniquely process some antigens presented by HLA class I molecules', *Proceedings of the National Academy of Sciences*. Proceedings of the National Academy of Sciences, 107(43), pp. 18599–18604. doi: 10.1073/pnas.1009778107.

Gundermann, S. *et al.* (2014) 'A comprehensive analysis of primary acute myeloid leukemia identifies biomarkers predicting susceptibility to human allogeneic Vγ9Vδ2 T cells.', *Journal of immunotherapy*. J Immunother, 37(6), pp. 321–30. doi: 10.1097/CJI.0000000000000043.

Hamann, D. *et al.* (1997) 'Phenotypic and Functional Separation of Memory and Effector Human CD8+ T Cells', *Journal of Experimental Medicine*. The Rockefeller University Press, 186(9), pp. 1407–1418. doi: 10.1084/jem.186.9.1407.

Hamann, D., Roos, M. T. . L. and van Lier, R. A. W. (1999) 'Faces and phases of human CD8+ T-cell development', *Immunology Today*. Elsevier Current Trends, 20(4), pp. 177–180. doi: 10.1016/S0167-5699(99)01444-9.

Hammarström, M. *et al.* (2002) 'Rapid screening for improved solubility of small human proteins produced as fusion proteins in Escherichia coli.', *Protein science : a publication of the Protein Society*, 11(2), pp. 313–21. doi: 10.1110/ps.22102.

Hammarström, S. *et al.* (1982) 'Mitogenic leukoagglutinin from Phaseolus vulgaris binds to a pentasaccharide unit in N-acetyllactosamine-type glycoprotein glycans.', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 79(5), pp. 1611–5. doi: 10.1073/pnas.79.5.1611.

Hanahan, D. and Weinberg, R. A. (2011) 'Hallmarks of cancer: the next generation.', *Cell*. Elsevier, 144(5), pp. 646–74. doi: 10.1016/j.cell.2011.02.013.

Harding, C. V and Unanue, E. R. (1990) 'Cellular mechanisms of antigen processing and the function of class I and II major histocompatibility complex molecules.', *Cell Regulation*. American Society for Cell Biology (ASCB), 1(7), pp. 499–509. doi: 10.1091/mbc.1.7.499.

Harland, K. L. *et al.* (2014) 'Epigenetic plasticity of Cd8a locus during CD8+ T-cell development and effector differentiation and reprogramming', *Nature Communications*. Nature Publishing Group, 5(1), p. 3547. doi: 10.1038/ncomms4547.

Harly, C. *et al.* (2012) 'Key implication of CD277/butyrophilin-3 (BTN3A) in cellular stress sensing by a major human γδ T-cell subset', *Blood*. American Society of Hematology, 120(11), pp. 2269–2279. doi: 10.1182/blood-2012-05-430470.

Harris, D. T. and Kranz, D. M. (2016) 'Adoptive T Cell Therapies: A Comparison of T Cell Receptors and Chimeric Antigen Receptors', *Trends in pharmacological sciences*. 2015/12/17, 37(3), pp. 220–230. doi: 10.1016/j.tips.2015.11.004.

Harrop, R. *et al.* (2008) 'Vaccination of colorectal cancer patients with TroVax given alongside chemotherapy (5-fluorouracil, leukovorin and irinotecan) is safe and induces potent immune responses', *Cancer Immunology, Immunotherapy*, 57(7), pp. 977–986. doi: 10.1007/s00262-007-0428-7.

Hattab, Georges *et al.* (no date) 'Membrane Protein Production in Escherichia coli: Overview and Protocols 4.1 Introduction'. doi: 10.1007/978-1-4939-0662-8_4.

Hawiger, D. *et al.* (2001) 'Dendritic cells induce peripheral T cell unresponsiveness under steady state conditions in vivo', *The Journal of experimental medicine*. The Rockefeller University Press, 194(6), pp. 769–779. doi: 10.1084/jem.194.6.769.

Hayday, A. C. (2000) 'γδ Cells: A Right Time and a Right Place for a Conserved Third Way of Protection', *Annual Review of Immunology*. Annual Reviews 4139 El Camino Way, P.O. Box 10139, Palo Alto, CA 94303-0139, USA, 18(1), pp. 975–1026. doi: 10.1146/annurev.immunol.18.1.975.

Hayday, A. C. (2009) 'γδ T Cells and the Lymphoid Stress-Surveillance Response', *Immunity*, 31(2), pp. 184–196. doi: 10.1016/j.immuni.2009.08.006.

Hayday, A. and Vantourout, P. (2013) 'A long-playing CD about the γδ TCR repertoire.', *Immunity*. Elsevier, 39(6), pp. 994–6. doi: 10.1016/j.immuni.2013.11.016.

He, Y. *et al.* (2014) 'γδ T cell and other immune cells crosstalk in cellular immunity.', *Journal of immunology research*. Hindawi Publishing Corporation, 2014, p. 960252. doi: 10.1155/2014/960252.

Van Hede, D. *et al.* (2017) 'Human papillomavirus oncoproteins induce a reorganization of epithelial-associated γδ T cells promoting tumor formation', *Proceedings of the National Academy of Sciences of the United States of America*. 2017/10/10. National Academy of Sciences, 114(43), pp. E9056–E9065. doi: 10.1073/pnas.1712883114.

Hedges, J. F., Lubick, K. J. and Jutila, M. A. (2005) 'T Cells Respond Directly to Pathogen-Associated Molecular Patterns', *The Journal of Immunology*. American Association of Immunologists, 174(10), pp. 6045–6053. doi: 10.4049/jimmunol.174.10.6045.

Helft, J. *et al.* (2015) 'GM-CSF Mouse Bone Marrow Cultures Comprise a Heterogeneous Population of CD11c+MHCII+ Macrophages and Dendritic Cells', *Immunity*. Elsevier BV, 42(6), pp. 1197–1211. doi: 10.1016/j.immuni.2015.05.018.

Henson, S. M. and Akbar, A. N. (2009) 'KLRG1--more than a marker for T cell senescence.', *Age (Dordrecht, Netherlands)*. Age (Dordr), 31(4), pp. 285–91. doi: 10.1007/s11357-009-9100-9.

Hiasa, A. *et al.* (2009) 'Rapid αβ TCR-mediated responses in γδ T cells transduced with cancer-specific TCR genes', *Gene Therapy*. Springer Science and Business Media LLC, 16(5), pp. 620–628. doi: 10.1038/gt.2009.6.

Himoudi, N. *et al.* (2012) 'Human γδ T Lymphocytes Are Licensed for Professional Antigen Presentation by Interaction with Opsonized Target Cells', *The Journal of Immunology*. The American Association of Immunologists, 188(4), pp. 1708–1716. doi: 10.4049/jimmunol.1102654.

Ho, N. I. *et al.* (2018) 'Adjuvants Enhancing Cross-Presentation by Dendritic Cells: The Key to More Effective Vaccines?', *Frontiers in Immunology*. Frontiers, 9, p. 2874. doi: 10.3389/fimmu.2018.02874.

Hoeres, T. *et al.* (2018) 'Improving the Efficiency of Vγ9Vδ2 T-Cell Immunotherapy in Cancer', *Frontiers in immunology*. Frontiers Media S.A., 9, p. 800. doi: 10.3389/fimmu.2018.00800.

Hoeres, T. *et al.* (2019) 'PD-1 signaling modulates interferon-γ production by Gamma Delta (γδ) T-Cells in response to leukemia', *OncoImmunology*, 8. doi: 10.1080/2162402X.2018.1550618.

Hoffmann, M. M. and Slansky, J. E. (2020) 'T-cell receptor affinity in the age of cancer immunotherapy', *Molecular Carcinogenesis*. John Wiley & Sons, Ltd, 59(7), pp. 862–870. doi: 10.1002/mc.23212.

Holderness, J. *et al.* (2007) 'Select plant tannins induce IL-2Ralpha up-regulation and augment cell division in gammadelta T cells.', *Journal of immunology (Baltimore, Md. : 1950)*. American Association of Immunologists, 179(10), pp. 6468–78. doi:

10.4049/jimmunol.179.10.6468.

Hole, N. and Stern, P. L. (1988) 'A 72 kD trophoblast glycoprotein defined by a monoclonal antibody.', *British journal of cancer*. Nature Publishing Group, 57(3), pp. 239–246. doi: 10.1038/bjc.1988.53.

Hole, N. and Stern, P. L. (1990) 'Isolation and characterization of 5T4, a tumour-associated antigen', *International Journal of Cancer*, 45(1), pp. 179–184. doi: 10.1002/ijc.2910450132.

Hosking, M. P., Flynn, C. T. and Whitton, J. L. (2014) 'Antigen-Specific Naive CD8 + T Cells Produce a Single Pulse of IFN-γ In Vivo within Hours of Infection, but without Antiviral Effect', *The Journal of Immunology*. American Association of Immunologists, 193(4), pp. 1873–1885. doi: 10.4049/jimmunol.1400348.

Houde, M. *et al.* (2003) 'Phagosomes are competent organelles for antigen cross-presentation', *Nature*. Nature Publishing Group, 425(6956), pp. 402–406. doi: 10.1038/nature01912.

Howard, J. *et al.* (2017) 'The Antigen-Presenting Potential of Vγ39Vδ 2 T Cells during Plasmodium falciparum Blood-Stage Infection', *Journal of Infectious Diseases*. Oxford University Press (OUP), 215(10), pp. 1569–1579. doi: 10.1093/infdis/jix149.

Hu, C. *et al.* (2012) 'Antigen-presenting effects of effector memory Vγ9VδT cells in rheumatoid arthritis', *Cellular and Molecular Immunology*, 9(3), pp. 245–254. doi: 10.1038/cmi.2011.50.

Hu, M. and Polyak, K. (2008) 'Microenvironmental regulation of cancer development', *Current opinion in genetics & development*. 2008/02/20, 18(1), pp. 27–34. doi: 10.1016/j.gde.2007.12.006.

Hua, F. *et al.* (2013) 'Potential regulatory role of in vitro-expanded Vδ1 T cells from human peripheral blood.', *Immunologic research*. Immunol Res, 56(1), pp. 172–80. doi: 10.1007/s12026-013-8390-2.

Huang, C.-J. *et al.* (2007) 'Expression and purification of the cancer antigen SSX2: A potential cancer vaccine', *Protein Expression and Purification*, 56(2), pp. 212–219. doi: 10.1016/j.pep.2007.07.009.

Huber, A. *et al.* (2018) 'Current State of Dendritic Cell-Based Immunotherapy: Opportunities for in vitro Antigen Loading of Different DC Subsets?', *Frontiers in immunology*. Frontiers Media S.A., 9, p. 2804. doi: 10.3389/fimmu.2018.02804.

Hudspeth, K., Silva-Santos, B. and Mavilio, D. (2013) 'Natural cytotoxicity receptors: broader expression patterns and functions in innate and adaptive immune cells.', *Frontiers in immunology*. Front Immunol, 4, p. 69. doi: 10.3389/fimmu.2013.00069.

Idrees, A. S. M. *et al.* (2013) 'Comparison of γδ T cell responses and farnesyl diphosphate synthase inhibition in tumor cells pretreated with zoledronic acid.', *Cancer science*. Cancer Sci, 104(5), pp. 536–42. doi: 10.1111/cas.12124.

Iezzi, G., Scheidegger, D. and Lanzavecchia, A. (2001) 'Migration and function of antigen-primed nonpolarized T lymphocytes in vivo.', *The Journal of experimental medicine*. The Rockefeller University Press, 193(8), pp. 987–93. doi: 10.1084/jem.193.8.987.

Ishikawa, T. *et al.* (2003) 'Tumor-specific Immunological Recognition of Frameshift-mutated Peptides in Colon Cancer with Microsatellite Instability', *Cancer research*. American Association for Cancer Research, 63(17), pp. 5564–5572. Available at: https://cancerres.aacrjournals.org/content/63/17/5564.article-info (Accessed: 4 March 2021).

Ismaili, J. *et al.* (2002) 'Human gamma delta T cells induce dendritic cell maturation.', *Clinical immunology (Orlando, Fla.)*. Clin Immunol, 103(3 Pt 1), pp. 296–302. doi: 10.1006/clim.2002.5218.

Iyoda, T. *et al.* (2002) 'The CD8 Dendritic Cell Subset Selectively Endocytoses Dying Cells in Culture and In Vivo', *J. Exp. Med.* ☐ *The*. Rockefeller University Press, 195(10), pp. 1289–1302. doi: 10.1084/jem.20020161.

Jäger, E. *et al.* (1998) 'Simultaneous humoral and cellular immune response against cancer-testis antigen NY-ESO-1: definition of human histocompatibility leukocyte antigen (HLA)-A2-binding peptide epitopes.', *The Journal of experimental medicine*. Rockefeller University Press, 187(2), pp. 265–70. doi: 10.1084/jem.187.2.265.

Jäger, E. *et al.* (2000a) 'Induction of primary NY-ESO-1 immunity: CD8+ T lymphocyte and antibody responses in peptide-vaccinated patients with NY-ESO-1+ cancers', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 97(9), pp. 4760–5. doi: 10.1073/pnas.97.9.4760.

Jäger, E. *et al.* (2000b) 'Monitoring CD8 T cell responses to NY-ESO-1: correlation of humoral and cellular immune

responses.', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 97(9), pp. 4760–5. doi: 10.1073/pnas.97.9.4760.

Jameson, S. C. and Masopust, D. (2018) 'Understanding Subset Diversity in T Cell Memory', *Immunity*. Cell Press, 48(2), pp. 214–226. doi: 10.1016/J.IMMUNI.2018.02.010.

Janikashvili, N., Larmonier, N. and Katsanis, E. (2010) 'Personalized dendritic cell-based tumor immunotherapy.', *Immunotherapy*. NIH Public Access, 2(1), pp. 57–68. doi: 10.2217/imt.09.78.

Jauhiainen, M. *et al.* (2009) 'Analysis of endogenous ATP analogs and mevalonate pathway metabolites in cancer cell cultures using liquid chromatography–electrospray ionization mass spectrometry', *Journal of Chromatography B*. Elsevier, 877(27), pp. 2967–2975. doi: 10.1016/j.jchromb.2009.07.010.

Jenne, L. *et al.* (2000) 'Dendritic cells containing apoptotic melanoma cells prime human CD8+ T cells for efficient tumor cell lysis', *Cancer Research*, 60(16), pp. 4446–4452.

Joffre, O. P. *et al.* (2012) 'Cross-presentation by dendritic cells', *Nature Reviews Immunology*. Nature Publishing Group, 12(8), pp. 557–569. doi: 10.1038/nri3254.

Johnson, D. R. (2000) 'Differential expression of human major histocompatibility class I loci: HLA-A, -B, and -C', *Human Immunology*, 61(4), pp. 389–396. doi: 10.1016/S0198-8859(99)00186-X.

Jungbluth, A. A. *et al.* (2001) 'Immunohistochemical analysis of NY-ESO-1 antigen expression in normal and malignant human tissues', *International Journal of Cancer*. John Wiley & Sons, Ltd, 92(6), pp. 856–860. doi: 10.1002/ijc.1282.

Junqueira, C. *et al.* (2021) 'γδ T cells suppress Plasmodium falciparum blood-stage infection by direct killing and phagocytosis', *Nature Immunology*. Nature Publishing Group, 22(3), pp. 347–357. doi: 10.1038/s41590-020-00847-4.

Jürgen, B. *et al.* (2010) 'Quality control of inclusion bodies in Escherichia coli', *Microbial Cell Factories*. BioMed Central, 9(1), p. 41. doi: 10.1186/1475-2859-9-41.

Van Kaer, L. *et al.* (2019) 'Role of autophagy in MHC class I-restricted antigen presentation', *Molecular Immunology*. Elsevier, 113(October 2017), pp. 2–5. doi: 10.1016/j.molimm.2017.10.021.

Kagermeier-Schenk, B. *et al.* (2011) 'Waif1/5T4 inhibits Wnt/β-catenin signaling and activates noncanonical Wnt pathways by modifying LRP6 subcellular localization.', *Developmental cell*. Elsevier, 21(6), pp. 1129–43. doi: 10.1016/j.devcel.2011.10.015.

Kalyan, S. *et al.* (2013) 'Neutrophil uptake of nitrogen-bisphosphonates leads to the suppression of human peripheral blood γδ T cells', *Cellular and Molecular Life Sciences*. Springer Science and Business Media LLC, 71(12), pp. 2335–2346. doi: 10.1007/s00018-013-1495-x.

Kambayashi, T. and Laufer, T. M. (2014) 'Atypical MHC class II-expressing antigen-presenting cells: Can anything replace a dendritic cell?', *Nature Reviews Immunology*. Nature Publishing Group, 14(11), pp. 719–730. doi: 10.1038/nri3754.

Kamei, R. *et al.* (2018) 'Expression levels of UL16 binding protein 1 and natural killer group 2 member D affect overall survival in patients with gastric cancer following gastrectomy.', *Oncology letters*. Oncol Lett, 15(1), pp. 747–754. doi: 10.3892/ol.2017.7354.

Kamigaki, T. *et al.* (2013) 'Zoledronate-pulsed dendritic cell-based anticancer vaccines.', *Oncoimmunology*. Taylor & Francis, 2(9), p. e25636. doi: 10.4161/onci.25636.

Kantoff, P. W. *et al.* (2010) 'Sipuleucel-T Immunotherapy for Castration-Resistant Prostate Cancer', *New England Journal of Medicine*. Massachusetts Medical Society, 363(20), pp. 1966–1968. doi: 10.1056/NEJMc1009982.

Karin, M., Lawrence, T. and Nizet, V. (2006) 'Innate Immunity Gone Awry: Linking Microbial Infections to Chronic Inflammation and Cancer', *Cell*. Elsevier BV, 124(4), pp. 823–835. doi: 10.1016/j.cell.2006.02.016.

Karunakaran, M. M. *et al.* (2020) 'Butyrophilin-2A1 Directly Binds Germline-Encoded Regions of the Vγ9Vδ2 TCR and Is Essential for Phosphoantigen Sensing', *Immunity*. Elsevier, 52(3), pp. 487-498.e6. doi: 10.1016/j.immuni.2020.02.014.

Kashem, S. W., Haniffa, M. and Kaplan, D. H. (2017) 'Antigen-Presenting Cells in the Skin', *Annual Review of Immunology*, 35(1), pp. 469–499. doi: 10.1146/annurev-immunol-051116-052215.

Kazen, A. R. and Adams, E. J. (2011) 'Evolution of the V, D, and J gene segments used in the primate gammadelta T-cell receptor reveals a dichotomy of conservation and diversity', *Proceedings of the National Academy of Sciences of the United States of America*. 2011/07/05. National Academy of Sciences, 108(29), pp. E332–E340. doi: 10.1073/pnas.1105105108.

Kelly, A. and Trowsdale, J. (2019) 'Genetics of antigen processing and presentation', *Immunogenetics*. Springer Berlin Heidelberg, 71(3), pp. 161–170. doi: 10.1007/s00251-018-1082-2.

Keppler, S. J. *et al.* (2012) 'Signal 3 Cytokines as Modulators of Primary Immune Responses during Infections: The Interplay of Type I IFN and IL-12 in CD8 T Cell Responses', *PLoS ONE*. Edited by R. Tripp. Public Library of Science, 7(7), p. e40865. doi: 10.1371/journal.pone.0040865.

Khan, M. W. A. *et al.* (2014) 'Expanded Human Blood-Derived γδT Cells Display Potent Antigen-Presentation Functions.', *Frontiers in immunology*. Frontiers, 5, p. 344. doi: 10.3389/fimmu.2014.00344.

Khan, M. W. A., Eberl, M. and Moser, B. (2014) 'Potential Use of γδ T Cell-Based Vaccines in Cancer Immunotherapy.', *Frontiers in Immunology*. Frontiers, 5, p. 512. doi: 10.3389/fimmu.2014.00512.

Kim, D. W. *et al.* (2010) 'TroVax, a recombinant modified vaccinia Ankara virus encoding 5T4: lessons learned and future development.', *Human vaccines*, 6(10), pp. 784–91. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20975327 (Accessed: 19 June 2017).

Kim, M. *et al.* (2006) 'Antigen dose governs the shaping of CTL repertoires in vitro and in vivo.', *International immunology*, 18(3), pp. 435–44. doi: 10.1093/intimm/dxh383.

Kim, R., Emi, M. and Tanabe, K. (2007) 'Cancer immunoediting from immune surveillance to immune escape', *Immunology*, 121(1), pp. 1–14. doi: 10.1111/j.1365-2567.2007.02587.x.

Kisielow, J. *et al.* (2011) 'Evidence for the divergence of innate and adaptive T-cell precursors before commitment to the αβ and γδ lineages', *Blood*. American Society of Hematology, 118(25), pp. 6591–6600. doi: 10.1182/blood-2011-05-352732.

Klebanoff, C. A. *et al.* (2010) 'Therapeutic cancer vaccines: are we there yet?', *Immunological Reviews*. Wiley, 239(1), pp. 27–44. doi: 10.1111/j.1600-065x.2010.00979.x.

Klebanoff, C. A. *et al.* (2016) 'Memory T cell-driven differentiation of naive cells impairs adoptive immunotherapy.', *The Journal of clinical investigation*, 126(1), pp. 318–34. doi: 10.1172/JCI81217.

Klein, L. *et al.* (2014) 'Positive and negative selection of the T cell repertoire: what thymocytes see (and don't see).', *Nature reviews. Immunology*, 14(6), pp. 377–91. doi: 10.1038/nri3667.

Kon, E. and Benhar, I. (2019) 'Immune checkpoint inhibitor combinations: Current efforts and important aspects for success', *Drug Resistance Updates*. Churchill Livingstone, 45, pp. 13–29. doi: 10.1016/J.DRUP.2019.07.004.

Kondo, M. *et al.* (2011) 'Expansion of human peripheral blood γδ T cells using zoledronate', *Journal of visualized experiments : JoVE*. MyJove Corporation, 2(55), p. 3182. doi: 10.3791/3182.

Kong, Y. *et al.* (2009) 'The NKG2D ligand ULBP4 binds to TCRγ9/δ2 and induces cytotoxicity to tumor cells through both TCRγδ and NKG2D', *Blood*. American Society of Hematology, 114(2), pp. 310–317. doi: 10.1182/blood-2008-12-196287.

Kramer, R. M. *et al.* (2012) 'Toward a Molecular Understanding of Protein Solubility: Increased Negative Surface Charge Correlates with Increased Solubility', *Biophysical Journal*. The Biophysical Society, 102(8). Available at: http://www.ncbi.nlm.nih.gov/pubmed/22768947 (Accessed: 6 May 2019).

Kryczek, I. *et al.* (2005) 'CXCL12 and vascular endothelial growth factor synergistically induce neonaniogenisis in human ovarian cancers', *Cancer Research*, 65(2), pp. 465–472.

Kulig, P. *et al.* (2015) 'IL17A-Mediated Endothelial Breach Promotes Metastasis Formation', *Cancer Immunology Research*. American Association for Cancer Research (AACR), 4(1), pp. 26–32. doi: 10.1158/2326-6066.cir-15-0154.

Kunzmann, V., Bauer, E. and Wilhelm, M. (1999) 'γ/δ T-Cell Stimulation by Pamidronate', *New England Journal of Medicine*. Massachusetts Medical Society, 340(9), pp. 737–738. doi: 10.1056/NEJM199903043400914.

Kurts, C. and Wagner, H. (2011) 'Grand Challenges in Molecular Antigen-presenting Cell Biology', *Frontiers in Immunology*.

Frontiers, 2, p. 8. doi: 10.3389/fimmu.2011.00008.

Laconi, E. (2007) 'The evolving concept of tumor microenvironments', *BioEssays*. Wiley, 29(8), pp. 738–744. doi: 10.1002/bies.20606.

Lafont, V. *et al.* (2001) 'Production of TNF-α by Human Vγ9Vδ2 T Cells Via Engagement of FcγRIIIA, the Low Affinity Type 3 Receptor for the Fc Portion of IgG, Expressed upon TCR Activation by Nonpeptidic Antigen', *The Journal of Immunology*, 166(12), pp. 7190–7199. doi: 10.4049/jimmunol.166.12.7190.

Lafont, V. *et al.* (2014) 'Plasticity of γδ T Cells: Impact on the Anti-Tumor Response', *Frontiers in Immunology*. Frontiers, 5, p. 622. doi: 10.3389/fimmu.2014.00622.

Laird, R. M. *et al.* (2013) 'γδ T cells acquire effector fates in the thymus and differentiate into cytokine-producing effectors in a Listeria model of infection independently of CD28 costimulation', *PloS one*. Public Library of Science, 8(5), pp. e63178–e63178. doi: 10.1371/journal.pone.0063178.

Lalor, S. J. and McLoughlin, R. M. (2016) 'Memory γδ T Cells–Newly Appreciated Protagonists in Infection and Immunity', *Trends in Immunology*, 37(10), pp. 690–702. doi: 10.1016/j.it.2016.07.006.

Lança, T. *et al.* (2010) 'The MHC class Ib protein ULBP1 is a nonredundant determinant of leukemia/lymphoma susceptibility to γδ T-cell cytotoxicity', *Blood*. American Society of Hematology, 115(12), pp. 2407–2411. doi: 10.1182/blood-2009-08-237123.

Landmeier, S. *et al.* (2009) 'Activated Human γδ T Cells as Stimulators of Specific CD8+ T-cell Responses to Subdominant Epstein Barr Virus Epitopes', *Journal of Immunotherapy*, 32(3), pp. 310–321. doi: 10.1097/CJI.0b013e31819b7c30.

Laplane, L. *et al.* (2019) 'Beyond the tumour microenvironment', *International Journal of Cancer*. doi: 10.1002/ijc.32343.

Lauber, K. *et al.* (2012) 'Dying cell clearance and its impact on the outcome of tumor radiotherapy', *Frontiers in oncology*. Frontiers Research Foundation, 2, p. 116. doi: 10.3389/fonc.2012.00116.

Laugel, B., Price, D. A., *et al.* (2007) 'CD8 exerts differential effects on the deployment of cytotoxic T lymphocyte effector functions', *European Journal of Immunology*. Wiley, 37(4), pp. 905–913. doi: 10.1002/eji.200636718.

Laugel, B., van den Berg, H. A., *et al.* (2007) 'Different T Cell Receptor Affinity Thresholds and CD8 Coreceptor Dependence Govern Cytotoxic T Lymphocyte Activation and Tetramer Binding Properties', *Journal of Biological Chemistry*. American Society for Biochemistry and Molecular Biology, 282(33), pp. 23799–23810. doi: 10.1074/jbc.M700976200.

Laugel, B. *et al.* (2011) 'The multiple roles of the CD8 coreceptor in T cell biology: opportunities for the selective modulation of self-reactive cytotoxic T cells', *Journal of Leukocyte Biology*. Wiley, 90(6), pp. 1089–1099. doi: 10.1189/jlb.0611316.

LaVallie, Edward R. *et al.* (1993) 'A Thioredoxin Gene Fusion Expression System That Circumvents Inclusion Body Formation in the *E. coli* Cytoplasm', *Bio/Technology*. Nature Publishing Group, 11(2), pp. 187–193. doi: 10.1038/nbt0293-187.

LaVallie, Edward R *et al.* (1993) 'A thioredoxin gene fusion expression system that cirformation in the *E. coli* cytoplasm', *Bio/Technology*, 11(2), pp. 187–193. doi: 10.1038/nbt0293-187.

LaVallie, E. R. *et al.* (no date) 'Thioredoxin and Related Proteins as Multifunctional Fusion Tags for Soluble Expression in *E. coli*', in *E. coli Gene Expression Protocols*. New Jersey: Humana Press, pp. 119–140. doi: 10.1385/1-59259-301-1:119.

Lawand, M., Déchanet-Merville, J. and Dieu-Nosjean, M.-C. (2017) 'Key Features of Gamma-Delta T-Cell Subsets in Human Diseases and Their Immunotherapeutic Implications', *Frontiers in immunology*. Frontiers Media S.A., 8, p. 761. doi: 10.3389/fimmu.2017.00761.

Lee, S. J. *et al.* (2013) '4-1BB signal stimulates the activation, expansion, and effector functions of γδ T cells in mice and humans', *European Journal of Immunology*. Wiley, 43(7), pp. 1839–1848. doi: 10.1002/eji.201242842.

Leggatt, G. R. *et al.* (2004) 'Changes to peptide structure, not concentration, contribute to expansion of the lowest avidity cytotoxic T lymphocytes.', *Journal of leukocyte biology*. Society for Leukocyte Biology, 76(4), pp. 787–95. doi: 10.1189/jlb.0104026.

Lever, M. *et al.* (2014) 'Phenotypic models of T cell activation', *Nature Reviews Immunology*. Nature Publishing Group, 14(9), pp. 619–629. doi: 10.1038/nri3728.

Li, H., Fan, X. and Houghton, J. (2007) 'Tumor microenvironment: The role of the tumor stroma in cancer', *Journal of Cellular Biochemistry*. Wiley, 101(4), pp. 805–815. doi: 10.1002/jcb.21159.

Li, W. *et al.* (2010) 'Effect of IL-18 on Expansion of γδ T Cells Stimulated by Zoledronate and IL-2', *Journal of Immunotherapy*. Ovid Technologies (Wolters Kluwer Health), 33(3), pp. 287–296. doi: 10.1097/cji.0b013e3181c80ffa.

Li, Y., Bleakley, M. and Yee, C. (2005) 'IL-21 Influences the Frequency, Phenotype, and Affinity of the Antigen-Specific CD8 T Cell Response', *The Journal of Immunology*, 175(4), pp. 2261–2269. doi: 10.4049/jimmunol.175.4.2261.

Li, Z. *et al.* (2011) 'IFN-γ enhances HOS and U2OS cell lines susceptibility to γδ T cell-mediated killing through the Fas/Fas ligand pathway', *International Immunopharmacology*. Elsevier BV, 11(4), pp. 496–503. doi: 10.1016/j.intimp.2011.01.001.

Lindahl-Kiessling, K. (1972) 'Mechanism of phytohemagglutinin (PHA) action. V. PHA compared with concanavalin A (Con A)', *Experimental Cell Research*, 70(1), pp. 17–26. doi: 10.1016/0014-4827(72)90176-0.

Littman, D. R. and Singh, H. (2007) 'Asymmetry and Immune Memory', *Science*, 315(5819), pp. 1673–1674. doi: 10.1126/science.1141184.

Liu, S. *et al.* (2007) 'IL-21 synergizes with IL-7 to augment expansion and anti-tumor function of cytotoxic T cells', *International Immunology*. Oxford Academic, 19(10), pp. 1213–1221. doi: 10.1093/intimm/dxm093.

Liu, Z. *et al.* (2015) 'IL-2, IL-15 and IL-21 expand T cells for targeted adoptive therapy', *Journal for ImmunoTherapy of Cancer*, 3(Suppl 2), p. P31. doi: 10.1186/2051-1426-3-s2-p31.

Long, A. H. *et al.* (2015) '4-1BB costimulation ameliorates T cell exhaustion induced by tonic signaling of chimeric antigen receptors', *Nature medicine*. 2015/05/04, 21(6), pp. 581–590. doi: 10.1038/nm.3838.

Lopez de Castro, J. A. (1989) 'Co-operative interactions in the recognition of target cells by cytotoxic T lymphocytes: Role of CD8', *Biochemical Society Transactions*, 17(6), pp. 985–988. doi: 10.1042/bst0170985.

Di Lorenzo, B., Déchanet-Merville, J. and Silva-Santos, B. (2017) 'Peripheral clonal selection shapes the human γδ T-cell repertoire', *Cellular & Molecular Immunology*. Nature Publishing Group, 14(9), pp. 733–735. doi: 10.1038/cmi.2017.51.

Lowe, A. J. *et al.* (2011) 'Expression and purification of cGMP grade NY-ESO-1 for clinical trials', *Biotechnology Progress*, 27(2), pp. 435–441. doi: 10.1002/btpr.552.

Lu, B.-J. *et al.* (2004) 'Gastric medullary carcinoma, a distinct entity associated with microsatellite instability-H, prominent intraepithelial lymphocytes and improved prognosis', *Histopathology*. Wiley, 45(5), pp. 485–492. doi: 10.1111/j.1365-2559.2004.01998.x.

Lu, J. V., Chen, H. C. and Walsh, C. M. (2014) 'Necroptotic signaling in adaptive and innate immunity', *Seminars in Cell and Developmental Biology*. Elsevier Ltd, 35, pp. 33–39. doi: 10.1016/j.semcdb.2014.07.003.

Lubick, K. and Jutila, M. A. (2006) 'LTA recognition by bovine T cells involves CD36', *Journal of Leukocyte Biology*. John Wiley & Sons, Ltd, 79(6), pp. 1268–1270. doi: 10.1189/jlb.1005616.

Lucas, P. J. *et al.* (1995) 'Naive CD28-deficient T cells can initiate but not sustain an in vitro antigen-specific immune response.', *Journal of immunology (Baltimore, Md. : 1950)*. American Association of Immunologists, 154(11), pp. 5757–68. Available at: http://www.ncbi.nlm.nih.gov/pubmed/7751626 (Accessed: 12 October 2020).

Luescher, I. F. *et al.* (1995) 'CD8 modulation of T-cell antigen receptor–ligand interactions on living cytotoxic T lymphocytes', *Nature*. Nature Publishing Group, 373(6512), pp. 353–356. doi: 10.1038/373353a0.

Lugli, E. *et al.* (2013) 'Superior T memory stem cell persistence supports long-lived T cell memory.', *The Journal of clinical investigation*. American Society for Clinical Investigation, 123(2), pp. 594–9. doi: 10.1172/JCI66327.

Lugli, E. *et al.* (2020) 'Stem, Effector, and Hybrid States of Memory CD8+ T Cells', *Trends in Immunology*. Elsevier Inc., 41(1), pp. 17–28. doi: 10.1016/j.it.2019.11.004.

Ma, C. *et al.* (2012) 'Tumor-infiltrating γδ T lymphocytes predict clinical outcome in human breast cancer', *Journal of immunology (Baltimore, Md. : 1950)*. 2012/10/03, 189(10), pp. 5029–5036. doi: 10.4049/jimmunol.1201892.

Ma, S. *et al.* (2014) 'IL-17A Produced by γδ T Cells Promotes Tumor Growth in Hepatocellular Carcinoma', *Cancer Research*. American Association for Cancer Research (AACR), 74(7), pp. 1969–1982. doi: 10.1158/0008-5472.can-13-2534.

Mahnke, Y. D. *et al.* (2013) 'The who's who of T-cell differentiation: Human memory T-cell subsets', *European Journal of Immunology*. Wiley, 43(11), pp. 2797–2809. doi: 10.1002/eji.201343751.

Makino, S., Reynolds, J. A. and Tanford, C. (1973) 'The binding of deoxycholate and Triton X 100 to proteins', *Journal of Biological Chemistry*.

Maman, S. and Witz, I. P. (2018) 'A history of exploring cancer in context', *Nature Reviews Cancer*. Springer Science and Business Media LLC, 18(6), pp. 359–376. doi: 10.1038/s41568-018-0006-7.

Maniar, A. *et al.* (2010) 'Human gammadelta T lymphocytes induce robust NK cell-mediated antitumor cytotoxicity through CD137 engagement', *Blood*. 2010/06/02. American Society of Hematology, 116(10), pp. 1726–1733. doi: 10.1182/blood-2009-07-234211.

Marchand, M. *et al.* (1999) 'Tumor regressions observed in patients with metastatic melanoma treated with an antigenic peptide encoded by geneMAGE-3 and presented by HLA-A1', *International Journal of Cancer*. Wiley, 80(2), pp. 219–230. doi: 10.1002/(sici)1097-0215(19990118)80:2<219::aid-ijc10>3.0.co;2-s.

Di Marco Barros, R. *et al.* (2016) 'Epithelia Use Butyrophilin-like Molecules to Shape Organ-Specific γδ T Cell Compartments', *Cell*. 2016/09/15. Elsevier, 167(1), pp. 203-218.e17. doi: 10.1016/j.cell.2016.08.030.

Martin, B. *et al.* (2009) 'Interleukin-17-Producing γδ T Cells Selectively Expand in Response to Pathogen Products and Environmental Signals', *Immunity*. Elsevier BV, 31(2), pp. 321–330. doi: 10.1016/j.immuni.2009.06.020.

Di Martino, J. S., Mondal, C. and Bravo-Cordero, J. J. (2019) 'Textures of the tumour microenvironment', *Essays in Biochemistry*. Edited by A. Malliri et al. Portland Press, 63(5), pp. 619–629. doi: 10.1042/EBC20190019.

Mastelic-Gavillet, B. *et al.* (2019) 'Personalized Dendritic Cell Vaccines-Recent Breakthroughs and Encouraging Clinical Results', *Frontiers in immunology*. Frontiers Media S.A., 10, p. 766. doi: 10.3389/fimmu.2019.00766.

Mazzucchelli, R. and Durum, S. K. (2007) 'Interleukin-7 receptor expression: Intelligent design', *Nature Reviews Immunology*, 7(2), pp. 144–154. doi: 10.1038/nri2023.

McGilvray, I. D. *et al.* (2000) 'Nonopsonic monocyte/macrophage phagocytosis of Plasmodium falciparum-parasitized erythrocytes: a role for CD36 in malarial clearance.', *Blood*. Elsevier, 96(9), pp. 3231–40. doi: 10.1182/BLOOD.V96.9.3231.

Melandri, D. *et al.* (2018) 'The γδTCR combines innate immunity with adaptive immunity by utilizing spatially distinct regions for agonist selection and antigen responsiveness', *Nature Immunology*. Nature Publishing Group, 19(12), pp. 1352–1365. doi: 10.1038/s41590-018-0253-5.

Melero, I. *et al.* (2014) 'Therapeutic vaccines for cancer: an overview of clinical trials', *Nature Reviews Clinical Oncology*. Springer Science and Business Media LLC, 11(9), pp. 509–524. doi: 10.1038/nrclinonc.2014.111.

Meraviglia, S. *et al.* (2010) 'In vivo manipulation of Vgamma9Vdelta2 T cells with zoledronate and low-dose interleukin-2 for immunotherapy of advanced breast cancer patients.', *Clinical and experimental immunology*. Clin Exp Immunol, 161(2), pp. 290–7. doi: 10.1111/j.1365-2249.2010.04167.x.

Meuter, S., Eberl, M. and Moser, B. (2010) 'Prolonged antigen survival and cytosolic export in cross-presenting human γδ T cells', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 107(19), pp. 8730–8735. doi: 10.1073/pnas.1002769107.

Mintern, J. D. *et al.* (2015) 'Differential use of autophagy by primary dendritic cells specialized in cross-presentation', *Autophagy*, 11(6), pp. 906–917. doi: 10.1080/15548627.2015.1045178.

Mintern, J. D., Macri, C. and Villadangos, J. A. (2015) 'Modulation of antigen presentation by intracellular trafficking', *Current Opinion in Immunology*. Elsevier Ltd, 34(Mhc I), pp. 16–21. doi: 10.1016/j.coi.2014.12.006.

Miroux, B. and Walker, J. E. (1996) *Over-production of Proteins in Escherichia coli: Mutant Hosts that Allow Synthesis of some Membrane Proteins and Globular Proteins at High Levels*, *J. Mol. Biol.* Available at:

http://wolfson.huji.ac.il/expression/local/bacteria/c41-c43-1996.pdf (Accessed: 24 May 2019).

Moens, E. *et al.* (2011) 'IL-23R and TCR signaling drives the generation of neonatal V 9V 2 T cells expressing high levels of cytotoxic mediators and producing IFN- and IL-17', *Journal of Leukocyte Biology*. Wiley, 89(5), pp. 743–752. doi: 10.1189/jlb.0910501.

Mokuno, Y. *et al.* (2000) 'Expression of Toll-Like Receptor 2 on γδ T Cells Bearing Invariant Vγ6/Vδ1 Induced byEscherichia coliInfection in Mice', *The Journal of Immunology*. The American Association of Immunologists, 165(2), pp. 931–940. doi: 10.4049/jimmunol.165.2.931.

Mönkkönen, H. *et al.* (2008) 'Bisphosphonate-induced ATP analog formation and its effect on inhibition of cancer cell growth', *Anti-Cancer Drugs*. Anticancer Drugs, 19(4), pp. 391–399. doi: 10.1097/CAD.0b013e3282f632bf.

Montigny, C. *et al.* (2004) 'Overcoming the toxicity of membrane peptide expression in bacteria by upstream insertion of Asp-Pro sequence', *Biochimica et Biophysica Acta (BBA) - Biomembranes*, 1660(1), pp. 53–65. doi: 10.1016/j.bbamem.2003.10.013.

Mookerjee-Basu, J. *et al.* (2010) 'F1-Adenosine Triphosphatase Displays Properties Characteristic of an Antigen Presentation Molecule for Vγ9Vδ2 T Cells', *The Journal of Immunology*. The American Association of Immunologists, 184(12), pp. 6920–6928. doi: 10.4049/jimmunol.0904024.

Morita, C. T. *et al.* (1995) 'Direct Presentation of Nonpeptide Prenyl Pryophosphate Antigens to Human gamma delta T cells', *Immunity*, 3, pp. 495–507.

Morita, C. T. *et al.* (2007) 'Nonpeptide antigens, presentation mechanisms, and immunological memory of human Vγ2Vδ2 T cells: Discriminating friend from foe through the recognition of prenyl pyrophosphate antigens', *Immunological Reviews*, 215(1), pp. 59–76. doi: 10.1111/j.1600-065X.2006.00479.x.

Moroz, A. *et al.* (2004) ' IL-21 Enhances and Sustains CD8 + T Cell Responses to Achieve Durable Tumor Immunity: Comparative Evaluation of IL-2, IL-15, and IL-21 ', *The Journal of Immunology*, 173(2), pp. 900–909. doi: 10.4049/jimmunol.173.2.900.

Moulin, M. *et al.* (2017) 'Vγ9Vδ2 T cell activation by strongly agonistic nucleotidic phosphoantigens', *Cellular and Molecular Life Sciences*. Springer Science and Business Media LLC, 74(23), pp. 4353–4367. doi: 10.1007/s00018-017-2583-0.

Myers, K. A. *et al.* (1994) 'Isolation of a cDNA Encoding 5T4 Oncofetal Trophoblast Glycoprotein', *Journal of biochemistry*, 269(12). Available at: http://www.jbc.org/content/269/12/9319.full.pdf (Accessed: 22 June 2017).

Nagarsheth, N. *et al.* (2016) 'PRC2 Epigenetically Silences Th1-Type Chemokines to Suppress Effector T-Cell Trafficking in Colon Cancer', *Cancer research*. 2015/11/13. American Association for Cancer Research, 76(2), pp. 275–282. doi: 10.1158/0008-5472.CAN-15-1938.

Nagarsheth, N., Wicha, M. S. and Zou, W. (2017) 'Chemokines in the cancer microenvironment and their relevance in cancer immunotherapy', *Nature Reviews Immunology*. Nature Publishing Group, 17(9), pp. 559–572. doi: 10.1038/nri.2017.49.

Naito, Y. *et al.* (1998) 'CD8+ T cells infiltrated within cancer cell nests as a prognostic factor in human colorectal cancer', *Cancer Research*, 58(16), pp. 3491–3494.

Nakamura, M. *et al.* (2015) 'A genome-wide analysis identifies a notch-RBP-Jκ-IL-7Rα axis that controls IL-17-producing γδ T cell homeostasis in mice.', *Journal of immunology (Baltimore, Md. : 1950)*. American Association of Immunologists, 194(1), pp. 243–51. doi: 10.4049/jimmunol.1401619.

Nakata, B. *et al.* (2002) 'Prognostic value of microsatellite instability in resectable pancreatic cancer.', *Clinical cancer research : an official journal of the American Association for Cancer Research*. Clin Cancer Res, 8(8), pp. 2536–40. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12171881 (Accessed: 9 March 2021).

Naoe, M. *et al.* (2010) 'Zoledronate stimulates gamma delta T cells in prostate cancer patients.', *Oncology research*. Oncol Res, 18(10), pp. 493–501. doi: 10.3727/096504010x12671222663638.

Nedellec, S. *et al.* (2010) 'NKG2D costimulates human V gamma 9V delta 2 T cell antitumor cytotoxicity through protein kinase C theta-dependent modulation of early TCR-induced calcium and transduction signals.', *Journal of immunology (Baltimore,*

Md. : 1950). J Immunol, 185(1), pp. 55–63. doi: 10.4049/jimmunol.1000373.

Nedellec, S., Bonneville, M. and Scotet, E. (2010) 'Human Vγ9Vδ2 T cells: From signals to functions', *Seminars in Immunology*. Elsevier BV, 22(4), pp. 199–206. doi: 10.1016/j.smim.2010.04.004.

Neefjes, J. *et al.* (2011) 'Towards a systems understanding of MHC class I and MHC class II antigen presentation', *Nature Reviews Immunology*. Nature Publishing Group, 11(12), pp. 823–836. doi: 10.1038/nri3084.

Neller, M. A. *et al.* (2014) 'High Efficiency Ex Vivo Cloning of Antigen-Specific Human Effector T Cells', *PLoS ONE*. Edited by D. Unutmaz. Public Library of Science, 9(11), p. e110741. doi: 10.1371/journal.pone.0110741.

Nerdal, P. T. *et al.* (2016) 'Butyrophilin 3A/CD277-Dependent Activation of Human γδ T Cells: Accessory Cell Capacity of Distinct Leukocyte Populations.', *Journal of immunology (Baltimore, Md. : 1950)*. American Association of Immunologists, 197(8), pp. 3059–3068. doi: 10.4049/jimmunol.1600913.

Ness-Schwickerath, K. J., Jin, C. and Morita, C. T. (2010) 'Cytokine requirements for the differentiation and expansion of IL-17A- and IL-22-producing human Vgamma2Vdelta2 T cells', *Journal of immunology (Baltimore, Md. : 1950)*. 2010/05/14, 184(12), pp. 7268–7280. doi: 10.4049/jimmunol.1000600.

van Niel, G. *et al.* (2006) 'Dendritic Cells Regulate Exposure of MHC Class II at Their Plasma Membrane by Oligoubiquitination', *Immunity*, 25(6), pp. 885–894. doi: 10.1016/j.immuni.2006.11.001.

Niu, C. *et al.* (2017) 'Low-dose bortezomib increases the expression of NKG2D and DNAM-1 ligands and enhances induced NK and γδ T cell-mediated lysis in multiple myeloma.', *Oncotarget*. Oncotarget, 8(4), pp. 5954–5964. doi: 10.18632/oncotarget.13979.

Le Nours, J. *et al.* (2019) 'A class of γδ T cell receptors recognize the underside of the antigen-presenting molecule MR1.', *Science (New York, N.Y.)*. American Association for the Advancement of Science, 366(6472), pp. 1522–1527. doi: 10.1126/science.aav3900.

Nussbaumer, O. *et al.* (2011) 'DC-like cell-dependent activation of human natural killer cells by the bisphosphonate zoledronic acid is regulated by γδ T lymphocytes', *Blood*. American Society of Hematology, 118(10), pp. 2743–2751. doi: 10.1182/blood-2011-01-328526.

O'Beirne, J., Farzaneh, F. and Harrison, P. M. (2010) 'Generation of functional CD8+ T Cells by human dendritic cells expressing glypican-3 epitopes', *Journal of Experimental & Clinical Cancer Research*. BioMed Central, 29(1), p. 48. doi: 10.1186/1756-9966-29-48.

Obar, J. J. and Lefrancois, L. (2010) 'Early events governing memory CD8+ T-cell differentiation', *International Immunology*. Oxford Academic, 22(8), pp. 619–625. doi: 10.1093/intimm/dxq053.

Oberg, H.-H. *et al.* (2011) 'Regulation of T cell activation by TLR ligands', *European Journal of Cell Biology*. Elsevier BV, 90(6–7), pp. 582–592. doi: 10.1016/j.ejcb.2010.11.012.

Oh, D. S. and Lee, H. K. (2019) 'Autophagy protein ATG5 regulates CD36 expression and anti-tumor MHC class II antigen presentation in dendritic cells', *Autophagy*. Taylor & Francis, 15(12), pp. 2091–2106. doi: 10.1080/15548627.2019.1596493.

Oh, J. Y. *et al.* (2019) 'Synergistic Autophagy Effect of miR-212-3p in Zoledronic Acid-Treated In Vitro and Orthotopic In Vivo Models and in Patient-Derived Osteosarcoma Cells', *Cancers*. Multidisciplinary Digital Publishing Institute, 11(11), p. 1812. doi: 10.3390/cancers11111812.

Ohlendieck, K. (1996) 'Removal of Detergent from Protein Fractions', in *Protein Purification Protocols*. New Jersey: Humana Press, pp. 305–312. doi: 10.1385/0-89603-336-8:305.

Olins, P. O. and Rangwala, S. H. (1989) 'A novel sequence element derived from bacteriophage T7 mRNA acts as an enhancer of translation of the lacZ gene in Escherichia coli.', *The Journal of biological chemistry*, 264(29), pp. 16973–6. Available at: http://www.ncbi.nlm.nih.gov/pubmed/2676996 (Accessed: 28 May 2019).

Paleja, B. *et al.* (2013) 'Decreased functional response to Toll like receptor ligands in patients with oral cancer', *Human Immunology*. Elsevier BV, 74(8), pp. 927–936. doi: 10.1016/j.humimm.2013.04.018.

Palucka, K. and Banchereau, J. (2013) 'Dendritic-Cell-Based Therapeutic Cancer Vaccines', *Immunity*. Cell Press, 39(1), pp. 38–48. doi: 10.1016/j.immuni.2013.07.004.

Pamer, E. and Cresswell, P. (1998) 'MECHANISMS OF MHC CLASS I–RESTRICTED ANTIGEN PROCESSING', *Annual Review of Immunology*. Annual Reviews, 16(1), pp. 323–358. doi: 10.1146/annurev.immunol.16.1.323.

Papotto, P. H., Ribot, J. C. and Silva-Santos, B. (2017) 'IL-17+ γδ T cells as kick-starters of inflammation', *Nature Immunology*, 18(6), pp. 604–611. doi: 10.1038/ni.3726.

Pardoll, D. M. (2012) 'The blockade of immune checkpoints in cancer immunotherapy.', *Nature reviews. Cancer*. Springer Science and Business Media LLC, 12(4), pp. 252–64. doi: 10.1038/nrc3239.

Parish, I. A. and Kaech, S. M. (2009) 'Diversity in CD8+ T cell differentiation', *Current Opinion in Immunology*. Elsevier Current Trends, 21(3), pp. 291–297. doi: 10.1016/J.COI.2009.05.008.

Park, J.-H. *et al.* (2007) '"Coreceptor tuning": cytokine signals transcriptionally tailor CD8 coreceptor expression to the self-specificity of the TCR', *Nature Immunology*. Nature Publishing Group, 8(10), pp. 1049–1059. doi: 10.1038/ni1512.

Park, T. S., Rosenberg, S. A. and Morgan, R. A. (2011) 'Treating cancer with genetically engineered T cells', *Trends in Biotechnology*. Elsevier Ltd, 29(11), pp. 550–557. doi: 10.1016/j.tibtech.2011.04.009.

Paul, S. and Lal, G. (2016) 'Regulatory and effector functions of gamma-delta (γδ) T cells and their therapeutic potential in adoptive cellular therapy for cancer', *International Journal of Cancer*, 139(5), pp. 976–985. doi: 10.1002/ijc.30109.

Paulos, C. M. *et al.* (2007) 'Toll-like receptors in tumor immunotherapy', *Clinical cancer research : an official journal of the American Association for Cancer Research*, 13(18 Pt 1), pp. 5280–5289. doi: 10.1158/1078-0432.CCR-07-1378.

Pauza, C. D. and Cairo, C. (2015) 'Evolution and function of the TCR Vgamma9 chain repertoire: It's good to be public', *Cellular immunology*. 2015/03/04. Academic Press, 296(1), pp. 22–30. doi: 10.1016/j.cellimm.2015.02.010.

Peng, D. *et al.* (2015) 'Epigenetic silencing of TH1-type chemokines shapes tumour immunity and immunotherapy', *Nature*. 2015/10/26. Nature Publishing Group, 527(7577), pp. 249–253. doi: 10.1038/nature15520.

Peng, G. *et al.* (2005) 'Toll-Like Receptor 8-Mediated Reversal of CD4+ Regulatory T Cell Function', *Science*. American Association for the Advancement of Science (AAAS), 309(5739), pp. 1380–1384. doi: 10.1126/science.1113401.

Peng, G. *et al.* (2007) 'Tumor-infiltrating gammadelta T cells suppress T and dendritic cell function via mechanisms controlled by a unique toll-like receptor signaling pathway.', *Immunity*. Immunity, 27(2), pp. 334–48. doi: 10.1016/j.immuni.2007.05.020.

Penn, I. (1988) 'Tumors of the Immunocompromised Patient', *Annual Review of Medicine*, 39(1), pp. 63–73. doi: 10.1146/annurev.me.39.020188.000431.

Pereira, M. S. *et al.* (2018) 'Glycans as Key Checkpoints of T Cell Activity and Function', *Frontiers in Immunology*. Frontiers Media SA, 9, p. 2754. doi: 10.3389/fimmu.2018.02754.

Perry, J. S. A. *et al.* (2018) 'Transfer of Cell-Surface Antigens by Scavenger Receptor CD36 Promotes Thymic Regulatory T Cell Receptor Repertoire Development and Allo-tolerance.', *Immunity*. Elsevier, 48(5), pp. 923-936.e4. doi: 10.1016/j.immuni.2018.04.007.

Peters, C. *et al.* (2014) 'Phenotype and regulation of immunosuppressive Vδ2-expressing γδ T cells', *Cellular and Molecular Life Sciences*. Cell Mol Life Sci, 71(10), pp. 1943–1960. doi: 10.1007/s00018-013-1467-1.

Peters, C. *et al.* (2016) 'Human Vδ2 T cells are a major source of interleukin-9', *Proceedings of the National Academy of Sciences of the United States of America*. 2016/10/17. National Academy of Sciences, 113(44), pp. 12520–12525. doi: 10.1073/pnas.1607136113.

Peti, W. and Page, R. (2007) 'Strategies to maximize heterologous protein expression in Escherichia coli with minimal cost.', *Protein expression and purification*, 51(1), pp. 1–10. doi: 10.1016/j.pep.2006.06.024.

Petrie, H. T. *et al.* (1993) 'Multiple rearrangements in T cell receptor alpha chain genes maximize the production of useful thymocytes.', *The Journal of Experimental Medicine*. The Rockefeller University Press, 178(2), pp. 615–622. doi:

10.1084/jem.178.2.615.

Pettenati, C. and Ingersoll, M. A. (2018) 'Mechanisms of BCG immunotherapy and its outlook for bladder cancer', *Nature Reviews Urology*. Nature Publishing Group, 15(10), pp. 615–625. doi: 10.1038/s41585-018-0055-4.

Picker, L. J. *et al.* (1993) 'Control of lymphocyte recirculation in man. I. Differential regulation of the peripheral lymph node homing receptor L-selectin on T cells during the virgin to memory cell transition.', *Journal of immunology (Baltimore, Md. : 1950)*. J Immunol, 150(3), pp. 1105–21. Available at: http://www.ncbi.nlm.nih.gov/pubmed/7678616 (Accessed: 5 October 2020).

Pietschmann, K. *et al.* (2009) 'Toll-Like Receptor Expression and Function in Subsets of Human γδ T Lymphocytes', *Scandinavian Journal of Immunology*. Wiley, 70(3), pp. 245–255. doi: 10.1111/j.1365-3083.2009.02290.x.

Pittet, M. J. *et al.* (1999) 'High frequencies of naive Melan-A/MART-1-specific CD8+ T cells in a large proportion of human histocompatibility leukocyte antigen (HLA)-A2 individuals', *Journal of Experimental Medicine*, 190(5), pp. 705–715. doi: 10.1084/jem.190.5.705.

Platt, C. D. *et al.* (2010) 'Mature dendritic cells use endocytic receptors to capture and present antigens', *Proceedings of the National Academy of Sciences of the United States of America*, 107(9), pp. 4287–4292. doi: 10.1073/pnas.0910609107.

Poggi, A. *et al.* (2004) 'Vδ1 T Lymphocytes from B-CLL Patients Recognize ULBP3 Expressed on Leukemic B Cells and Up-Regulated by Trans-Retinoic Acid', *Cancer Research*. American Association for Cancer Research (AACR), 64(24), pp. 9172–9179. doi: 10.1158/0008-5472.can-04-2417.

Pollack, S. M. *et al.* (2014) 'Tetramer guided, cell sorter assisted production of clinical grade autologous NY-ESO-1 specific CD8+ T cells', *Journal for ImmunoTherapy of Cancer*. BioMed Central Ltd., 2(1), p. 36. doi: 10.1186/s40425-014-0036-y.

Pollok, K. E. *et al.* (1993) 'Inducible T cell antigen 4-1BB. Analysis of expression and function.', *Journal of immunology (Baltimore, Md. : 1950)*. American Association of Immunologists, 150(3), pp. 771–81. Available at: http://www.ncbi.nlm.nih.gov/pubmed/7678621 (Accessed: 1 March 2021).

Potts, W. K. and Slev, P. R. (1995) 'Pathogen-Based Models Favoring MHC Genetic Diversity', *Immunological Reviews*. Wiley, 143(1), pp. 181–197. doi: 10.1111/j.1600-065x.1995.tb00675.x.

Poupot, M., Pont, F. and Fournié, J.-J. (2005) 'Profiling blood lymphocyte interactions with cancer cells uncovers the innate reactivity of human gamma delta T cells to anaplastic large cell lymphoma.', *Journal of immunology (Baltimore, Md. : 1950)*. American Association of Immunologists, 174(3), pp. 1717–22. doi: 10.4049/jimmunol.174.3.1717.

Powell, D. J. *et al.* (2005) 'Transition of late-stage effector T cells to CD27+ CD28+ tumor-reactive effector memory T cells in humans after adoptive cell transfer therapy.', *Blood*. NIH Public Access, 105(1), pp. 241–50. doi: 10.1182/blood-2004-06-2482.

Pozzi, L.-A. M., Maciaszek, J. W. and Rock, K. L. (2005) 'Both Dendritic Cells and Macrophages Can Stimulate Naive CD8 T Cells In Vivo to Proliferate, Develop Effector Function, and Differentiate into Memory Cells', *The Journal of Immunology*. American Association of Immunologists, 175(4), pp. 2071–2081. doi: 10.4049/JIMMUNOL.175.4.2071.

Lo Presti, E., Dieli, F. and Meraviglia, S. (2014) 'Tumor-Infiltrating γδ T Lymphocytes: Pathogenic Role, Clinical Significance, and Differential Programing in the Tumor Microenvironment', *Frontiers in Immunology*. Frontiers, 5, p. 607. doi: 10.3389/fimmu.2014.00607.

Propper, D. J. *et al.* (2003) 'Low-Dose IFN-γ Induces Tumor MHC Expression in Metastatic Malignant Melanoma', *Clinical Cancer Research*, 9(1), pp. 84–92.

Puan, K.-J. *et al.* (2007) 'Preferential recognition of a microbial metabolite by human V 2V 2 T cells', *International Immunology*. Oxford University Press (OUP), 19(5), pp. 657–673. doi: 10.1093/intimm/dxm031.

Pufnock, J. S. *et al.* (2011) 'Priming CD8+ T cells with dendritic cells matured using TLR4 and TLR7/8 ligands together enhances generation of CD8+ T cells retaining CD28', *Blood*, 117(24), pp. 6542–6551. doi: 10.1182/blood-2010-11-317966.

Quintarelli, C. *et al.* (2018) 'Choice of costimulatory domains and of cytokines determines CAR T-cell activity in neuroblastoma', *Oncoimmunology*. Taylor & Francis, 7(6), pp. e1433518–e1433518. doi: 10.1080/2162402X.2018.1433518.

Raines, R. T. *et al.* (2000) '[23] The S·tag fusion system for protein purification', *Methods in Enzymology*. Academic Press, 326, pp. 362–376. doi: 10.1016/S0076-6879(00)26065-5.

Rajasekaran, K. *et al.* (2010) 'Functional Dichotomy between NKG2D and CD28-Mediated Co-Stimulation in Human CD8+ T Cells', *PLoS ONE*. Edited by D. Unutmaz. Public Library of Science, 5(9), p. e12635. doi: 10.1371/journal.pone.0012635.

Raulet, D. H. and Guerra, N. (2009) 'Oncogenic stress sensed by the immune system: role of natural killer cell receptors', *Nature reviews. Immunology*. Nature Publishing Group, 9(8), pp. 568–580. doi: 10.1038/nri2604.

Ravens, S. *et al.* (2020) 'Microbial exposure drives polyclonal expansion of innate γδ T cells immediately after birth', *Proceedings of the National Academy of Sciences of the United States of America*, 117(31), pp. 18649–18660. doi: 10.1073/pnas.1922588117.

Reinicke, A. T. *et al.* (2019) 'Deubiquitinating Enzyme UCH-L1 Promotes Dendritic Cell Antigen Cross-Presentation by Favoring Recycling of MHC Class I Molecules', *The Journal of Immunology*. American Association of Immunologists, 203(7), pp. 1730–1742. doi: 10.4049/jimmunol.1801133.

Reynolds, J. M. *et al.* (2012) 'Toll-like receptor 4 signaling in T cells promotes autoimmune inflammation.', *Proceedings of the National Academy of Sciences of the United States of America*. Proc Natl Acad Sci U S A, 109(32), pp. 13064–9. doi: 10.1073/pnas.1120585109.

Reynolds, J. M. and Dong, C. (2013) 'Toll-like receptor regulation of effector T lymphocyte function', *Trends in Immunology*. Elsevier BV, 34(10), pp. 511–519. doi: 10.1016/j.it.2013.06.003.

Rhodes, D. A. *et al.* (2015) 'Activation of human γδ T cells by cytosolic interactions of BTN3A1 with soluble phosphoantigens and the cytoskeletal adaptor periplakin', *Journal of immunology (Baltimore, Md. : 1950)*. 2015/01/30. AAI, 194(5), pp. 2390–2398. doi: 10.4049/jimmunol.1401064.

Rhodes, D. A. *et al.* (2018) 'Regulation of Human γδ T Cells by BTN3A1 Protein Stability and ATP-Binding Cassette Transporters', *Frontiers in Immunology*. Frontiers, 9, p. 662. doi: 10.3389/fimmu.2018.00662.

Rhodes, D. A., de Bono, B. and Trowsdale, J. (2005) 'Relationship between SPRY and B30.2 protein domains. Evolution of a component of immune defence?', *Immunology*. John Wiley & Sons, Ltd, 0(0), p. 051025020346011. doi: 10.1111/j.1365-2567.2005.02248.x.

Ribas, A. and Wolchok, J. D. (2018) 'Cancer immunotherapy using checkpoint blockade', *Science*, 359(6382), pp. 1350–1355. doi: 10.1126/science.aar4060.

Ribeiro, S. T. S. T., Ribot, J. C. and Silva-Santos, B. (2015) 'Five Layers of Receptor Signaling in γδ T-Cell Differentiation and Activation', *Frontiers in Immunology*. Frontiers, 6, p. 15. doi: 10.3389/fimmu.2015.00015.

Ribot, J. C. *et al.* (2010) 'Cutting edge: adaptive versus innate receptor signals selectively control the pool sizes of murine IFN-γ- or IL-17-producing γδ T cells upon infection', *Journal of immunology (Baltimore, Md. : 1950)*. 2010/10/29, 185(11), pp. 6421–6425. doi: 10.4049/jimmunol.1002283.

Ribot, J. C. *et al.* (2012) 'B7–CD28 Costimulatory Signals Control the Survival and Proliferation of Murine and Human γδ T Cells via IL-2 Production', *The Journal of Immunology*. The American Association of Immunologists, 189(3), pp. 1202–1208. doi: 10.4049/jimmunol.1200268.

Ribot, J. C. *et al.* (2014) 'Human γδ thymocytes are functionally immature and differentiate into cytotoxic type 1 effector T cells upon IL-2/IL-15 signaling.', *Journal of immunology (Baltimore, Md. : 1950)*. American Association of Immunologists, 192(5), pp. 2237–43. doi: 10.4049/jimmunol.1303119.

Ribot, J. C., deBarros, A. and Silva-Santos, B. (2011) 'Searching for "signal 2": costimulation requirements of γδ T cells', *Cellular and Molecular Life Sciences*. Springer Science and Business Media LLC, 68(14), pp. 2345–2355. doi: 10.1007/s00018-011-0698-2.

Ridnour, L. A. *et al.* (2013) 'Molecular pathways: toll-like receptors in the tumor microenvironment--poor prognosis or new therapeutic opportunity', *Clinical cancer research : an official journal of the American Association for Cancer Research*. 2012/12/27, 19(6), pp. 1340–1346. doi: 10.1158/1078-0432.CCR-12-0408.

Riganti, C. *et al.* (2012) 'Human γδ T-cell responses in infection and immunotherapy: common mechanisms, common mediators?', *European journal of immunology*. Eur J Immunol, 42(7), pp. 1668–76. doi: 10.1002/eji.201242492.

Rigau, M. *et al.* (2020) 'Butyrophilin 2A1 is essential for phosphoantigen reactivity by γδ T cells.', *Science (New York, N.Y.)*. American Association for the Advancement of Science, 367(6478). doi: 10.1126/science.aay5516.

Rincon-Orozco, B. *et al.* (2005) 'Activation of Vγ9Vδ2 T Cells by NKG2D', *The Journal of Immunology*. The American Association of Immunologists, 175(4), pp. 2144–2151. doi: 10.4049/jimmunol.175.4.2144.

Ring, A. M. *et al.* (2012) 'Mechanistic and structural insight into the functional dichotomy between IL-2 and IL-15', *Nature Immunology*. Nature Publishing Group, 13(12), pp. 1187–1195. doi: 10.1038/ni.2449.

Rius, C. *et al.* (2018) 'Peptide–MHC Class I Tetramers Can Fail To Detect Relevant Functional T Cell Clonotypes and Underestimate Antigen-Reactive T Cell Populations', *The Journal of Immunology*, 200(7), pp. 2263–2279. doi: 10.4049/jimmunol.1700242.

Rivoltini, L. *et al.* (1995) 'Induction of tumor-reactive CTL from peripheral blood and tumor- infiltrating lymphocytes of melanoma patients by in vitro stimulation with an immunodominant peptide of the human melanoma antigen MART-1', *Journal of immunology*, 154(5), pp. 2257–65. Available at: https://keio.pure.elsevier.com/en/publications/induction-of-tumor-reactive-ctl-from-peripheral-blood-and-tumor-i.

Robbins, P. F. *et al.* (2008) 'Single and Dual Amino Acid Substitutions in TCR CDRs Can Enhance Antigen-Specific T Cell Functions', *The Journal of Immunology*. The American Association of Immunologists, 180(9), pp. 6116–6131. doi: 10.4049/jimmunol.180.9.6116.

Roberto, A. *et al.* (2015) 'Role of naive-derived T memory stem cells in T-cell reconstitution following allogeneic transplantation', *Blood*. American Society of Hematology, 125(18), pp. 2855–2864. doi: 10.1182/blood-2014-11-608406.

Robinson, J. and Marsh, S. G. E. E. (2003) 'HLA Informatics: Accessing HLA Sequences from Sequence Databases', *MHC Protocols*. Humana Press, 210, pp. 3–22. doi: 10.1385/1-59259-291-0:03.

Robinson, M. S. (1994) 'The role of clathrin, adaptors and dynamin in endocytosis', *Current Opinion in Cell Biology*. Elsevier Current Trends, 6(4), pp. 538–544. doi: 10.1016/0955-0674(94)90074-4.

Rock, E. P. *et al.* (1994) 'CDR3 length in antigen-specific immune receptors.', *Journal of Experimental Medicine*. The Rockefeller University Press, 179(1), pp. 323–8. doi: 10.1084/jem.179.1.323.

Rock, K. L. and Goldberg, A. L. (1999) 'DEGRADATION OF CELL PROTEINS AND THE GENERATION OF MHC CLASS I-PRESENTED PEPTIDES', *Annual Review of Immunology*. Annual Reviews 4139 El Camino Way, P.O. Box 10139, Palo Alto, CA 94303-0139, USA , 17(1), pp. 739–779. doi: 10.1146/annurev.immunol.17.1.739.

Rodríguez-Pinto, D. (2005) 'B cells as antigen presenting cells', *Cellular Immunology*. Academic Press, 238(2), pp. 67–75. doi: 10.1016/J.CELLIMM.2006.02.005.

Roelofs, A. J. *et al.* (2009) 'Peripheral blood monocytes are responsible for γδ T cell activation induced by zoledronic acid through accumulation of IPP/DMAPP', *British Journal of Haematology*, 144(2), pp. 245–250. doi: 10.1111/j.1365-2141.2008.07435.x.

Rohaan, M. W. *et al.* (2018) 'Adoptive transfer of tumor-infiltrating lymphocytes in melanoma: a viable treatment option', *Journal for immunotherapy of cancer*. BioMed Central, 6(1), p. 102. doi: 10.1186/s40425-018-0391-1.

Romero, P. *et al.* (2007) 'Four Functionally Distinct Populations of Human Effector-Memory CD8 + T Lymphocytes', *The Journal of Immunology*. American Association of Immunologists, 178(7), pp. 4112–4119. doi: 10.4049/jimmunol.178.7.4112.

De Rosa, S. C. *et al.* (2004) 'Ontogeny of gamma delta T cells in humans.', *Journal of immunology (Baltimore, Md. : 1950)*. American Association of Immunologists, 172(3), pp. 1637–45. doi: 10.4049/jimmunol.172.3.1637.

Rosenberg, S. A. *et al.* (1985) 'Observations on the systemic administration of autologous lymphokine-activated killer cells and recombinant interleukin-2 to patients with metastatic cancer.', *The New England journal of medicine*. United States, 313(23), pp. 1485–1492. doi: 10.1056/NEJM198512053132327.

Rosenberg, S. A., Yang, J. C. and Restifo, N. P. (2004) 'Cancer immunotherapy: moving beyond current vaccines', *Nature Medicine*. Springer Science and Business Media LLC, 10(9), pp. 909–915. doi: 10.1038/nm1100.

Rowe, J. and Cen, P. (2014) 'TroVax in colorectal cancer.', *Human vaccines & immunotherapeutics*. Taylor & Francis, 10(11), pp. 3196–200. doi: 10.4161/21645515.2014.973323.

Rowshanravan, B., Halliday, N. and Sansom, D. M. (2018) 'CTLA-4: a moving target in immunotherapy.', *Blood*. Blood, 131(1), pp. 58–67. doi: 10.1182/blood-2017-06-741033.

Roy, S. *et al.* (2011) 'Defective dendritic cell generation from monocytes is a potential reason for poor therapeutic efficacy of interferon α2b (IFNα2b) in cervical cancer.', *Translational research : the journal of laboratory and clinical medicine*. Transl Res, 158(4), pp. 200–13. doi: 10.1016/j.trsl.2011.03.003.

Rudolph, M. G., Stanfield, R. L. and Wilson, I. A. (2006) 'HOW TCRS BIND MHCS, PEPTIDES, AND CORECEPTORS', *Annual Review of Immunology*. Annual Reviews, 24(1), pp. 419–466. doi: 10.1146/annurev.immunol.23.021704.115658.

Rufer, N. *et al.* (2003) 'Ex vivo characterization of human CD8+ T subsets with distinct replicative history and partial effector functions', *Blood*. American Society of Hematology, 102(5), pp. 1779–1787. doi: 10.1182/blood-2003-02-0420.

Ryan, B. J. *et al.* (2013) 'Overview of Approaches to Preventing and Avoiding Proteolysis During Expression and Purification of Proteins', in *Current Protocols in Protein Science*. Hoboken, NJ, USA, NJ, USA: John Wiley & Sons, Inc., pp. 5.25.1-5.25.7. doi: 10.1002/0471140864.ps0525s71.

Ryan, P. L. *et al.* (2016) 'Heterogeneous yet stable Vδ2(+) T-cell profiles define distinct cytotoxic effector potentials in healthy human individuals', *Proceedings of the National Academy of Sciences of the United States of America*. 2016/11/28. National Academy of Sciences, 113(50), pp. 14378–14383. doi: 10.1073/pnas.1611098113.

Saeterdal, I. *et al.* (2001) 'Frameshift-mutation-derived peptides as tumor-specific antigens in inherited and spontaneous colorectal cancer', *Proceedings of the National Academy of Sciences of the United States of America*. 2001/10/30. The National Academy of Sciences, 98(23), pp. 13255–13260. doi: 10.1073/pnas.231326898.

Sakamoto, M. *et al.* (2011) 'Adoptive immunotherapy for advanced non-small cell lung cancer using zoledronate-expanded γδTcells: a phase I clinical study.', *Journal of immunotherapy (Hagerstown, Md. : 1997)*. J Immunother, 34(2), pp. 202–11. doi: 10.1097/CJI.0b013e318207ecfb.

Salgaller, M. L. *et al.* (1996) 'Immunization against epitopes in the human melanoma antigen gp100 following patient immunization with synthetic peptides', *Cancer Research*, 56(20), pp. 4749–4757.

Salimu, J. *et al.* (2015) 'Cross-Presentation of the Oncofetal Tumor Antigen 5T4 from Irradiated Prostate Cancer Cells--A Key Role for Heat-Shock Protein 70 and Receptor CD91', *Cancer Immunology Research*. American Association for Cancer Research, 3(6), pp. 678–688. doi: 10.1158/2326-6066.CIR-14-0079.

Sallusto, F. *et al.* (1999) 'Two subsets of memory T lymphocytes with distinct homing potentials and effector functions', *Nature*, 401(6754), pp. 708–712. doi: 10.1038/44385.

Sallusto, F., Geginat, J. and Lanzavecchia, A. (2004) 'Central Memory and Effector Memory T Cell Subsets: Function, Generation, and Maintenance', *Annual Review of Immunology*. Annual Reviews, 22(1), pp. 745–763. doi: 10.1146/annurev.immunol.22.012703.104702.

Sallusto, F. and Lanzavecchia, A. (1994) 'Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha.', *The Journal of Experimental Medicine*, 179(4), pp. 1109–1118. doi: 10.1084/jem.179.4.1109.

Salter, R. D. and Cresswell, P. (1986) 'Impaired assembly and transport of HLA-A and -B antigens in a mutant TxB cell hybrid.', *The EMBO journal*. European Molecular Biology Organization, 5(5), pp. 943–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/3522223 (Accessed: 15 March 2019).

Salter, R. D., Howell, D. N. and Cresswell, P. (1985) 'Genes regulating HLA class I antigen expression in T-B lymphoblast hybrids', *Immunogenetics*, 21(3), pp. 235–246. doi: 10.1007/BF00375376.

Sánchez-Paulete, A. R. *et al.* (2017) 'Antigen cross-presentation and T-cell cross-priming in cancer immunology and

immunotherapy', *Annals of Oncology*. Narnia, 28(suppl_12), pp. xii44–xii55. doi: 10.1093/annonc/mdx237.

Sanders, M. E., Makgoba, M. W. and Shaw, S. (1988) 'Human naive and memory T cells: reinterpretation of helper-inducer and suppressor-inducer subsets', *Immunology Today*. Elsevier Current Trends, 9(7–8), pp. 195–199. doi: 10.1016/0167-5699(88)91212-1.

Sandstrom, A. *et al.* (2014) 'The intracellular B30.2 domain of butyrophilin 3A1 binds phosphoantigens to mediate activation of human Vγ9Vδ2 T cells', *Immunity*. 2014/04/03, 40(4), pp. 490–500. doi: 10.1016/j.immuni.2014.03.003.

Santin, A. D. *et al.* (1996) 'Effects of irradiation on the expression of surface antigens in human ovarian cancer', *Gynecologic Oncology*, 60(3), pp. 468–474. doi: 10.1006/gyno.1996.0075.

Saxena, M. *et al.* (2018) 'Towards superior dendritic-cell vaccines for cancer therapy', *Nature biomedical engineering*. 2018/06/11, 2(6), pp. 341–346. doi: 10.1038/s41551-018-0250-x.

Saxena, M. and Bhardwaj, N. (2017) 'Turbocharging vaccines: emerging adjuvants for dendritic cell based therapeutic cancer vaccines', *Current opinion in immunology*. 2017/07/18. Elsevier Current Trends, 47, pp. 35–43. doi: 10.1016/j.coi.2017.06.003.

Saxena, M. and Bhardwaj, N. (2018) 'Re-Emergence of Dendritic Cell Vaccines for Cancer Treatment', *Trends in cancer*. Elsevier Inc., 4(2), pp. 119–137. doi: 10.1016/j.trecan.2017.12.007.

Schäfer, M. and Werner, S. (2008) 'Cancer as an overhealing wound: an old hypothesis revisited', *Nature Reviews Molecular Cell Biology*. Springer Science and Business Media LLC, 9(8), pp. 628–638. doi: 10.1038/nrm2455.

Schein, C. H. (1991) 'Optimizing protein folding to the native state in bacteria', *Current Opinion in Biotechnology*. Elsevier Current Trends, 2(5), pp. 746–750. doi: 10.1016/0958-1669(91)90046-8.

Schilbach, K. *et al.* (2020) 'Suppressive activity of Vδ2 + γδ T cells on αβ T cells is licensed by TCR signaling and correlates with signal strength', *Cancer Immunology, Immunotherapy*. Springer Berlin Heidelberg, 69(4), pp. 593–610. doi: 10.1007/s00262-019-02469-8.

Schmidt, M. E. and Varga, S. M. (2018) 'The CD8 T cell response to respiratory virus infections', *Frontiers in Immunology*, 9(APR). doi: 10.3389/fimmu.2018.00678.

Schneiders, F. L. *et al.* (2014) 'CD1d-Restricted Antigen Presentation by V 9V 2-T Cells Requires Trogocytosis', *Cancer Immunology Research*. American Association for Cancer Research, 2(8), pp. 732–740. doi: 10.1158/2326-6066.CIR-13-0167.

Schnurr, M. *et al.* (2005) 'Tumor antigen processing and presentation depend critically on dendritic cell type and the mode of antigen delivery'. doi: 10.1182/blood- 2004-08-3105.

Schodin, B. A., Tsomides, T. J. and Kranz, D. M. (1996) 'Correlation between the number of T cell receptors required for T cell activation and TCR-ligand affinity', *Immunity*, 5(2), pp. 137–146. doi: 10.1016/S1074-7613(00)80490-2.

Scholtalbers, J. *et al.* (2015) 'TCLP: an online cancer cell line catalogue integrating HLA type, predicted neo-epitopes, virus and gene expression', *Genome Medicine*. BioMed Central, 7(1), p. 118. doi: 10.1186/s13073-015-0240-5.

Schubert, U. *et al.* (2000) 'Rapid degradation of a large fraction of newly synthesized proteins by proteasomes', *Nature*, 404(6779), pp. 770–774. doi: 10.1038/35008096.

Schuijs, M. J., Hammad, H. and Lambrecht, B. N. (2019) 'Professional and "Amateur" Antigen-Presenting Cells In Type 2 Immunity', *Trends in Immunology*. Elsevier Current Trends, 40(1), pp. 22–34. doi: 10.1016/J.IT.2018.11.001.

Schultz-Thater, E. *et al.* (2000) 'NY-ESO-1 tumour associated antigen is a cytoplasmic protein detectable by specific monoclonal antibodies in cell lines and clinical specimens.', *British journal of cancer*. Nature Publishing Group, 83(2), pp. 204–208. doi: 10.1054/bjoc.2000.1251.

Schwacha, M. G. *et al.* (2013) 'Mitochondrial damage-associated molecular patterns activate γδ T-cells', *Innate Immunity*. SAGE Publications, 20(3), pp. 261–268. doi: 10.1177/1753425913488969.

Schwacha, M. G. and Daniel, T. (2008) 'Up-regulation of cell surface Toll-like receptors on circulating gammadelta T-cells following burn injury.', *Cytokine*. Cytokine, 44(3), pp. 328–34. doi: 10.1016/j.cyto.2008.09.001.

Schwartz, R. H. (1992) 'Costimulation of T lymphocytes: the role of CD28, CTLA-4, and B7/BB1 in interleukin-2 production and immunotherapy.', *Cell*. Cell, 71(7), pp. 1065–8. doi: 10.1016/s0092-8674(05)80055-8.

Scotton, C. J. *et al.* (2001) 'Epithelial cancer cell migration: A role for chemokine receptors?', *Cancer Research*, 61(13), pp. 4961–4965.

Scurr, M. *et al.* (2017) 'MVA-5T4 immunotherapy and low-dose cyclophosphamide for advanced colorectal cancer (TaCTiCC): An open-label, randomized phase I/II trial.', in *MVA-5T4 immunotherapy and low-dose cyclophosphamide for advanced colorectal cancer (TaCTiCC): An open-label, randomized phase I/II trial.* ASCO Journals. Available at: http://meetinglibrary.asco.org/record/140965/abstract (Accessed: 27 June 2017).

Sebestyen, Z. *et al.* (2016) 'RhoB Mediates Phosphoantigen Recognition by Vγ9Vδ2 T Cell Receptor', *Cell Reports*. Cell Press, 15(9), pp. 1973–1985. Available at: http://linkinghub.elsevier.com/retrieve/pii/S2211124716305265 (Accessed: 6 July 2017).

Sebestyen, Z. *et al.* (2020) 'Translating gammadelta (γδ) T cells and their receptors into cancer cell therapies', *Nature Reviews Drug Discovery*. Nature Publishing Group, 19(3), pp. 169–184. doi: 10.1038/s41573-019-0038-z.

Serrano, R., Wesch, D. and Kabelitz, D. (2020) 'Activation of Human γδ T Cells: Modulation by Toll-Like Receptor 8 Ligands and Role of Monocytes', *Cells*. Multidisciplinary Digital Publishing Institute, 9(3), p. 713. doi: 10.3390/cells9030713.

Shao, Z. and Schwarz, H. (2010) 'CD137 ligand, a member of the tumor necrosis factor family, regulates immune responses via reverse signal transduction', *Journal of Leukocyte Biology*. Wiley, 89(1), pp. 21–29. doi: 10.1189/jlb.0510315.

Shayan, G. *et al.* (2018) 'Phase Ib Study of Immune Biomarker Modulation with Neoadjuvant Cetuximab and TLR8 Stimulation in Head and Neck Cancer to Overcome Suppressive Myeloid Signals', *Clinical cancer research : an official journal of the American Association for Cancer Research*. 2017/10/23, 24(1), pp. 62–72. doi: 10.1158/1078-0432.CCR-17-0357.

Shen, H. *et al.* (2006) 'Enhanced and prolonged cross-presentation following endosomal escape of exogenous antigens encapsulated in biodegradable nanoparticles', *Immunology*. Wiley/Blackwell (10.1111), 117(1), pp. 78–88. doi: 10.1111/j.1365-2567.2005.02268.x.

Shingler, W. H. *et al.* (2008) 'Identification and functional validation of MHC class I epitopes in the tumor-associated antigen 5T4', *International Immunology*, 20(8), pp. 1057–1066. doi: 10.1093/intimm/dxn063.

Shires, J., Theodoridis, E. and Hayday, A. C. (2001) 'Biological insights into TCRgammadelta+ and TCRalphabeta+ intraepithelial lymphocytes provided by serial analysis of gene expression (SAGE).', *Immunity*. Elsevier, 15(3), pp. 419–34. doi: 10.1016/s1074-7613(01)00192-3.

Shojaei, H. *et al.* (2009) 'Toll-like receptors 3 and 7 agonists enhance tumor cell lysis by human gammadelta T cells.', *Cancer research*. Cancer Res, 69(22), pp. 8710–7. doi: 10.1158/0008-5472.CAN-09-1602.

Siemann, D. W. (2010) *Tumor Microenvironment*, *Tumor Microenvironment*. Edited by D. W. Siemann. Chichester, UK: John Wiley & Sons, Ltd. doi: 10.1002/9780470669891.

Silva-Santos, B., Serre, K. and Norell, H. (2015) 'γδ T cells in cancer', *Nature Reviews Immunology*. Nature Publishing Group, 15(11), pp. 683–691. doi: 10.1038/nri3904.

Silva-Santos, B. and Strid, J. (2018) 'Working in "NK Mode": Natural Killer Group 2 Member D and Natural Cytotoxicity Receptors in Stress-Surveillance by γδ T Cells', *Frontiers in Immunology*. Frontiers, 9, p. 851. doi: 10.3389/fimmu.2018.00851.

Silverstein, R. L. and Febbraio, M. (2009) 'CD36, a scavenger receptor involved in immunity, metabolism, angiogenesis, and behavior.', *Science signaling*. NIH Public Access, 2(72), p. re3. doi: 10.1126/scisignal.272re3.

Simpson, A. J. G. *et al.* (2005) 'Cancer/testis antigens, gametogenesis and cancer', *Nature Reviews Cancer*. Springer Science and Business Media LLC, 5(8), pp. 615–625. doi: 10.1038/nrc1669.

Sivashanmugam, A. *et al.* (2009) 'Practical protocols for production of very high yields of recombinant proteins using *Escherichia coli*', *Protein Science*. Wiley Subscription Services, Inc., A Wiley Company, 18(5), pp. 936–948. doi: 10.1002/pro.102.

Smith, S. M. (2011) 'Strategies for the Purification of Membrane Proteins', in. Humana Press, pp. 485–496. doi: 10.1007/978-1-

60761-913-0_29.

Smits, E. L. J. M. *et al.* (2008) 'The Use of TLR7 and TLR8 Ligands for the Enhancement of Cancer Immunotherapy', *The Oncologist*. Wiley, 13(8), pp. 859–875. doi: 10.1634/theoncologist.2008-0097.

Smyth, L. J. *et al.* (2006) 'Cd8 T-cell recognition of human 5T4 oncofetal antigen', *International Journal of Cancer*. John Wiley & Sons, Ltd, 119(7), pp. 1638–1647. doi: 10.1002/ijc.22018.

Snauwaert, S. *et al.* (2014) 'In vitro generation of mature, naive antigen-specific CD8+ T cells with a single T-cell receptor by agonist selection', *Leukemia*. Nature Publishing Group, 28(4), pp. 830–841. doi: 10.1038/leu.2013.285.

Somerville, R. P. T. *et al.* (2012) 'Clinical scale rapid expansion of lymphocytes for adoptive cell transfer therapy in the WAVE® bioreactor', *Journal of Translational Medicine*. Springer Science and Business Media LLC, 10(1), p. 69. doi: 10.1186/1479-5876-10-69.

Sørensen, Hans *et al.* (2005) 'Soluble expression of recombinant proteins in the cytoplasm of Escherichia coli', *Microbial Cell Factories*. BioMed Central, 4(1), p. 1. doi: 10.1186/1475-2859-4-1.

Sorkin, A. (2004) 'Cargo recognition during clathrin-mediated endocytosis: a team effort', *Current Opinion in Cell Biology*. Elsevier BV, 16(4), pp. 392–399. doi: 10.1016/j.ceb.2004.06.001.

Southall, P. J. *et al.* (1990) 'Immunohistological distribution of 5T4 antigen in normal and malignant tissues', *British Journal of Cancer*. Nature Publishing Group, 61(1), pp. 89–95. doi: 10.1038/bjc.1990.20.

Spear, T. T. *et al.* (2019) 'Understanding TCR affinity, antigen specificity, and cross-reactivity to improve TCR gene-modified T cells for cancer immunotherapy', *Cancer Immunology, Immunotherapy*. Springer, 68(11), pp. 1881–1889. doi: 10.1007/s00262-019-02401-0.

Spranger, S., Bao, R. and Gajewski, T. F. (2015) 'Melanoma-intrinsic β-catenin signalling prevents anti-tumour immunity', *Nature*. Springer Science and Business Media LLC, 523(7559), pp. 231–235. doi: 10.1038/nature14404.

Sprent, J. (1995) 'Antigen-presenting cells. Professionals and amateurs.', *Current biology : CB*. Elsevier, 5(10), pp. 1095–7. doi: 10.1016/s0960-9822(95)00219-3.

Spriestersbach, A. *et al.* (2015) 'Purification of His-Tagged Proteins'. doi: 10.1016/bs.mie.2014.11.003.

Starzynska, T., Rahi, V. and Stern, P. L. (1992) 'The expression of 5T4 antigen in colorectal and gastric carcinoma.', *British journal of cancer*. Nature Publishing Group, 66(5), pp. 867–9. doi: 10.1038/bjc.1992.375.

Steinbach, K., Vincenti, I. and Merkler, D. (2018) 'Resident-Memory T Cells in Tissue-Restricted Immune Responses: For Better or Worse?', *Frontiers in Immunology*. Frontiers, 9, p. 2827. doi: 10.3389/fimmu.2018.02827.

Steinle, A. and Schendel, D. J. (1994) 'HLA class I alleles of LCL 721 and 174XCEM.T2 (T2)', *Tissue Antigens*. John Wiley & Sons, Ltd (10.1111), 44(4), pp. 268–270. doi: 10.1111/j.1399-0039.1994.tb02394.x.

Stern, P. L. *et al.* (2014) 'Understanding and exploiting 5T4 oncofoetal glycoprotein expression', *Seminars in Cancer Biology*. Academic Press, 29, pp. 13–20. doi: 10.1016/j.semcancer.2014.07.004.

Stockert, E. *et al.* (1998) 'A Survey of the Humoral Immune Response of Cancer Patients to a Panel of Human Tumor Antigens', *Journal of Experimental Medicine*. The Rockefeller University Press, 187(8), pp. 1349–1354. doi: 10.1084/jem.187.8.1349.

Stone, J. D. *et al.* (2011) 'Opposite Effects of Endogenous Peptide–MHC Class I on T Cell Activity in the Presence and Absence of CD8', *The Journal of Immunology*, 186(9), pp. 5193–5200. doi: 10.4049/jimmunol.1003755.

Stone, J. D., Chervin, A. S. and Kranz, D. M. (2009) 'T-cell receptor binding affinities and kinetics: impact on T-cell activity and specificity', *Immunology*, 126(2), pp. 165–176. doi: 10.1111/j.1365-2567.2008.03015.x.

Stone, J. D., Harris, D. T. and Kranz, D. M. (2015) 'TCR affinity for p/MHC formed by tumor antigens that are self-proteins: impact on efficacy and toxicity', *Current Opinion in Immunology*, 33, pp. 16–22. doi: 10.1016/j.coi.2015.01.003.

Strid, J. *et al.* (2008) 'Acute upregulation of an NKG2D ligand promotes rapid reorganization of a local immune compartment with pleiotropic effects on carcinogenesis', *Nature Immunology*. Springer Science and Business Media LLC, 9(2), pp. 146–154.

doi: 10.1038/ni1556.

Su, Z. *et al.* (2015) 'Apoptosis, autophagy, necroptosis, and cancer metastasis', *Molecular Cancer*. BioMed Central, 14(1), p. 48. doi: 10.1186/s12943-015-0321-5.

Swann, J. B. and Smyth, M. J. (2007) 'Immune surveillance of tumors.', *The Journal of clinical investigation*. American Society for Clinical Investigation, 117(5), pp. 1137–46. doi: 10.1172/JCI31405.

Szmania, S., Tricot, G. and van Rhee, F. (2006) 'NY-ESO-1 immunotherapy for multiple myeloma', *Leukemia & Lymphoma*. Taylor & Francis, 47(10), pp. 2037–2048. doi: 10.1080/10428190600742292.

Takata, H. and Takiguchi, M. (2006) 'Three Memory Subsets of Human CD8+ T Cells Differently Expressing Three Cytolytic Effector Molecules', *The Journal of Immunology*. American Association of Immunologists, 177(7), pp. 4330–4340. doi: 10.4049/JIMMUNOL.177.7.4330.

Tan, M. P. *et al.* (2015) 'T cell receptor binding affinity governs the functional profile of cancer-specific CD8+ T cells', *Clinical and Experimental Immunology*. Blackwell Publishing Ltd, 180(2), pp. 255–270. doi: 10.1111/cei.12570.

Tanaka, Yoshimasa *et al.* (1995) 'Natural and synthetic non-peptide antigens recognized by human γδ T cells', *Nature*. Nature Publishing Group, 375(6527), pp. 155–158. doi: 10.1038/375155a0.

Tanyi, J. L. *et al.* (2018) 'Personalized cancer vaccine effectively mobilizes antitumor T cell immunity in ovarian cancer', *Science Translational Medicine*. American Association for the Advancement of Science (AAAS), 10(436), p. eaao5931. doi: 10.1126/scitranslmed.aao5931.

Tao, H. *et al.* (2010) 'Purifying natively folded proteins from inclusion bodies using sarkosyl, Triton X-100, and CHAPS', 48(1), pp. 61–64. doi: 10.2144/000113304.

Tassone, P. *et al.* (1998) 'CD36 is rapidly and transiently upregulated on phytohemagglutinin (PHA)-stimulated peripheral blood lymphocytes. Analysis by a new monoclonal antibody (UN7)', *Tissue Antigens*, 51(6), pp. 671–675. doi: 10.1111/j.1399-0039.1998.tb03013.x.

Theaker, S. M. *et al.* (2016) 'T-cell libraries allow simple parallel generation of multiple peptide-specific human T-cell clones', *Journal of Immunological Methods*. The Authors, 430, pp. 43–50. doi: 10.1016/j.jim.2016.01.014.

Thedrez, A. *et al.* (2009) 'IL-21-Mediated Potentiation of Antitumor Cytolytic and Proinflammatory Responses of Human Vγ9Vδ2 T Cells for Adoptive Immunotherapy', *The Journal of Immunology*. The American Association of Immunologists, 182(6), pp. 3423–3431. doi: 10.4049/jimmunol.0803068.

Thompson, K., Rojas-Navea, J. and Rogers, M. J. (2006) 'Alkylamines cause Vγ9Vδ2 T-cell activation and proliferation by inhibiting the mevalonate pathway', *Blood*. American Society of Hematology, 107(2), pp. 651–654. doi: 10.1182/blood-2005-03-1025.

Tian, Y. and Zajac, A. J. (2016) 'IL-21 and T Cell Differentiation: Consider the Context', *Trends in Immunology*. Elsevier Ltd, 37(8), pp. 557–568. doi: 10.1016/j.it.2016.06.001.

Timp, W. and Feinberg, A. P. (2013) 'Cancer as a dysregulated epigenome allowing cellular growth advantage at the expense of the host', *Nature reviews. Cancer*. 2013/06/13, 13(7), pp. 497–510. doi: 10.1038/nrc3486.

Todaro, M. *et al.* (2013) 'Combining conventional chemotherapy and γδ T cell-based immunotherapy to target cancer-initiating cells', *Oncoimmunology*. 2013/07/29. Landes Bioscience, 2(9), pp. e25821–e25821. doi: 10.4161/onci.25821.

Tomiyama, H. *et al.* (2004) 'Phenotypic classification of human CD8+ T cells reflecting their function: Inverse correlation between quantitative expression of CD27 and cytotoxic effector function', *European Journal of Immunology*. John Wiley & Sons, Ltd, 34(4), pp. 999–1010. doi: 10.1002/eji.200324478.

Tomiyama, H., Matsuda, T. and Takiguchi, M. (2002) 'Differentiation of Human CD8 + T Cells from a Memory to Memory/Effector Phenotype', *The Journal of Immunology*. American Association of Immunologists, 168(11), pp. 5538–5550. doi: 10.4049/jimmunol.168.11.5538.

Tonegawa, S. *et al.* (1989) 'Diversity, Development, Ligands, and Probable Functions of gammadelta T Cells', *Cold Spring*

Harbor Symposia on Quantitative Biology. Cold Spring Harbor Laboratory Press, 54, pp. 31–44. doi: 10.1101/SQB.1989.054.01.005.

Tosolini, M. *et al.* (2017) 'Assessment of tumor-infiltrating TCRVγ9Vδ2 γδ lymphocyte abundance by deconvolution of human cancers microarrays', *OncoImmunology*. Taylor & Francis, 6(3), pp. 1–10. doi: 10.1080/2162402X.2017.1284723.

Tran, K. Q. *et al.* (2008) 'Minimally Cultured Tumor-infiltrating Lymphocytes Display Optimal Characteristics for Adoptive Cell Therapy', *Journal of Immunotherapy*. Ovid Technologies (Wolters Kluwer Health), 31(8), pp. 742–751. doi: 10.1097/cji.0b013e31818403d5.

Treilleux, I. *et al.* (2004) 'Dendritic Cell Infiltration and Prognosis of Early Stage Breast Cancer', *Clinical Cancer Research*. American Association for Cancer Research (AACR), 10(22), pp. 7466–7474. doi: 10.1158/1078-0432.ccr-04-0684.

Triantafilou, M. *et al.* (2006) 'Membrane Sorting of Toll-like Receptor (TLR)-2/6 and TLR2/1 Heterodimers at the Cell Surface Determines Heterotypic Associations with CD36 and Intracellular Targeting', *Journal of Biological Chemistry*. JBC Papers in Press, 281(41), pp. 31002–31011. doi: 10.1074/jbc.M602794200.

Trombetta, E. S. and Mellman, I. (2005) 'Cell Biology of Antigen Processing in Vitro and in Vivo', *Annual Review of Immunology*. Annual Reviews, 23(1), pp. 975–1028. doi: 10.1146/annurev.immunol.22.012703.104538.

Troutt, A. B. and Kelso, A. (1992) 'Enumeration of lymphokine mRNA-containing cells in vivo in a murine graft-versus-host reaction using the PCR.', 89(12). doi: 10.1073/pnas.89.12.5276.

Tsang, K. Y., Jochems, C. and Schlom, J. (2015) 'Insights on Peptide Vaccines in Cancer Immunotherapy', *Cancer Drug Discovery and Development*. Springer International Publishing, pp. 1–27. doi: 10.1007/978-3-319-21167-1_1.

Tsuda, J. *et al.* (2011) 'Involvement of CD56brightCD11c+ Cells in IL-18–Mediated Expansion of Human γδ T Cells', *The Journal of Immunology*. The American Association of Immunologists, 186(4), pp. 2003–2012. doi: 10.4049/jimmunol.1001919.

Tungatt, K. *et al.* (2015) 'Antibody Stabilization of Peptide–MHC Multimers Reveals Functional T Cells Bearing Extremely Low-Affinity TCRs', *The Journal of Immunology*. The American Association of Immunologists, 194(1), pp. 463–474. doi: 10.4049/jimmunol.1401785.

Tykodi, S. S. *et al.* (2012) 'CD8+ T-cell Clones Specific for the 5T4 Antigen Target Renal Cell Carcinoma Tumor-initiating Cells in a Murine Xenograft Model', *Journal of Immunotherapy*. NIH Public Access, 35(7), pp. 523–533. doi: 10.1097/CJI.0b013e318261d630.

Tyler, C. J. *et al.* (2015) 'Human Vγ9/Vδ2 T cells: Innate adaptors of the immune system', *Cellular Immunology*, 296(1), pp. 10–21. doi: 10.1016/j.cellimm.2015.01.008.

Tyler, C. J. *et al.* (2017) 'Antigen-Presenting Human γδ T Cells Promote Intestinal CD4 + T Cell Expression of IL-22 and Mucosal Release of Calprotectin', *The Journal of Immunology*, 198(9), pp. 3417–3425. doi: 10.4049/jimmunol.1700003.

Urban, S. L., Berg, L. J. and Welsh, R. M. (2016) 'Type 1 interferon licenses naïve CD8 T cells to mediate anti-viral cytotoxicity.', *Virology*. NIH Public Access, 493, pp. 52–9. doi: 10.1016/j.virol.2016.03.005.

Urban, S. L. and Welsh, R. M. (2014) 'Out-of-Sequence Signal 3 as a Mechanism for Virus-Induced Immune Suppression of CD8 T Cell Responses', *PLoS Pathogens*. Edited by C. M. Walker. Public Library of Science, 10(9), p. e1004357. doi: 10.1371/journal.ppat.1004357.

US National Library of Medicine (no date) *ClinicalTrials.gov*.

Valmori, D. *et al.* (1999) *Optimal activation of tumor-reactive T cells by selected antigenic peptide analogues*, *International Immunology*. Available at: https://watermark.silverchair.com/111971.pdf?token=AQECAHi208BE49Ooan9kkhW_Ercy7Dm3ZL_9Cf3qfKAc485ysgAAAmQwggJgBgkqhkiG9w0BBwagggJRMIICTQIBADCCAkYGCSqGSIb3DQEHATAeBglghkgBZQMEAS4wEQQM8-X5nxoTnO0Iq1jOAgEQgIICF7ZcS4ATGXDOZshdUlkuKDKZG-ilgZ0ZM3tfEFapke3bvcMu5DVRCWnJCpusgxIaxyhXb0YZhaxt4gM-yjmzCEZQL-O0o9uyT9t4ILjCxQaDz4Zk2bGTRW7sGnzk5K_-DOoODujXL77Tzxiuht8Ikc2dKOfx5pJnl8yzICd-

_czOP7AsV74BguB3CSA8ioJhSoVXLLVHRHr36-MuPJFBHZJJSxNW1xXZnmQuovbXnmPW030IkylAXslz4ieXmrrLytn8M5QygPjKEfzpBnRyrTfPv90p7W-0jMEdhkA5G4gm04_vqbXGpTTJ4rA6-aeRPDFJnd1YBHanjF57nutrmB10iG-XREdGBluhwOwa_7U9FXaZKRkHZCgd9r__DoJmL4zriR22mZHHN6VjmragU1PmRFQ960-5MCt4dusNXjbCs0a4iXAbsfNOmffLkVT8Iirzz9Irq7bGqQ6K_DqsXykvzj4JIE-rXnRIqPEHtHUAZZxlJLqomAfRQM5rFMq5kV1Vdt_R-P7c89piWY38v6RKtiFqUViMvEjUoyoUfN4f7yzmpDZFC9Ksb4UQiPW36h9dWrmmwAeWpomxnaopRxGDToVGM1IEL1ekqMqPzTTfakItFOhIoitfoMOf1aH52iN03lkNA7CoJ_vaUCZBX7ykjesR4NR0lPsODBdk6fkznmoahzexXuXWS3WvJ_09yxfOYeIwhUk (Accessed: 20 January 2020).

Valmori, D. *et al.* (2000) 'Naturally Occurring Human Lymphocyte Antigen-A2 Restricted CD8 T-Cell Response to the Cancer Testis Antigen NY-ESO-1 in Melanoma Patients', *Cancer Res*. American Association for Cancer Research, 59(9), pp. 2167–2173. Available at: http://cancerres.aacrjournals.org/content/60/16/4499.long (Accessed: 14 May 2019).

Vantourout, P. *et al.* (2009) 'Specific requirements for Vgamma9Vdelta2 T cell stimulation by a natural adenylated phosphoantigen', *Journal of immunology (Baltimore, Md. : 1950)*. 2009/08/26. American Association of Immunologists, 183(6), pp. 3848–3857. doi: 10.4049/jimmunol.0901085.

Vantourout, P. *et al.* (2018) 'Heteromeric interactions regulate butyrophilin (BTN) and BTN-like molecules governing γδ T cell biology.', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 115(5), pp. 1039–1044. doi: 10.1073/pnas.1701237115.

Vantourout, P. and Hayday, A. (2013) 'Six-of-the-best: unique contributions of γδ T cells to immunology', *Nature Reviews Immunology*. Nature Publishing Group, 13(2), pp. 88–100. doi: 10.1038/nri3384.

Vera, A. *et al.* (2005) 'Lon and ClpP proteases participate in the physiological disintegration of bacterial inclusion bodies', *Journal of Biotechnology*. Elsevier, 119(2), pp. 163–171. doi: 10.1016/J.JBIOTEC.2005.04.006.

Vermijlen, D. *et al.* (2007) 'Distinct cytokine-driven responses of activated blood gammadelta T cells: insights into unconventional T cell pleiotropy', *Journal of immunology (Baltimore, Md. : 1950)*, 178(7), pp. 4304–4314. doi: 10.4049/jimmunol.178.7.4304.

Vermijlen, D. *et al.* (2018) 'γδ T cell responses: How many ligands will it take till we know?', *Seminars in Cell & Developmental Biology*. Elsevier BV, 84, pp. 75–86. doi: 10.1016/j.semcdb.2017.10.009.

Villaverde, A. and Carrió, M. M. (2003) 'Protein aggregation in recombinant bacteria: biological role of inclusion bodies.', *Biotechnology letters*, 25(17), pp. 1385–95. Available at: https://link.springer.com/content/pdf/10.1023%2FA%3A1025024104862.pdf (Accessed: 24 May 2019).

Vyas, J. M., Van Der Veen, A. G. and Ploegh, H. L. (2008) 'The known unknowns of antigen processing and presentation', *Nature Reviews Immunology*, 8(8), pp. 607–618. doi: 10.1038/nri2368.

Wakita, D. *et al.* (2010) 'Tumor-infiltrating IL-17-producing γδ T cells support the progression of tumor by promoting angiogenesis', *European Journal of Immunology*. Wiley, 40(7), pp. 1927–1937. doi: 10.1002/eji.200940157.

Wang, B. *et al.* (2001) 'Multiple paths for activation of naive CD8+ T cells: CD4-independent help.', *Journal of immunology (Baltimore, Md. : 1950)*. American Association of Immunologists, 167(3), pp. 1283–9. doi: 10.4049/jimmunol.167.3.1283.

Warren, E. H., Greenberg, P. D. and Riddell, S. R. (1998) 'Cytotoxic T-Lymphocyte–Defined Human Minor Histocompatibility Antigens With a Restricted Tissue Distribution', *Blood*, 91(6). Available at: http://www.bloodjournal.org/content/91/6/2197.long?sso-checked=true#sec-1 (Accessed: 5 April 2017).

Weber, J. S. *et al.* (1999) 'A Phase I Trial of an HLA-A1 Restricted MAGE-3 Epitope Peptide with Incomplete Freund's Adjuvant in Patients with Resected High-Risk Melanoma', *Journla of Immunotherapy*. Ovid Technologies (Wolters Kluwer Health), 22(5), pp. 431–440. doi: 10.1097/00002371-199909000-00007.

Weekes, M. P. *et al.* (2014) 'Quantitative temporal viromics: an approach to investigate host-pathogen interaction.', *Cell*. Elsevier, 157(6), pp. 1460–1472. doi: 10.1016/j.cell.2014.04.028.

Weinreich, M. A. *et al.* (2010) 'T cells expressing the transcription factor PLZF regulate the development of memory-like CD8+ T cells.', *Nature immunology*. Nat Immunol, 11(8), pp. 709–16. doi: 10.1038/ni.1898.

Welton, J. L. *et al.* (2013) 'Monocytes and γδ T cells control the acute-phase response to intravenous zoledronate: Insights from a phase IV safety trial', *Journal of Bone and Mineral Research*. Wiley, 28(3), pp. 464–471. doi: 10.1002/jbmr.1797.

Wencker, M. *et al.* (2014) 'Innate-like T cells straddle innate and adaptive immunity by altering antigen-receptor responsiveness', *Nature Immunology*. Nature Publishing Group, 15(1), pp. 80–87. doi: 10.1038/ni.2773.

Wesch, D. *et al.* (2006) 'Direct Costimulatory Effect of TLR3 Ligand Poly(I:C) on Human γδ T Lymphocytes', *The Journal of Immunology*. The American Association of Immunologists, 176(3), pp. 1348–1354. doi: 10.4049/jimmunol.176.3.1348.

Wesch, D. *et al.* (2011) 'Modulation of γδ T cell responses by TLR ligands', *Cellular and Molecular Life Sciences*. Springer Science and Business Media LLC, 68(14), pp. 2357–2370. doi: 10.1007/s00018-011-0699-1.

Wesch, D., Glatzel, A. and Kabelitz, D. (2001) 'Differentiation of Resting Human Peripheral Blood γδ T Cells toward Th1- or Th2-Phenotype', *Cellular Immunology*. Elsevier BV, 212(2), pp. 110–117. doi: 10.1006/cimm.2001.1850.

White, C. B. *et al.* (1995) 'A novel activity of OmpT. Proteolysis under extreme denaturing conditions.', *The Journal of biological chemistry*. American Society for Biochemistry and Molecular Biology, 270(22), pp. 12990–4. doi: 10.1074/jbc.270.22.12990.

White, J. T., Cross, E. W. and Kedl, R. M. (2017) 'Antigen-inexperienced memory CD8+ T cells: Where they come from and why we need them', *Nature Reviews Immunology*. Nature Publishing Group, 17(6), pp. 391–400. doi: 10.1038/nri.2017.34.

Whiteside, T. L. (2008) 'The tumor microenvironment and its role in promoting tumor growth', *Oncogene*, 27(45), pp. 5904–5912. doi: 10.1038/onc.2008.271.

Wieczorek, M. *et al.* (2017) 'Major Histocompatibility Complex (MHC) Class I and MHC Class II Proteins: Conformational Plasticity in Antigen Presentation', *Frontiers in Immunology*. Frontiers, 8, p. 292. doi: 10.3389/fimmu.2017.00292.

Wilgenhof, S. *et al.* (2016) 'Phase II Study of Autologous Monocyte-Derived mRNA Electroporated Dendritic Cells (TriMixDC-MEL) Plus Ipilimumab in Patients With Pretreated Advanced Melanoma', *Journal of Clinical Oncology*. American Society of Clinical Oncology (ASCO), 34(12), pp. 1330–1338. doi: 10.1200/jco.2015.63.4121.

Willcox, C. R. *et al.* (2012) 'Cytomegalovirus and tumor stress surveillance by binding of a human γδ T cell antigen receptor to endothelial protein C receptor', *Nature Immunology*. Nat Immunol, 13(9), pp. 872–879. doi: 10.1038/ni.2394.

Willcox, C. R., Davey, M. S. and Willcox, B. E. (2018) 'Development and Selection of the Human Vγ9Vδ2+ T-Cell Repertoire', *Frontiers in Immunology*. Frontiers, 9, p. 1501. doi: 10.3389/fimmu.2018.01501.

Witz, I. P. (2008) 'Tumor–Microenvironment Interactions: Dangerous Liaisons', in *Advances in Cancer Research*. Elsevier, pp. 203–229. doi: 10.1016/S0065-230X(08)00007-9.

Wölfl, M. *et al.* (2011) 'Primed tumor-reactive multifunctional CD62L+ human CD8 + T cells for immunotherapy', *Cancer Immunology, Immunotherapy*, 60(2), pp. 173–186. doi: 10.1007/s00262-010-0928-8.

Wong, P. and Pamer, E. G. (2003) 'CD8 T cell responses to infectious pathogens', *Annual Review of Immunology*, 21(1), pp. 29–70. doi: 10.1146/annurev.immunol.21.120601.141114.

Wooldridge, L. *et al.* (2009) 'Tricks with tetramers: how to get the most from multimeric peptide-MHC', *Immunology*, 126(2), pp. 147–164. doi: 10.1111/j.1365-2567.2008.02848.x.

Wooldridge, L. *et al.* (2012) 'A Single Autoimmune T Cell Receptor Recognizes More Than a Million Different Peptides*', *Journal of Biological Chemistry*. Elsevier BV, 287(2), pp. 1168–1177. doi: 10.1074/jbc.m111.289488.

Wosen, J. E. *et al.* (2018) 'Epithelial MHC Class II Expression and Its Role in Antigen Presentation in the Gastrointestinal and Respiratory Tracts', *Frontiers in Immunology*. Frontiers, 9, p. 2144. doi: 10.3389/fimmu.2018.02144.

Wrobel, P. *et al.* (2007) 'Lysis of a Broad Range of Epithelial Tumour Cells by Human ?? T Cells: Involvement of NKG2D ligands and T-cell Receptor- versus NKG2D-dependent Recognition', *Scandinavian Journal of Immunology*. Wiley, 66(2–3), pp. 320–328. doi: 10.1111/j.1365-3083.2007.01963.x.

Wu, P. *et al.* (2014) 'γδT17 Cells Promote the Accumulation and Expansion of Myeloid-Derived Suppressor Cells in Human Colorectal Cancer', *Immunity*. Elsevier, 40(5), pp. 785–800. doi: 10.1016/j.immuni.2014.03.013.

Wu, Y. *et al.* (2009) 'Human γδ T Cells: A Lymphoid Lineage Cell Capable of Professional Phagocytosis', *The Journal of Immunology*. The American Association of Immunologists, 183(9), pp. 5622–5629. doi: 10.4049/jimmunol.0901772.

Xiang, Z. *et al.* (2014) 'Targeted Activation of Human Vγ9Vδ2-T Cells Controls Epstein-Barr Virus-Induced B Cell Lymphoproliferative Disease', *Cancer Cell*, 26(4), pp. 565–576. doi: 10.1016/j.ccr.2014.07.026.

Xiao, L. *et al.* (2018) 'Large-scale expansion of Vγ9Vδ2 T cells with engineered K562 feeder cells in G-Rex vessels and their use as chimeric antigen receptor–modified effector cells', *Cytotherapy*. Elsevier Inc., 20(3), pp. 420–435. doi: 10.1016/j.jcyt.2017.12.014.

Xu, Y. *et al.* (2014) 'Closely related T-memory stem cells correlate with in vivo expansion of CAR.CD19-T cells and are preserved by IL-7 and IL-15.', *Blood*. The American Society of Hematology, 123(24), pp. 3750–9. doi: 10.1182/blood-2014-01-552174.

Yang, X. A. *et al.* (2004) 'Purification and refolding of a novel cancer/testis antigen BJ-HCC-2 expressed in the inclusion bodies of Escherichia coli', *Protein Expression and Purification*, 33(2), pp. 332–338. doi: 10.1016/j.pep.2003.10.006.

Yazdanifar, M. *et al.* (2020) 'γδ T Cells: The Ideal Tool for Cancer Immunotherapy', *Cells*. Multidisciplinary Digital Publishing Institute, 9(5), p. 1305. doi: 10.3390/cells9051305.

Ye, J. *et al.* (2013) 'Specific recruitment of γδ regulatory T cells in human breast cancer', *Cancer research*. 2013/08/19, 73(20), pp. 6137–6148. doi: 10.1158/0008-5472.CAN-13-0348.

Yee, C. *et al.* (1999) 'Isolation of high avidity melanoma-reactive CTL from heterogeneous populations using peptide-MHC tetramers.', *Journal of immunology (Baltimore, Md. : 1950)*, 162(4), pp. 2227–34. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9973498 (Accessed: 22 June 2017).

Yewdell, J. W., Reits, E. and Neefjes, J. (2003) 'Making sense of mass destruction: quantitating MHC class I antigen presentation', *Nature Reviews Immunology*. Nature Publishing Group, 3(12), pp. 952–961. doi: 10.1038/nri1250.

Young, C. L., Britton, Z. T. and Robinson, A. S. (2012) 'Recombinant protein expression and purification: A comprehensive review of affinity tags and microbial applications', *Biotechnology Journal*. WILEY-VCH Verlag, 7(5), pp. 620–634. doi: 10.1002/biot.201100155.

Yu, L. and Chen, S. (2008) 'Toll-like receptors expressed in tumor cells: targets for therapy', *Cancer Immunology, Immunotherapy*. Springer Science and Business Media LLC, 57(9), pp. 1271–1278. doi: 10.1007/s00262-008-0459-8.

Zehn, D., Lee, S. Y. and Bevan, M. J. (2009) 'Complete but curtailed T-cell response to very low-affinity antigen', *Nature*. Nature Publishing Group, 458(7235), pp. 211–214. doi: 10.1038/nature07657.

Zehner, M. *et al.* (2015) 'The Translocon Protein Sec61 Mediates Antigen Transport from Endosomes in the Cytosol for Cross-Presentation to CD8+ T Cells', *Immunity*, 42(5), pp. 850–863. doi: 10.1016/j.immuni.2015.04.008.

Zendman, A. J. W., Ruiter, D. J. and Van Muijen, G. N. P. (2003) 'Cancer/Testis-Associated Genes: Identification, Expression Profile, and Putative Function', *JOURNAL OF CELLULAR PHYSIOLOGY*, 194, pp. 272–288. doi: 10.1002/jcp.10215.

Zeng, R. *et al.* (2005) 'Synergy of IL-21 and IL-15 in regulating CD8+ T cell expansion and function', *Journal of Experimental Medicine*. Rockefeller University Press, 201(1), pp. 139–148. doi: 10.1084/jem.20041057.

Zhang, S.-Y. *et al.* (2007) 'TLR3 Deficiency in Patients with Herpes Simplex Encephalitis', *Science*. American Association for the Advancement of Science (AAAS), 317(5844), pp. 1522–1527. doi: 10.1126/science.1139522.

Zhang, S. *et al.* (2016) 'Direct measurement of T cell receptor affinity and sequence from naïve antiviral T cells', 8(341), pp. 1–9.

Zhang, Y. *et al.* (2010) 'Lipophilic pyridinium bisphosphonates: potent gammadelta T cell stimulators.', *Angewandte Chemie (International ed. in English)*. NIH Public Access, 49(6), pp. 1136–8. doi: 10.1002/anie.200905933.

Zhao, Y. *et al.* (2005) 'Primary Human Lymphocytes Transduced with NY-ESO-1 Antigen-Specific TCR Genes Recognize and Kill Diverse Human Tumor Cell Lines', *Journal of immunology*. American Association of Immunologists, 174(7), pp. 4415–4423. doi: 10.4049/jimmunol.174.7.4415.

Zhao, Y. *et al.* (2014) 'Structural insights into the inhibition of Wnt signaling by cancer antigen 5T4/Wnt-activated inhibitory factor 1.', *Structure (London, England : 1993)*. Elsevier, 22(4), pp. 612–20. doi: 10.1016/j.str.2014.01.009.

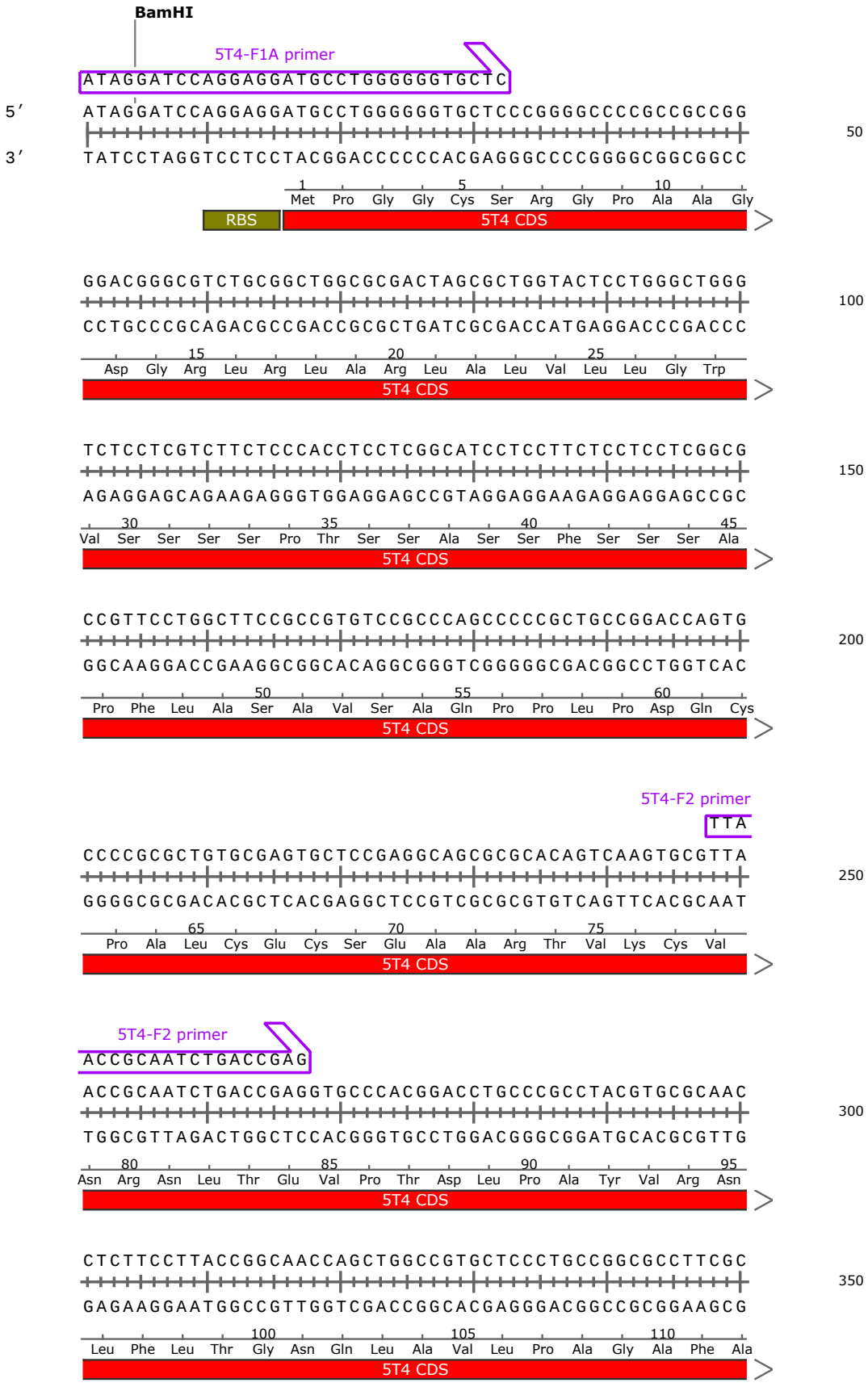
Zhao, Y. *et al.* (no date) *Structural insights into the inhibition of Wnt signaling by cancer antigen 5T4/Wnt-activated inhibitory factor 1*. Available at: https://www.cell.com/cms/10.1016/j.str.2014.01.009/attachment/9896bc13-c093-4a8a-a0c2-361e924e0e1e/mmc1.pdf (Accessed: 23 May 2019).

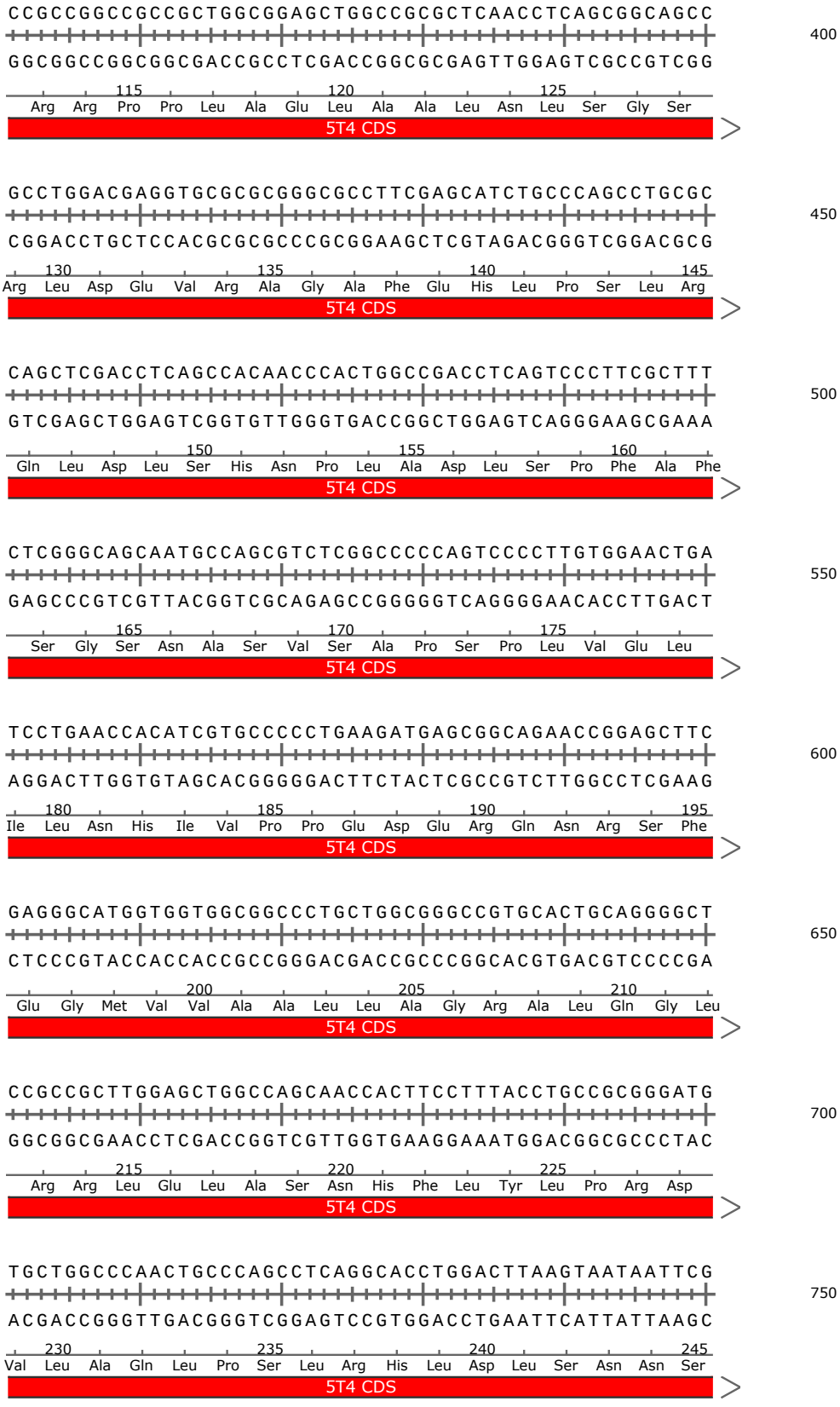
Zou, W. *et al.* (2001) 'Stromal-derived factor-1 in human tumors recruits and alters the function of plasmacytoid precursor dendritic cells', *Nature Medicine*. Springer Science and Business Media LLC, 7(12), pp. 1339–1346. doi: 10.1038/nm1201-1339.

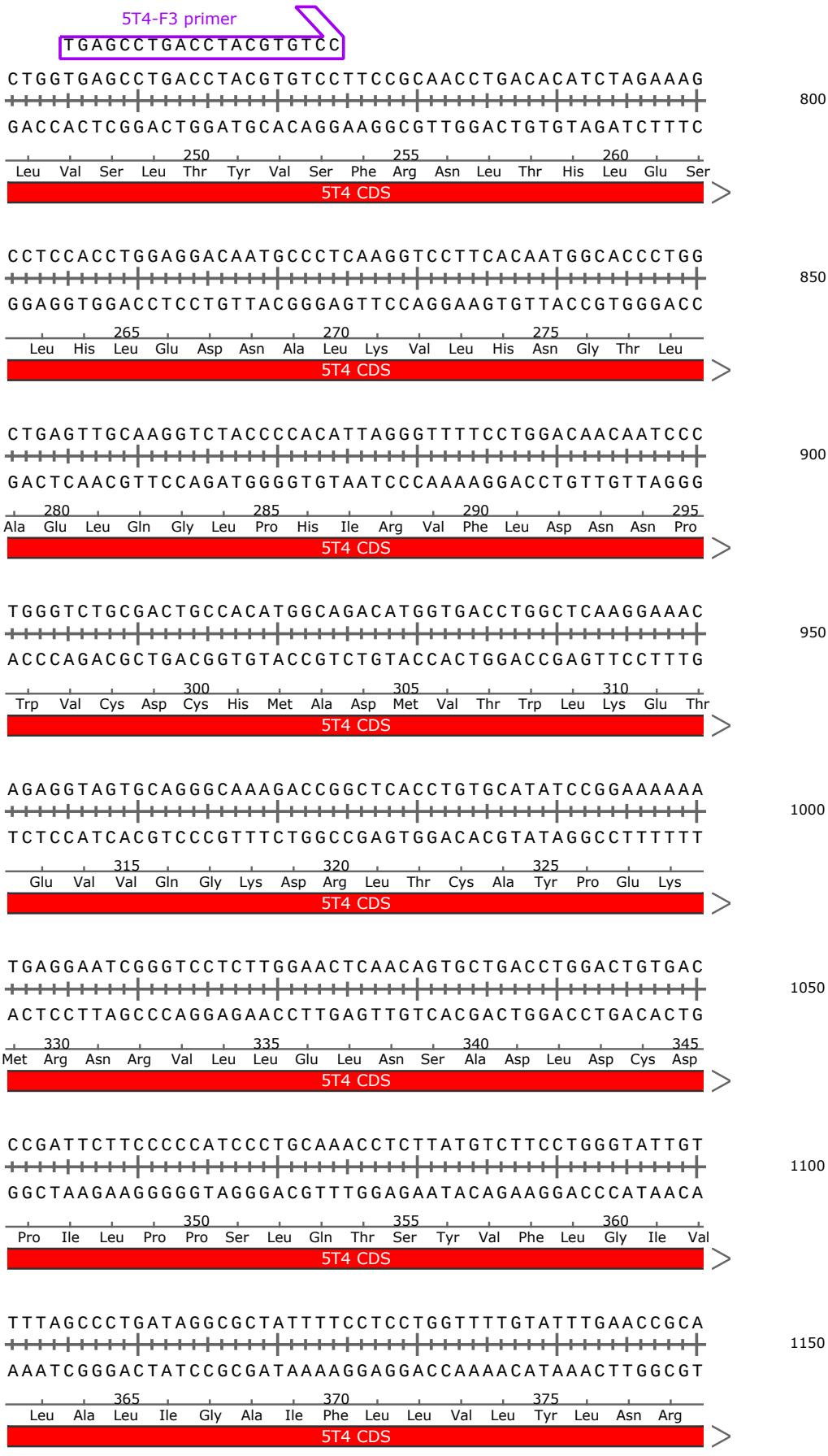
Zou, W. (2005) 'Immunosuppressive networks in the tumour environment and their therapeutic relevance', *Nature Reviews Cancer*. Springer Science and Business Media LLC, 5(4), pp. 263–274. doi: 10.1038/nrc1586.

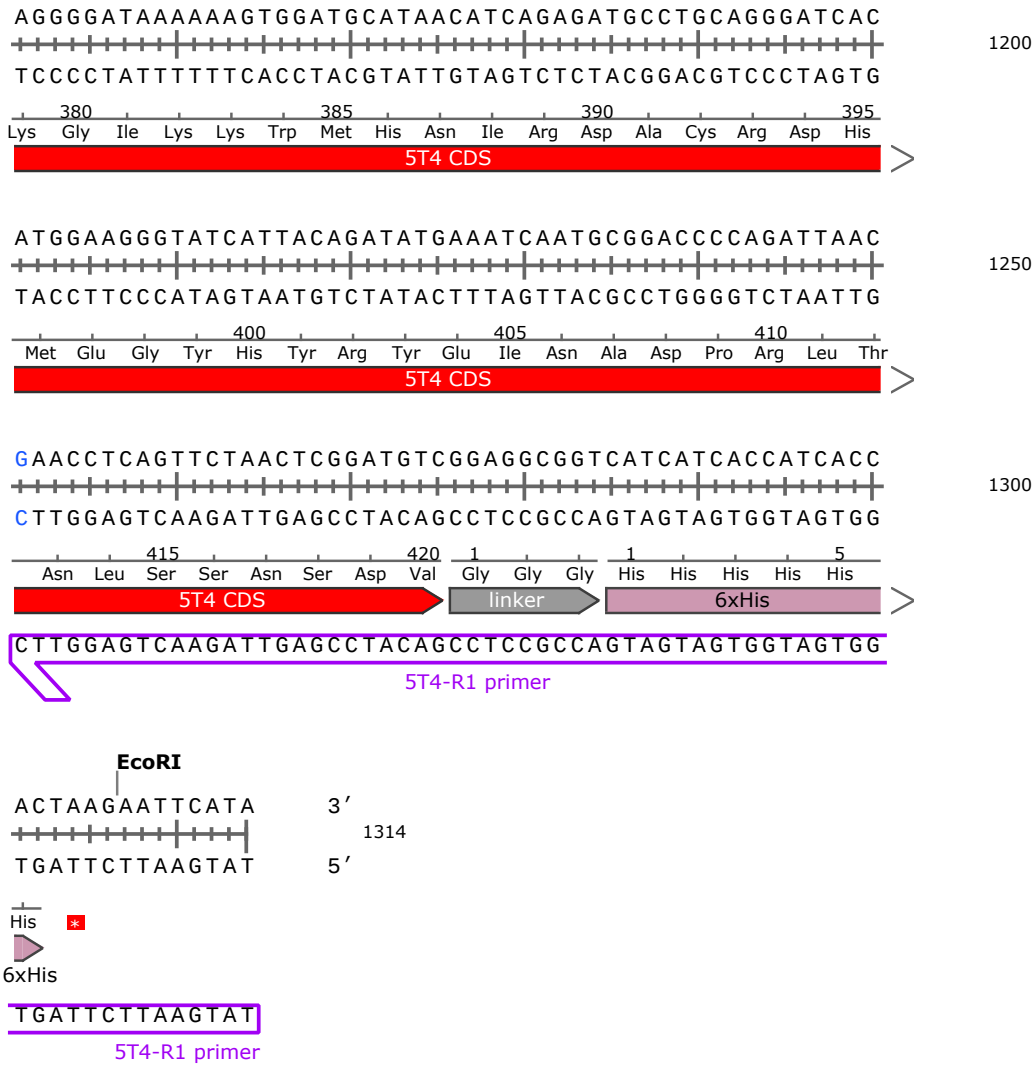
Zúñiga-Pflücker, J. C. (2004) 'T-cell development made simple', *Nature Reviews Immunology*. Nature Publishing Group, 4(1), pp. 67–72. doi: 10.1038/nri1257.

Appendix

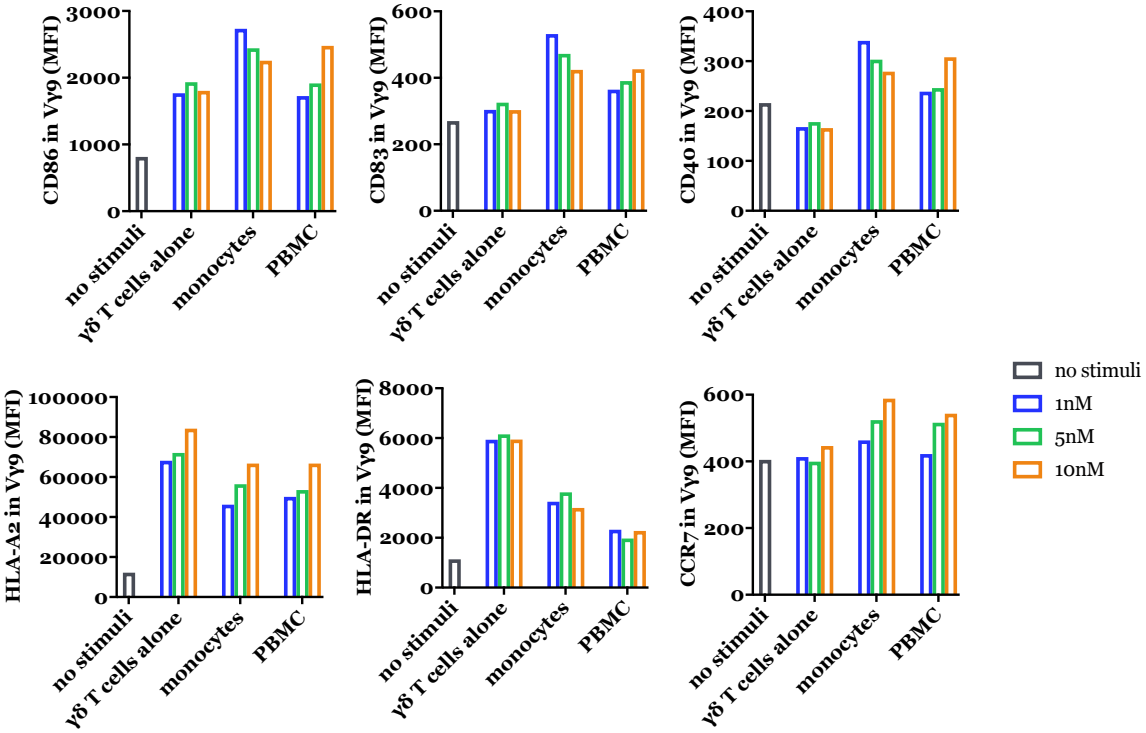

BamHI
5T4-F1A primer
ATAGGATCCAGGAGGATGCCTGGGGGGTGCTC
5′
ATAGGATCCAGGAGGATGCCTGGGGGGTGCTCCCGGGGCCCCGCCGCCGG
50
3′
TATCCTAGGTCCTCCTACGGACCCCCCACGAGGGCCCCGGGGCGGCGGCC
1 5 10
Met Pro Gly Gly Cys Ser Arg Gly Pro Ala Ala Gly
RBS
5T4 CDS
GGACGGGCGTCTGCGGCTGGCGCGACTAGCGCTGGTACTCCTGGGCTGGG
100
CCTGCCCGCAGACGCCGACCGCGCTGATCGCGACCATGAGGACCCGACCC
15 20 25
Asp Gly Arg Leu Arg Leu Ala Arg Leu Ala Leu Val Leu Leu Gly Trp
5T4 CDS
TCTCCTCGTCTTCTCCCACCTCCTCGGCATCCTCCTTCTCCTCCTCGGCG
150
AGAGGAGCAGAAGAGGGTGGAGGAGCCGTAGGAGGAAGAGGAGGAGCCGC
30 35 40 45
Val Ser Ser Ser Ser Pro Thr Ser Ser Ala Ser Ser Phe Ser Ser Ser Ala
5T4 CDS
CCGTTCCTGGCTTCCGCCGTGTCCGCCCAGCCCCCGCTGCCGGACCAGTG
200
GGCAAGGACCGAAGGCGGCACAGGCGGGTCGGGGGCGACGGCCTGGTCAC
50 55 60
Pro Phe Leu Ala Ser Ala Val Ser Ala Gln Pro Pro Leu Pro Asp Gln Cys
5T4 CDS
5T4-F2 primer
TTA
CCCCGCGCTGTGCGAGTGCTCCGAGGCAGCGCGCACAGTCAAGTGCGTTA
250
GGGGCGCGACACGCTCACGAGGCTCCGTCGCGCGTGTCAGTTCACGCAAT
65 70 75
Pro Ala Leu Cys Glu Cys Ser Glu Ala Ala Arg Thr Val Lys Cys Val
5T4 CDS
5T4-F2 primer
ACCGCAATCTGACCGAG
ACCGCAATCTGACCGAGGTGCCCACGGACCTGCCCGCCTACGTGCGCAAC
300
TGGCGTTAGACTGGCTCCACGGGTGCCTGGACGGGCGGATGCACGCGTTG
80 85 90 95
Asn Arg Asn Leu Thr Glu Val Pro Thr Asp Leu Pro Ala Tyr Val Arg Asn
5T4 CDS
CTCTTCCTTACCGGCAACCAGCTGGCCGTGCTCCCTGCCGGCGCCTTCGC
350
GAGAAGGAATGGCCGTTGGTCGACCGGCACGAGGGACGGCCGCGGAAGCG
100 105 110
Leu Phe Leu Thr Gly Asn Gln Leu Ala Val Leu Pro Ala Gly Ala Phe Ala
5T4 CDS


CCGCCGGCCGCCGCTGGCGGAGCTGGCCGCGCTCAACCTCAGCGGCAGCC
400
GGCGGCCGGCGGCGACCGCCTCGACCGGCGCGAGTTGGAGTCGCCGTCGG
115
120
125
Arg Arg Pro Pro Leu Ala Glu Leu Ala Ala Leu Asn Leu Ser Gly Ser
5T4 CDS
GCCTGGACGAGGTGCGCGCGGGCGCCTTCGAGCATCTGCCCAGCCTGCGC
450
CGGACCTGCTCCACGCGCGCCCGCGGAAGCTCGTAGACGGGTCGGACGCG
130
135
140
145
Arg Leu Asp Glu Val Arg Ala Gly Ala Phe Glu His Leu Pro Ser Leu Arg
5T4 CDS
CAGCTCGACCTCAGCCACAACCCACTGGCCGACCTCAGTCCCTTCGCTTT
500
GTCGAGCTGGAGTCGGTGTTGGGTGACCGGCTGGAGTCAGGGAAGCGAAA
150
155
160
Gln Leu Asp Leu Ser His Asn Pro Leu Ala Asp Leu Ser Pro Phe Ala Phe
5T4 CDS
CTCGGGCAGCAATGCCAGCGTCTCGGCCCCCAGTCCCCTTGTGGAACTGA
550
GAGCCCGTCGTTACGGTCGCAGAGCCGGGGGTCAGGGGAACACCTTGACT
165
170
175
Ser Gly Ser Asn Ala Ser Val Ser Ala Pro Ser Pro Leu Val Glu Leu
5T4 CDS
TCCTGAACCACATCGTGCCCCCTGAAGATGAGCGGCAGAACCGGAGCTTC
600
AGGACTTGGTGTAGCACGGGGGACTTCTACTCGCCGTCTTGGCCTCGAAG
180
185
190
195
Ile Leu Asn His Ile Val Pro Pro Glu Asp Glu Arg Gln Asn Arg Ser Phe
5T4 CDS
GAGGGCATGGTGGTGGCGGCCCTGCTGGCGGGCCGTGCACTGCAGGGGCT
650
CTCCCGTACCACCACCGCCGGGACGACCGCCCGGCACGTGACGTCCCCGA
200
205
210
Glu Gly Met Val Val Ala Ala Leu Leu Ala Gly Arg Ala Leu Gln Gly Leu
5T4 CDS
CCGCCGCTTGGAGCTGGCCAGCAACCACTTCCTTTACCTGCCGCGGGATG
700
GGCGGCGAACCTCGACCGGTCGTTGGTGAAGGAAATGGACGGCGCCCTAC
215
220
225
Arg Arg Leu Glu Leu Ala Ser Asn His Phe Leu Tyr Leu Pro Arg Asp
5T4 CDS
TGCTGGCCCAACTGCCCAGCCTCAGGCACCTGGACTTAAGTAATAATTCG
750
ACGACCGGGTTGACGGGTCGGAGTCCGTGGACCTGAATTCATTATTAAGC
230
235
240
245
Val Leu Ala Gln Leu Pro Ser Leu Arg His Leu Asp Leu Ser Asn Asn Ser
5T4 CDS


5T4-F3 primer
TGAGCCTGACCTACGTGTCC
CTGGTGAGCCTGACCTACGTGTCCTTCCGCAACCTGACACATCTAGAAAG
800
GACCACTCGGACTGGATGCACAGGAAGGCGTTGGACTGTGTAGATCTTTC
250 255 260
Leu Val Ser Leu Thr Tyr Val Ser Phe Arg Asn Leu Thr His Leu Glu Ser
5T4 CDS
CCTCCACCTGGAGGACAATGCCCTCAAGGTCCTTCACAATGGCACCCTGG
850
GGAGGTGGACCTCCTGTTACGGGAGTTCCAGGAAGTGTTACCGTGGGACC
265 270 275
Leu His Leu Glu Asp Asn Ala Leu Lys Val Leu His Asn Gly Thr Leu
5T4 CDS
CTGAGTTGCAAGGTCTACCCCACATTAGGGTTTTCCTGGACAACAATCCC
900
GACTCAACGTTCCAGATGGGGTGTAATCCCAAAAGGACCTGTTGTTAGGG
280 285 290 295
Ala Glu Leu Gln Gly Leu Pro His Ile Arg Val Phe Leu Asp Asn Asn Pro
5T4 CDS
TGGGTCTGCGACTGCCACATGGCAGACATGGTGACCTGGCTCAAGGAAAC
950
ACCCAGACGCTGACGGTGTACCGTCTGTACCACTGGACCGAGTTCCTTTG
300 305 310
Trp Val Cys Asp Cys His Met Ala Asp Met Val Thr Trp Leu Lys Glu Thr
5T4 CDS
AGAGGTAGTGCAGGGCAAAGACCGGCTCACCTGTGCATATCCGGAAAAAA
1000
TCTCCATCACGTCCCGTTTCTGGCCGAGTGGACACGTATAGGCCTTTTTT
315 320 325
Glu Val Val Gln Gly Lys Asp Arg Leu Thr Cys Ala Tyr Pro Glu Lys
5T4 CDS
TGAGGAATCGGGTCCTCTTGGAACTCAACAGTGCTGACCTGGACTGTGAC
1050
ACTCCTTAGCCCAGGAGAACCTTGAGTTGTCACGACTGGACCTGACACTG
330 335 340 345
Met Arg Asn Arg Val Leu Leu Glu Leu Asn Ser Ala Asp Leu Asp Cys Asp
5T4 CDS
CCGATTCTTCCCCCATCCCTGCAAACCTCTTATGTCTTCCTGGGTATTGT
1100
GGCTAAGAAGGGGGTAGGGACGTTTGGAGAATACAGAAGGACCCATAACA
350 355 360
Pro Ile Leu Pro Pro Ser Leu Gln Thr Ser Tyr Val Phe Leu Gly Ile Val
5T4 CDS
TTTAGCCCTGATAGGCGCTATTTTCCTCCTGGTTTTGTATTTGAACCGCA
1150
AAATCGGGACTATCCGCGATAAAAGGAGGACCAAAACATAAACTTGGCGT
365 370 375
Leu Ala Leu Ile Gly Ala Ile Phe Leu Leu Val Leu Tyr Leu Asn Arg
5T4 CDS

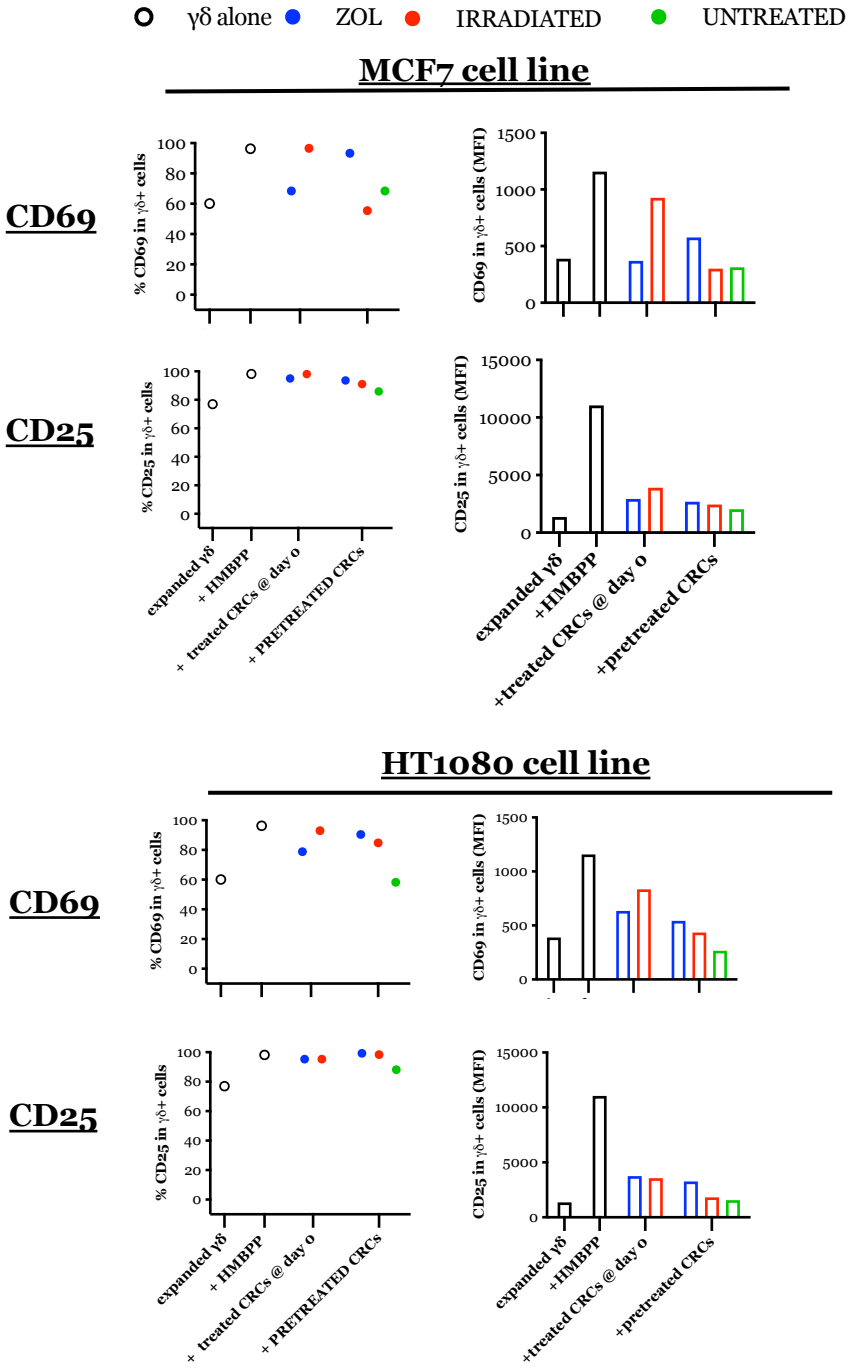


Appendix 1. Annotated full coding sequence and translation of the recombinant 5T4 gene.
Binding site of primers is shown in purple. * represents a stop codon.



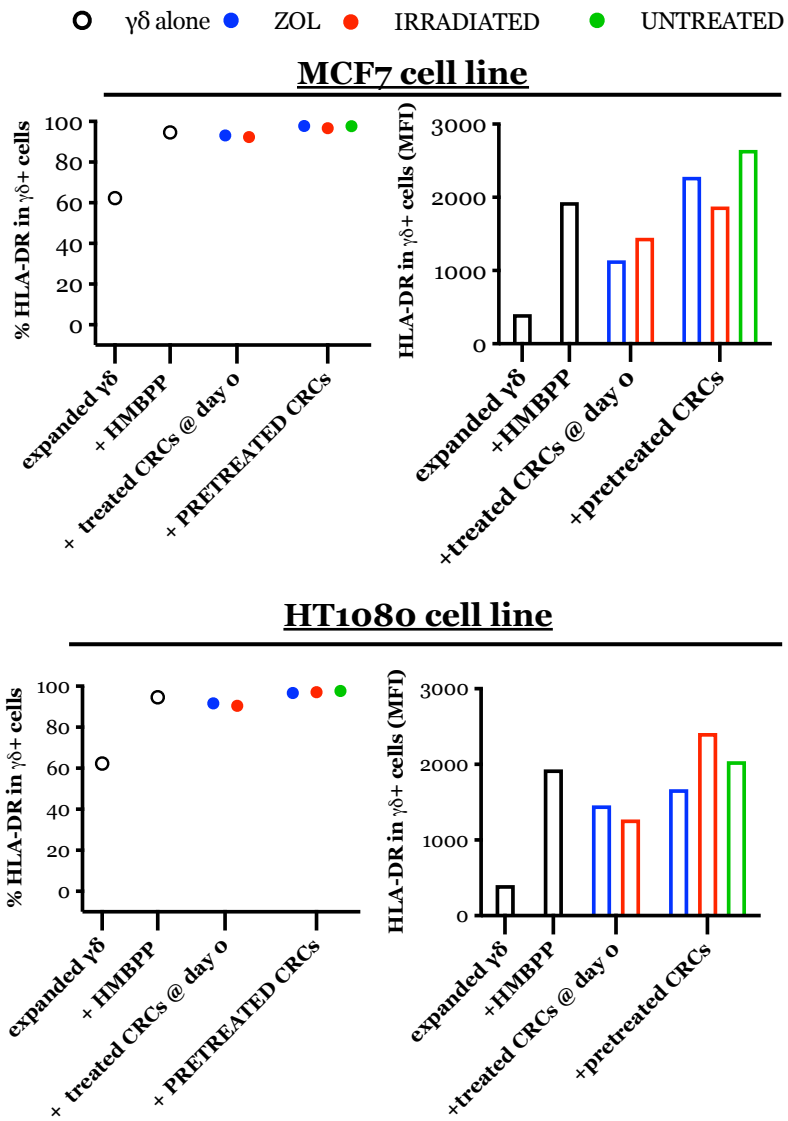
Appendix 2. Restimulation of expanded Vγ9/Vδ2 T cells: analysis of surface expression of APC molecules.

Expanded γδ T cells were re-stimulated in the presence or absence of allogenic irradiated accessory cells as described in **Figure 5.5**. Expression of costimulatory molecules (CD86, CD70, CD83, CD40), lymph node homing receptor (CCR7) and MHC molecules (HLA-DR, HLA-A2) is shown as MFI values in Vγ9+ cells.



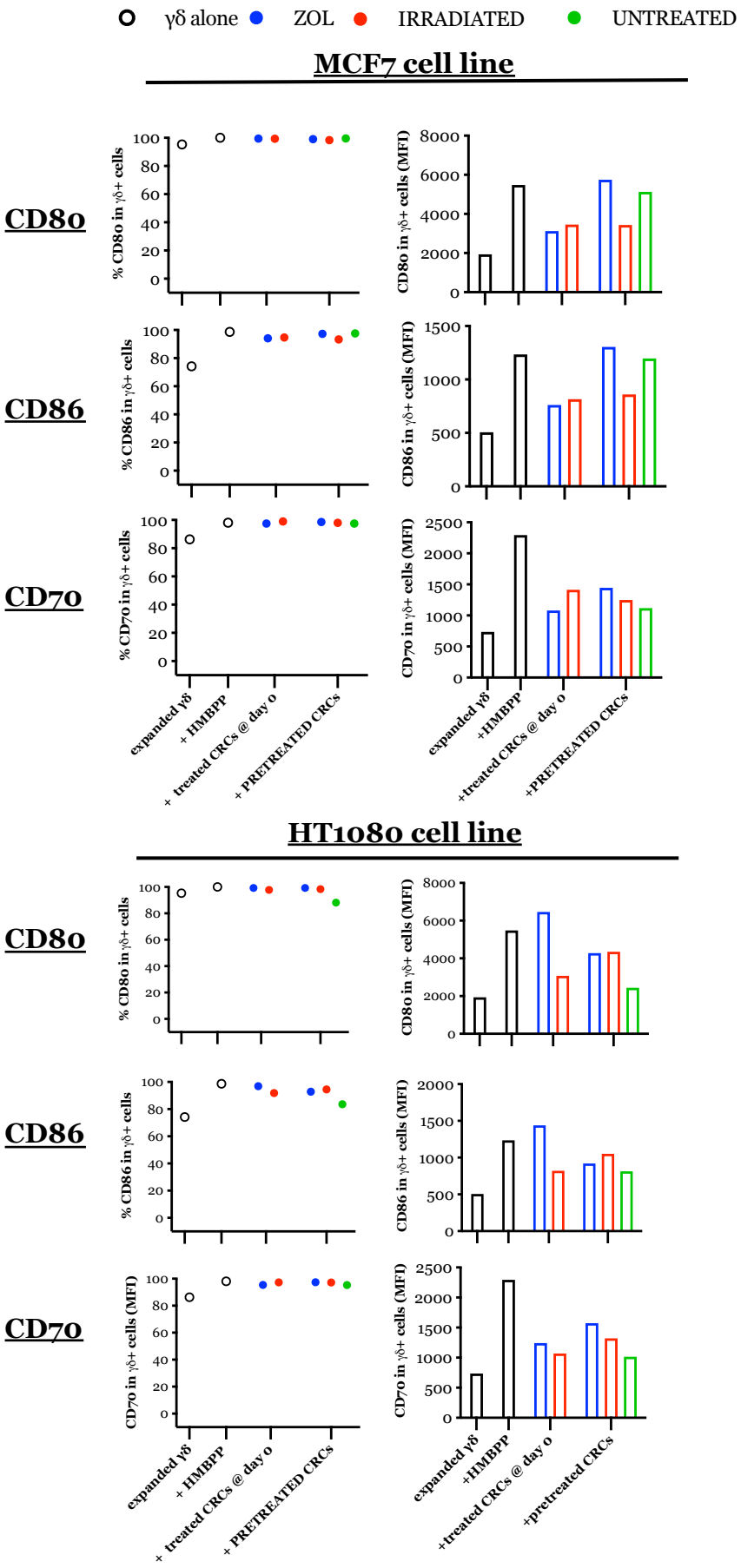
Appendix 3. HT1080 and MCF7 cancer cell lines induce activation of expanded γδ T cells.

Cancer cells were treated as described in **Figure 5.14**. γδ T cells were analysed by flow cytometry for expression of activation markers CD25 and CD69 expression 72 hours after co-culture with cancer cells. Expanded γδ T cells re-stimulated with HMBPP served as a positive control, and expanded γδ T cells maintained in complete RPMI with IL-2 and IL-15 served as a negative control. Data represents one experiment.



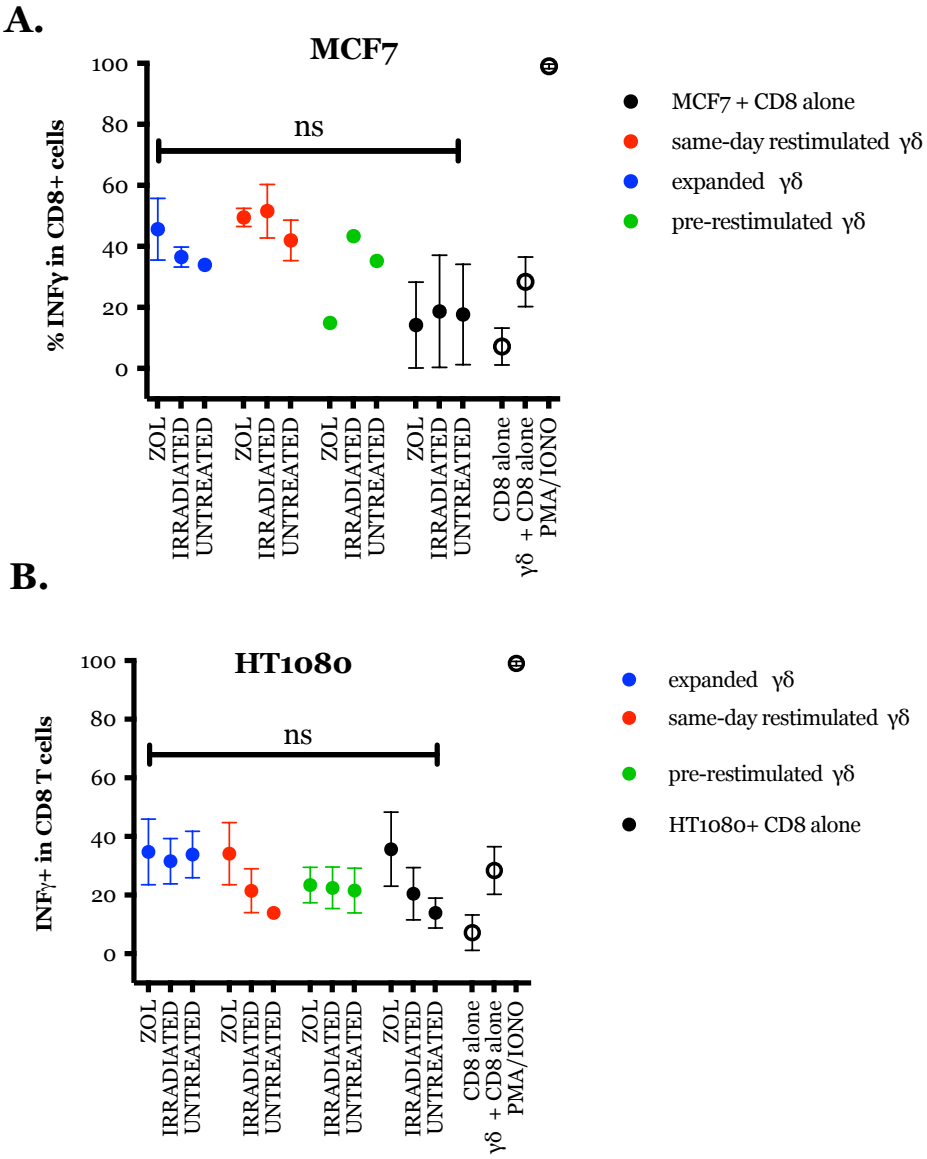
Appendix 4. MCF7 and HT1080 cancer cell lines enhance MHC class II expression in expanded γδ T cells.

Cancer cells were treated as described in **Figure 5.15**. γδ T cells were analysed by flow cytometry for expression of HLADR expression 72 hours after co-culture with cancer cells. Expanded γδ T cells re-stimulated with HMBPP served as a positive control, and expanded γδ T cells maintained in complete RPMI with IL-2 and IL-15 served as a negative control. Data represents one experiment.


γδ alone
ZOL
IRRADIATED
UNTREATED
MCF7 cell line
CD80
CD86
CD70
% CD80 in γδ+ cells
CD80 in γδ+ cells (MFI)
% CD86 in γδ+ cells
CD86 in γδ+ cells (MFI)
% CD70 in γδ+ cells
CD70 in γδ+ cells (MFI)
expanded γδ
+ HMBPP
+ treated CRCs @ day 0
+ PRETREATED CRCs
+pretreated CRCs
HT1080 cell line

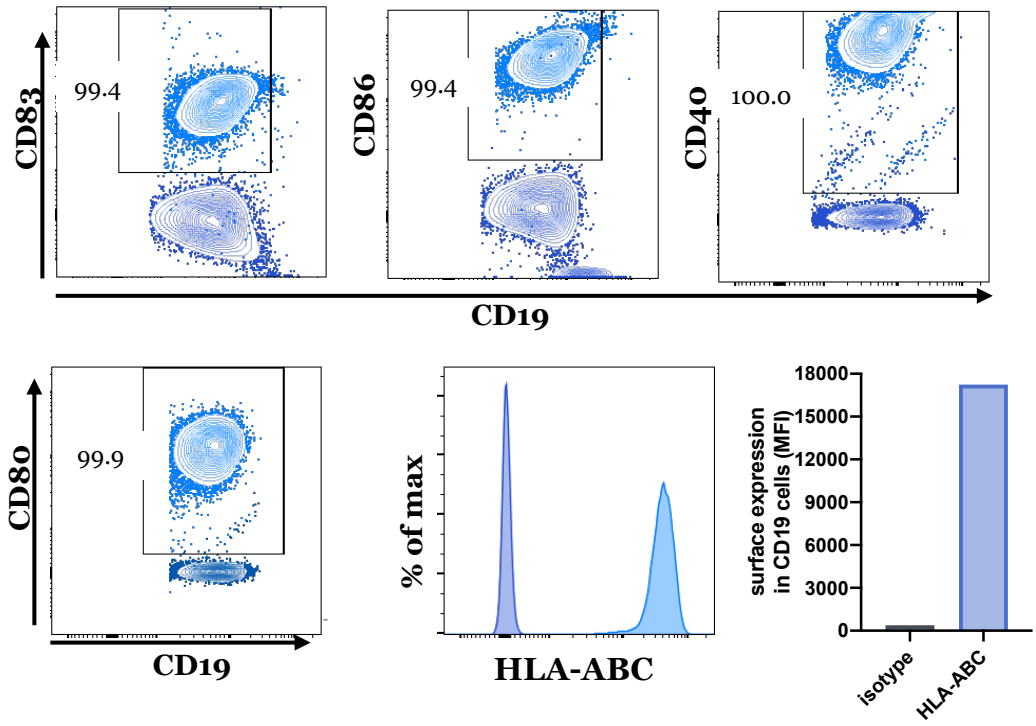
Appendix 5. MCF7 and HT1080 cancer cell lines enhance expression of costimulatory molecules in expanded γδ T cells.

Cancer cells were treated as described in **Figure 5.8**. γδ T cells were analysed by flow cytometry for expression of CD80, CD86, and CD70 72 hours after co-culture with cancer cells. Expanded γδ T cells re-stimulated with HMBPP served as a positive control, and expanded γδ T cells maintained in complete RPMI with IL-2 and IL-15 served as a negative control. Data represents one experiment.



Appendix 6. Cross-presentation of 5T4 antigen obtained from MCF7 and HT1080 cancer cell lines by Vγ9/Vδ2 T cells.

Experimental conditions were identical as described in **Figure 5.19**. Successful 5T4 antigen cross-presentation by γδ T cells was evaluated by measuring production of IFNγ in cognate CD8 T cells after an overnight coculture in the presence of BRE A. Data points represent mean ±SD of data from two individual experiments**.** X-axis describes conditions for cancer cell lines. PMA/iono, CD8 T cells stimulated with PMA/ionomycyin; ZOL, zoledronate; IRRADIATED, irradiated cancer cells; UNTREATED cancer cells. γδ + CD8 alone and CD8 alone represent negative controls. Significance of differences was calculated by two-way ANOVA followed by Dunnet's multiple comparison test. Significance displayed in comparison to cocultures with PC3 cells + CD8 T cell alone; ns, not significant.



Appendix 7. T2 cell phenotype analysis.
Cells were stained with fluorescently labelled antibodies and analysed by flow cytometry. (A) Shown are the expression CD40, CD80, CD83, CD86, HLA-ABC represented as percentage in CD19+ cells.HLA-ABC expression is also represented as MFI value. Isotype controls are shown in dark blue and the stained markers are shown in light blue. Gated on single, live, CD19+ cells.