Control of $CD4^+$ T Cell Responses by γδ T-APCs

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To my amazing parents and fiancée,

for your limitless support and faith

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'If you're going through hell, keep going'.

Summary

Human Vγ9Vδ2 T cells constitute a novel type of APC (γδ T-APCs) capable of stimulating $CD4^+$ T cell responses. The outcome of γδ T-APC induced $CD4^+$ T cell responses in terms of cytokine profiles, and the physiological implications for infection and autoimmunity, remain unknown.

This study demonstrates that γδ T cells are able to act as potent APCs, inducing proliferation in naive and memory $CD4^+$ T cell populations. Resulting cytokine responses triggered in naive $CD4^+$ T cells included production of IFN-γ and IL-22. Of note, γδ T cells had a greater capacity to promote production of IL-22 in naive and memory T cells than monocytes and monocyte-derived DCs in identical experiments. The microenvironment of γδ T-APCs played a major role in the subsequent polarisation of $CD4^+$ T cell responses, with APCs induced in the presence of IL-15 being superior in promoting IL-22 responses in naive $CD4^+$ T cells compared to γδ T-APCs generated in the presence of other cytokines. Unexpectedly, the IL-22 induction in $CD4^+$ T cells was IL-6 independent, but instead involved TNF-α and ICOS-L, both expressed by the γδ T-APCs. In addition, γδ T-APCs induced in the presence of IL-21 favoured increased induction of IL-10 in $CD4^+$ T cells.

The observation that γδ T-APCs are able to drive IL-22 responses in naive and memory T-cell populations presents a novel function for these APCs, with implications for a multitude of infection/disease scenarios. One such scenario is Inflammatory Bowel Disease (IBD), where IL-22 and γδ T-cells have previously been shown to play significant roles in disease pathogenesis and progression. Indeed, γδ T cells derived from intestinal biopsies are able to act as fully functional APCs. In summary, γδ T-APCs may be involved in the pathogenesis or maintenance of autoimmune inflammation in the intestine and other peripheral sites.

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List of Abbreviations

αGal-Cer - α-Galactosylceramide

AHR - Aryl Hydrocarbon Receptor

APC - Allophycocyanin

APC - Antigen Presenting Cell

BCL6 - B cell Lymphoma 6

BCG - Bacillus Calmette-Guerin

BSA - Bovine Serum Albumin

BTN3 - Butyrophilin 3

CD - Cluster of Differentiation

CFSE - Carboxyfluorescein Succinimidyl Ester

CMV - Cytomegalovirus

c-SMAC - Central-SMAC

CTL - Cytotoxic T Lymphocytes

Cy - Cyanine

DAMPS - Danger Associated Molecular Patterns

DC - Dendritic Cell

DMAPP - Dimethylallyl Pyrophosphate

DNA - Deoxyribonucleic Acid

DRiPS - Defective Ribosomal Products

EDTA - Ethylenediaminetetraacetic Acid

ELISA - Enzyme Linked Immunosorbent Assay

ER - Endoplasmic Reticulum

FACS - Fluorescence Activated Cell Sorting

FcR - Fc Receptor

FCS - Foetal Calf Serum

FICZ - 6-Formylindolo (3,2-b)carbazole

FITC - Fluorescein Isothiocyanate

FOXP3 - Forkhead Box P3

FPPS - Farnesyl Pyrophosphate

FSC - Forward Scatter

GATA3 - GATA Binding Protein 3

HAH - Halogenated Aromatic Hydrocarbons

HEV - High Endothelial Venules

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

IFN - Interferon

IL - Interleukin

IMDM - Iscove's Modified Dulbecco's Medium

IPP - Isopentenyl Pyrophosphate

IS - Immunological synapse

LFA-1 - Lymphocyte Function-Associated Antigen 1

LN - Lymph Node

LPS - Lipopolysaccharide

mAb - Monoclonal Antibody

MAdCAM-1 - Mucosal Addressin Cell Adhesion Molecule 1

mDC - Mature Dendritic Cell

memCD4 - Memory $CD4^+$ T cell

MFI - Median Fluorescence Intensity

MHC - Major Histocompatibility Complex

MLR - Mixed Lymphocyte Reaction

Mono - Monocyte

mo-DCs - Monocyte-Derived Dendritic Cell

nCD4 - Naive $CD4^+$ T Cell

NKG2D - Natural-Killer Group 2, Member D

NK T Cell - Natural Killer T Cell

OX40L - OX40 Ligand

PAH - Polycyclic Aromatic Hydrocarbons

PAMPS - Pathogen Associated Molecular Patterns

PBMC - Peripheral Blood Mononuclear Cells

PCR - Polymerase Chain Reaction

PE - Phycoerytherin

PerCP - Peridinin Chlorophyll

PGN - Peptidoglycan

PMA - Phorbol 12-myristate 13-acetate

PPD - Purified Protein Derivative

PRR - Pattern Recognition Receptor

qPCR - Quantitative PCR

RA - Retinoic Acid

RA - Rheumatoid Arthritis

RNA - Ribonucleic Acid

RORC - Nuclear Receptor ROR-gamma

RPMI - Roswell Park Memorial Institute

RPM - Revolutions Per Minute

RT-PCR - Real Time PCR

RT - Room Temperature

SSC - Side Scatter

TBX21 - T Box Transcription Factor 21

Tc cell - Cytotoxic T Cell

T_{CM} - T Central Memory Cell

TCR - T Cell Receptor

T_{EM} - T Effector Memory Cell

T_{EMRA} - Terminally Differentiated T cell

Tfh - T Follicular Helper Cell

TGF - Transforming Growth Factor

Th cell - T Helper Cell

Th1 - T Helper 1 Cell

Th2 - T Helper 2 Cell

Th17 - T Helper 17 Cell

Th22 - T Helper 22 Cell

Th9 - T Helper 9 Cell

TLR - Toll Like Receptor

TNF - Tumour Necrosis Factor

Treg - T Regulatory Cell

Chapter 1 - Introduction

1.1 - Mounting a Cellular Immune Response

1.1.1 - Innate and Adaptive Immunity

The human immune system evolved for the purpose of protecting the host from invading pathogens, a role performed by a complex network of cells and molecules acting cooperatively. Traditionally, the immune system has been divided into two conceptual groups; innate immunity and adaptive immunity, and these categories are commonly distinguished on the basis of their levels of specificity[1]. Optimal immunity against pathogens requires both of these types of responses.

The leukocytes which belong to the area of innate immunity, including neutrophils, macrophages, dendritic cells, and natural killer cells, possess receptors which recognise a limited number of ligands that are often evolutionarily conserved and expressed by wide ranges of pathogens and stressed tissues. Adaptive immunity is generally considered the domain of lymphocytes, with T lymphocytes and B lymphocytes expressing large repertoires of T cell antigen receptors and immunoglobulins, respectively. These receptors exhibit a vast diversity that is generated somatically through site-specific DNA recombination, allowing for the recognition of any possible antigen encountered. Each receptor of a particular specificity is in theory expressed by an individual lymphocyte clone.

Further characterisation of innate versus adaptive immunity can be defined by a rapid response versus a slower response, as well as short lived immunity versus immunological memory and 'recall' responses[2]. Recent discoveries have called this simplistic dichotomy of the immune systems into question, with the identification of innate cells that are capable of memory[3], and subsets of adaptive cells expressing conserved cell receptors[4]. What is certain is that as the mysteries of the immune system are gradually being unveiled, the complex network of immune components is moving beyond this simple characterisation of innate and adaptive immunity.

1.1.2 - Development of a Cellular Immune Response

The development of a cellular immune response is a complex process, involving multiple steps and different cell types. As a broad overview of the process, which is discussed in detail below, antigens from pathogenic organisms are taken up by cells called APCs (APCs), which process these antigens for presentation on their cell surface. Migration of APCs to the secondary lymphoid organs allows presentation of

antigens to clonally specific naive $CD4^+$ and $CD8^+$ T cells for their activation, allowing an adaptive immune response to be mounted. $CD4^+$ and $CD8^+$ T cells are then able to become effector cells to eradicate the infection, and subsequently become memory cells for rapid response to re-exposure to the antigen. These steps are discussed below.

1.1.3 - APCs; Determining When to Mount an Immune Response

Two fundamental questions exist in immunology. Firstly, how does the immune system decide when to respond to an antigen? And secondly, which kind of immune response should be generated? The first question will be examined here, the second in Section 1.2.

A belief long held by immunologists is that the immune system discriminates between 'self' and 'non-self', with foreign antigens eliciting an immune response and self antigens being subject to immunological tolerance[5]. However, this model has proven to be over-simplistic, not least in the lack of a consensus on what constitutes 'self'. More recent and seemingly more applicable models have since been proposed, termed the pattern recognition receptor model, and the danger model[6]. These models have a significant amount of overlap, and ultimately postulate that the immune system responds to stimuli determined to be 'dangerous' or 'damaging'. At the centre of both models are APCs, and it appears these cells are responsible for answering the question 'when should the immune system respond?'.

The pattern recognition receptor model (PRR) describes the activation of APCs, and innate cells in general, by conserved molecular patterns (PAMPs) expressed by broad ranges of pathogenic and non-pathogenic organisms[7]. Common PRRs include the Toll-like receptors (TLRs) which recognise a range of PAMPs including lipopolysaccharides (LPS), peptidoglycans (PGN), or unmethylated CpG sequences. Danger signals were first suggested to describe an immune system which responds to substances which cause damage, rather than are simply non-self[8]. These signals consist of molecules or molecular structures which cells undergoing stress or cell death produce or release. APCs can recognise these molecules to become activated and initiate adaptive immunity. Danger signals can include 'primal' signals, which initiate activation of innate cells, and feedback signals which modify or enhance ongoing immunity. Common danger signals include membrane expressed molecules such as CD40L, cytokines such as TNF-α, metabolites including ATP, and heat shock proteins[8]. The term DAMPs (damage associated molecular patterns) covers both danger signals and PAMPs, and as such a combination of both models

appears applicable to immune responses[9]. What is fundamentally clear is that APCs play a pivotal role in generating immune responses to pathogens.

1.1.4 - Antigen Presentation

The main function of APCs is to present antigens to responder cells to either initiate specific immunity to that specific antigen, or to tolerise cells to the antigen, in order to prevent inappropriate immunity. Once an antigen has been obtained, it is processed into short peptides and presented on the cell surface via major histocompatibility complex (MHC) molecules. A number of different pathways are available to cells to combine peptides with MHC molecules, and these are discussed below.

1.1.4.1 - MHC Class I Antigen Presentation

The classical pathway of MHC class I antigen presentation actually derives its peptides from intracellular pools of protein fragments. MHC class I molecules are expressed on all nucleated cells of the body, and as such all nucleated cells are able to present antigens on MHC class I molecules. Endogenous protein fragments originating in the cytosol or nucleus of cells are degraded by proteasomes to provide the peptides for presentation[10]. In fact, a large amount of protein produced by cells is immediately degraded after synthesis (referred to as DRiPs, defective ribosomal products), for reasons including defective transcription or translation, failed assembly into larger protein complexes, or incorporation of incorrect amino acids[11]. Upon degradation in the proteasome, resulting peptides are translocated into the endoplasmic reticulum (ER) by the TAP transporter protein. Inside the ER, MHC class I heterodimers are assembled from a polymorphic heavy chain, and a light chain referred to as β_2 microglobulin. The peptide combines with the MHC class I heterodimer, triggering chaperone molecules to release the fully assembled molecule for presentation on the cell surface[12]. Utilising this process, nucleated cells present steady state proteins and also viral proteins to signal to the immune system if the presenting cell is healthy, infected, or transformed.

1.1.4.2 - MHC Class II Presentation

MHC class II molecules are primarily expressed on professional APCs, such as dendritic cells, B cells, and macrophages. External protein complexes derived from pathogens or the general cellular microenvironment are taken up by APCs, and degraded in late endosomal compartments to provide peptides for presentation. Assembly of MHC class II molecules also occurs in the ER, where α and β chains

combine with the invariant chain Ii. The resulting complex is next transported to the MHC class II compartment (MIIC), a late endosomal compartment where Ii is digested to leave a class II associated Ii peptide (CLIP) in the binding groove of the MHC class II molecule. The molecule HLA-DM is required for the facilitation of CLIP exchange for an exogenous peptide. The MHC class II/peptide complex can then be transported to the cell surface for antigen presentation[12].

1.1.4.3 - Cross Presentation on MHC Class I Molecules

The restriction of the MHC class I loading pathway to endogenous antigens is insufficient in situations where naive $CD8^+$ T cells require activation by APCs before they can perform their cytotoxic functions[13]. Despite the fact that endogenous peptides are classically presented on MHC class I molecules and exogenous peptides are presented on MHC class II, an alternate pathway allows for the presentation of material taken up from the cellular microenvironment to be displayed on MHC class I molecules for presentation to $CD8^+$ T cells. This process is called cross-presentation, and allows for APCs to present antigens from pathogens which do not infect APCs, or alternatively to present tumour-derived antigens to $CD8^+$ T cells. Self antigens are also able to be presented in the same way, for the induction of tolerance in a process termed cross-tolerance[14]. Multiple pathways in which antigens are able to be cross-presented exist, the majority of which involve the capture of antigen by phagocytosis.

1.1.4.4 - Endogenous Antigen Presentation on MHC Class II Molecules

A further pathway allows for the presentation of endogenous peptides derived from the cytoplasm or nucleus to be displayed on the MHC class II molecules of APCs for presentation to $CD4^+$ T cells. This is, for instance, necessary for the induction of $CD4^+$ T cell immunity to viruses which may infect APCs, and as such antigens would be obtained from the intracellular rather than the extracellular compartment[15]. A process known as autophagy is responsible for this function[16]. Autophagy is defined as an auto-digestive process which performs the delivery of intracellular debris to lysosomal compartments for degradation and recycling. Via several distinct autophagy pathways[17], this allows for the presentation of endogenous peptides on MHC class II molecules.

1.1.5 - Migration of APCs to Lymph Nodes

An activated APC such as a mature dendritic cell (DC) will present antigens on its cell surface to activate naive T cells. However, in order for APCs to have maximal

contact with naive T cells and induce the appropriate clonal expansion, these cells must migrate to the draining lymph nodes where naive lymphocytes gather and search for antigen. This migration requires the function of a distinct set of molecules, known as chemokines.

Chemokines are a collection of small, structurally related molecules which regulate the trafficking of different subsets of leukocytes[18]. Via the action of corresponding chemokine receptors expressed on the surface of cells, chemokines direct each cell type to its appropriate location via a concentration gradient, and as such play pivotal roles during steady state and in inflammatory scenarios. The repertoire of chemokine receptors expressed on the surface of resting and activated cells is able to indicate their potential localisation and hence their likely role in immunity.

Lymph nodes, also known as secondary lymphoid organs, are a group of strategically positioned locales in which antigen presentation to naive T cells occurs[19]. Naive lymphocytes of both B and T subsets circulate in the blood and lymphatics between lymph nodes in search of their specific antigen. There exist two ports of entry into the lymph nodes, either through the afferent lymphatic vessels, or via high endothelial venules (HEVs)[20,21].The majority of lymphocytes utilise the HEVs for migration into the lymph nodes. A complex process involving a number of molecules is involved in the translocation of lymphocytes into the secondary lymphoid organs. The first molecule involved is the chemokine receptor CCR7, and its ligands CCL21 and CCL19. CCR7 is constitutively expressed by conventional naive T lymphocytes and directs these cells to localise at tissues possessing the relevant ligands[22–24]. CCL21 is constitutively expressed by HEVs, and CCL19 is expressed by the lymphatic endothelium and LN interstitial cells. As such, both chemokines are ideally located to direct naive lymphocyte migration to the secondary lymphoid organs. L-selectin, also referred to as CD62L, mediates tethering and rolling of lymphocytes along the endothelium via interaction with its ligand PNAd[19]. The lymphocytes are slowed down by this process, allowing CCR7 to bind to its ligand. Upon the ligation of CCR7, activation of integrins such as LFA-1 occurs, which allows it to interact with the intercellular adhesion molecule ICAM-1[25]. This interaction causes the arrest of rolling T cells, allowing for lymph node transmigration. Upon entering the lymph nodes, lymphocytes are directed to B or T cell areas where they attempt to encounter their specific antigen.

There are two main mechanisms by which antigens themselves are delivered to the secondary lymphoid organs. The first is when antigenic material enters the

lymphatics directly, independently of APCs, and makes its way to the lymph nodes where it can be taken up and processed by lymph node-resident DCs. The second is by transport from the tissue to the lymph node by migratory APCs themselves[26,27]. Regardless of the mechanism, an APC is required to process and present the antigen. Peripheral APCs are able to migrate to lymph nodes via the action of CCR7[28,29], as well as other chemokine receptors such as CXCR4 and CCR4. These chemokine receptors are upregulated upon maturation or activation of relevant APCs. While in transit, APCs upregulate the necessary molecules to attract and stimulate naive lymphocyte responses[30].

1.1.6 - Activation of αβ T Cell Responses

Once an activated APC has reached the secondary lymphoid organs, it is able to interact with naive $CD4^+$ or $CD8^+$ T cells to stimulate an adaptive immune response.

1.1.6.1 - The Immunological Synapse

T cell recognition of antigen presented by APCs requires intimate contact between cells, and as such the formation of an immunological synapse, which directs the redistribution of cell surface molecules towards the interface between cells[31,32]. This process is accompanied by cytoskeletal rearrangement and cellular polarisation. Molecules necessary for the formation of the immunological synapse and for T cell activation segregate into distinct areas within this interface. A clear identification of the mature synapse is the formation of a 'bulls eye' pattern of molecules, composed of a central supramolecular activation cluster (c-SMAC), surrounded by a peripheral adhesion ring (p-SMAC). The c-SMAC is enriched for molecules important in T cell signalling, such as the TCR, MHC-peptide, CD28, and CD2, whereas the p-SMAC is composed of the LFA-1 adhesion molecules and its ligand ICAM-1, and the cytoskeletal molecule talin. The formation of the immunological synapse is divided into several distinct stages; polarisation of T cell surface molecules, initial adhesion of cells, the initial signalling of synapse formation, and lastly the sustained signalling of a mature synapse. With the immunological synapse formed, three signals are provided by the APC to the naive T cell for optimal activation of T cell responses.

1.1.6.2 - Overview of 3 Signal Model

The activation and fate of naive T cells is determined by 3 signals, and is referred to as the three signal model[33]. Signal 1 is an antigen specific stimulatory signal, mediated via T cell receptor triggering upon interaction with the relevant peptide-MHC complex. Signal 2 is a co-stimulatory signal, provided by a variety of co-

stimulatory receptors and ligands expressed by the T cell and APC, respectively. Signal 3 is a polarising signal, and is responsible for directing the naive T cell differentiation towards the appropriate functional quality. Each signal is discussed in turn below.

1.1.6.3 - Signal 1: Peptide/MHC recognition

As previously stated, signal 1 for naive T cell activation is provided by TCR-peptide/MHC interaction. Despite this, TCR-pMHC interactions alone are not sufficient to provide signal 1 to the T cell[34,35]. Instead, the co-receptors CD4 or CD8 are involved in binding to the pMHC complex and in the downstream signalling. CD8 molecules specifically interact with MHC class I molecules, whereas CD4 interacts with MHC class II molecules. CD4 and CD8 are differentially expressed on subsets of T lymphocytes; CD4 expression denotes T helper cells, whereas CD8 is found on cytotoxic T cells. During T cell activation, the TCR initially binds to the peptide-MHC complex, upon which CD4 or CD8 can then bind to the MHC molecule[36], thereby stabilising the complex. The principal role of both molecules is in the recruitment of Lck, a Src tyrosine kinase, to the TCR-pMHC complex[37]. This complex and downstream signalling allows for signal 1 to be provided for naive T cell activation. Of note, it is currently unclear how provision of signal 1 occurs for "unconventional" non-MHC-restricted T cells that often lack both CD4 and CD8, such as NKT cells and other CD1-restricted T cells as well as γδ T cells.

1.1.6.4 - Signal 2: Costimulation

Signal 2, or the co-stimulatory signal, is fundamental for triggering a naive T cell response to antigen, and plays an important role in the ultimate outcome of the APC-T cell interaction. Signal 2 is actually able to provide stimulatory or inhibitory signals, thereby tipping the balance between immunity and tolerance depending on which co-stimulatory or co-inhibitory molecules are involved in the interaction[38]. Signal 2 also has a primary role in 'fine-tuning' adaptive immunity by affecting the magnitude and quality of the ensuing response. A large number of co-stimulatory molecules have been identified, and these can either be constitutively expressed on APCs or upregulated upon activation.

Perhaps the best known and most studied group of co-stimulatory molecules belongs to the immunoglobulin superfamily[39,40], comprising CD28 and CTLA-4 (CD152) with their ligands CD80 and CD86. CD28 is constitutively expressed on naive and activated T cells, whereas CTLA-4 becomes upregulated on activated T cells only; it is absent on naive and resting T cells. Both CD80 and CD86 become

upregulated on APCs following their activation. CD80 and CD86 alternatively bind CD28 during T cell activation to provide a co-stimulatory signal 2 to the naive T cell. Conversely, CD80/86 ligation with CTLA-4 provides an inhibitory signal to the T cell.

Other co-stimulatory molecules such as ICOS (inducible co-stimulator of signalling) become upregulated on activated T cells, and its ligand ICOSL allows for further manipulation of the T cell response once it has been initiated[41]. Lastly, adhesion molecules themselves have also been identified to play a co-stimulatory role. Such molecules include LFA-1 and its ligand ICAM-1, which in addition to forming the immunological synapse help stabilise the transmission of T cell receptor mediated signals[42,43]. Many more co-stimulatory and co-inhibitory molecules exist, with both overlapping and distinct roles in manipulating the adaptive immune response, and each individual effect on $CD4^+$ and $CD8^+$ T cell responses will be discussed below.

1.1.6.5 - Signal 3: Polarisation

Signal 3 is referred to as the polarising signal, and plays a major role in directing the type of immune response generated[33]. This signal is provided by the APC, and also from other locations and context dependent sources, predominantly in the form of polarising cytokines, such as IL-12 and IL-6. Many factors influence the types of polarising signals provided to T cells, not least the mechanism of activation of the APC itself. Signal 3 in the context of $CD4^+$ T cell (and $CD8^+$ T cell) polarisation is discussed in detail in a later section.

1.1.7 - Memory CD4 Subsets

Upon stimulation of naive $CD4^+$ T cell responses, clonally expanded cells can go on to become effector cells, producing cytokines which aid the immune response, and can also become memory cells, which exhibit rapid responses to re-infection. It has been demonstrated that $CD4^+$ T cells go through a number of stages of memory, characterised by their expression of the common leukocyte antigen CD45, and their chemokine receptor expression[44].

Naive $CD4^+$ T cells are cells which, by definition, have not encountered their specific antigen, and represent a population of cells that is able to give rise to effector and memory $CD4^+$ T cells[45]. CD45 is a tyrosine phosphatase which regulates the activation threshold of $CD4^+$ T cells, with the longer isoform CD45RA instilling a higher activation threshold on the expressing cell, and the shorter isoform CD45RO allowing for a lower activation threshold[46,47]. Naive $CD4^+$ T cells can be characterised by their expression of the longest isoform of CD45, known as

CD45RA, and expression of the lymph node homing molecules CCR7 and CD62L, which are essential for the circulation of those cells throughout the secondary lymphoid organs. Upon activation, naive $CD4^+$ T cells produce IL-2 but little other effector cytokines, and proliferate. This activation is dependent on the naive $CD4^+$ T cells receiving signals 1 and 2, and signal 3 for differentiation into effector cells. Naive $CD4^+$ T cells which receive signal 1 but no co-stimulation are rendered anergic and subsequently are unable to respond to antigen, and are hence classed as tolerised[44].

Memory $CD4^+$ T cells can be further differentiated into a number of memory subsets. This is due to the fact that some memory $CD4^+$ T cells mediate rapid responses to antigens in the periphery, whereas other $CD4^+$ memory cells continue to circulate the lymph nodes in their search for antigen. These subsets both express CD45RO, but differentially express CCR7, and to a lesser extent CD62L. Central memory cells (T_{CM}) are classed as memory cells which re-circulate through secondary lymphoid organs, following the migratory routes of naive cells, and as such retain expression of CCR7 and CD62L upon becoming memory cells. T_{CM} cells are phenotypically $CD45RO^+CCR7^+CD62L^+$. In contrast, effector memory cells (T_{EM}) are those which are found in the periphery, and as such do not express CCR7 and possess much lower levels of CD62L than T_{CM} cells.

In terms of activation threshold, T_{EM} cells are much more responsive to TCR stimulation in the presence or absence of co-stimulation than are T_{CM} cells, which in turn are much more responsive than naive $CD4^+$ T cells. T_{EM} cells also express a different repertoire of chemokine receptors ideally suited for direction to the peripheral tissues and inflammatory sites. This repertoire includes tissue specific receptors and integrins such as CD103/CLA and β1/β2 integrins, respectively[48], and inflammatory chemokine receptors such as CCR1, CCR3, and CCR5[49,50]. T_{CM} cells instead express intermediate levels of tissue integrins, as well as CCR4, CCR6, and CXCR3 to varying degrees. Upon stimulation of each memory subset, T_{CM} cells favour production of IL-2 only, whereas T_{EM} cells produce effector cytokines such as IFN-γ and IL-4.

Upon activation of each memory subset, cells are thought to mainly progress into the next memory subset in order. As such, naive cells potentially differentiate towards T_{CM}, and T_{CM} towards T_{EM} upon TCR ligation, though it is unclear whether this is the only possible pathway of memory cell differentiation. This observation is supported by assessing the length of telomeres, which shorten as a process of cell

division[51]. As such, naive cells possess much longer telomeres than do T_{EM} cells. This progressive differentiation of memory subsets identifies T_{CM} cells as a clonally expanded, ligand-primed population which can generate a new wave of effector cells, whereas T_{EM} cells represent a pool of cells able to rapidly enter peripheral tissues to mediate inflammation. Further dissection of memory subsets has also been achieved based on their expression of the co-stimulatory receptors CD27 and CD28[52]. The same subsets of memory cells can be identified in $CD8^+$ T cells as well, in addition to terminally differentiated (T_{EMRA}) cells, which display a $CD45RA^+CCR7^-$ phenotype[44].

1.1.7.1 - Asymmetric Division

An interesting phenomenon has been investigated for its potential role in generating different memory subsets of daughter cells from a single progeny. This process, known as asymmetric division, refers to the division of a cell whereby unequal inheritance of critical molecules is achieved in individual daughter cells, allowing for the divergence of cell fates[53]. This has been identified in studies of $CD4^+$ and $CD8^+$ T cells, and is a potential mechanism by which differential memory subsets can be generated. Indeed, 'proximal' and 'distal' daughter cells have been identified to possess different sets of memory markers, representing T_{EM} and T_{CM} cells, respectively.

1.2 - Polarisation of the T Lymphocyte Immune Response

The second question in immunology is: "How is the appropriate immune response generated for a particular pathogen or threat?" The process of T cell polarisation is able to, at least partially, answer this question. It has widely been accepted in immunology that no one effector response is able to deal with all forms and types of pathogens, for example an immune response against intracellular viruses must involve different cells and molecules than a response against extracellular bacteria or helminths. This observation led to the discovery that effector T helper cells could be categorised into functionally distinct subsets. The first evidence of this was reported in the form of the Th1-Th2 paradigm; two effector states of $CD4^+$ T cells which were functionally opposite to each other and were able to counter-regulate the opposing subset[54]. This phenomenon was first identified in murine T cell clones but subsequently reproduced with human T cells[55]. The control over CD4 functional fate was reported to be controlled by the antigen presenting cell, by a process called polarisation. Since this discovery, numerous factors have been identified as playing

a role in polarisation, forming a complex web or polarising factors. Arguably the most important ones amongst these are the polarising cytokines, which direct CD4 differentiation and have been extensively studied for this role. Since this discovery, many more T helper subtypes, or lineages, have been characterised to form an expanding view of the polarisation of $CD4^+$ T cells. Strong evidence supported the idea that these effector fates do not derive from distinct precursor cells but instead develop from single $CD4^+$ precursor populations[55].

Each $CD4^+$ T cell subset is characterised by a signature cytokine profile, which facilitates the characteristic immune effects related to each subset[45]. Further to this, each lineage has been reported to express a distinct chemokine receptor repertoire, aiding identification of lineages and indicating homing potential. Another important factor in defining a $CD4^+$ T cell as a particular lineage is built around the premise that each lineage expresses a 'master transcription factor'[56]. Each unique subset identified can be defined by a critical transcription factor which directs the cell fate, effector cytokine production, phenotype, and also inhibits differentiation towards alternate cell fates and lineages. Due to the distinct differences in each lineage, each is associated with a particular arm of immunity. The polarisation of CD4 responses in mice has been shown to be reasonably clear-cut, with either Th1 or Th2 responses observed in different experimental models. However, in human immunity the different subsets do not segregate as readily as in mice, with both Th1 and Th2 responses overlapping in numerous infection and disease scenarios[57]. This fact is observed with other T helper lineages as well. Given the discrepancies between mouse and human $CD4^+$ T cell polarisation mechanisms, the data discussed here are exclusively relevant for human immunity unless otherwise specified. A summary of $CD4^+$ T cell polarisation is displayed in Table 1.1, at the end of the section.

1.2.1 - The Th1 Cell Lineage

Th1 cells are characterised by the secretion of IFN-γ, representing their predominant effector cytokine, and expression of the master transcription factor, *TBX21*. The overall cytokine profile of human Th1 cells typically includes IFN-γ, IL-2 and TNF-β, as well as TNF-α and GM-CSF which are expressed across most T helper subtypes[55]. The main role of Th1 cells is in the combat of intracellular pathogens.

The polarisation of naive $CD4^+$ T cells towards a Th1 phenotype has been extensively shown to be mediated by the cytokine IL-12, which is secreted by activated DCs and macrophages during the activation of $CD4^+$ T cells[58]. IL-12 acts

via STAT4-mediated signalling, and induces the expression of IFN-γ. IFN-γ can then act in an autocrine fashion to induce the expression of TBX21 via STAT1 signalling[59]. IL-12 also promotes a Th1 response by the inhibition of Th2 responses and the reversal of Th2 polarisation in humans. While IFN-α and IFN-β, which are produced by many cell types including DCs and macrophages[60], also promote Th1 polarisation via STAT4[61], IL-12 is necessary for optimal Th1 induction[57] and induces much stronger Th1 responses than IFN-α/β. One potential explanation is that the IL-12-induced activation of STAT4 is longer lived, allowing full Th1 differentiation, whereas IFN-α/β activation appears more transient[61]. The growth factor IL-2, also important in optimal Th1 polarisation, synergises with IL-12 to prolong the activation of STAT4, and such a synergy is not seen with type 1 IFNs. Further to this, patients who exhibit deficiencies in IL-12 receptor-mediated signalling but possess intact type 1 IFN signalling pathways, have an impaired Th1 immunity against pathogens including *Mycobacteria* and *Salmonella* species[62,63].

As previously mentioned, Th1 cells are characterised by uniform expression of the transcription factor *TBX21*. This transcription factor is responsible for the upregulation of numerous genes important in Th1 regulation and immunity, and the suppression of genes important for alternative T helper lineage differentiation and function[59]. A further characterisation of human Th1 cells revealed the expression of chemokine receptors CXCR3 and CCR5, the combination of which is preferentially expressed by the Th1 lineage over other subtypes[64].

Th1 cells exert their immunomodulatory effects predominantly via the action of IFN-γ. This cytokine possesses far reaching effector functions to modulate a large number of immune and non-immune cells[65]. IFN-γ is able to induce the upregulation of APC markers such as MHC class I and II, and co-stimulatory molecules including CD80/86. A major role of IFN-γ during a Th1 immune response is the recruitment and activation of several immune cell subsets, such as macrophages and $CD8^+$ cytotoxic T cells, which function to combat intracellular bacteria[45], and IFN-γ can also act on APCs such as DCs to induce the production of IL-12, thereby forming a further positive feedback loop[65]. One final function of IFN-γ is in the promotion of antibody class switching, towards an IgG isotype response as opposed to IgE.

Th1 responses have been investigated and identified in a wide range of diseases, from infectious scenarios to autoimmunity. The types of responses induced by Th1 immunity have also been identified as optimal for the combating of various tumours, due to the ability to aid immunity against cellular targets.

1.2.2 - The Th2 Cell Lineage

The antagonistic lineage to Th1 cells was historically denoted as the Th2 subset. This lineage specialises in the production of the cytokines IL-4 (as its signature cytokine), as well as IL-5, IL-13 and, non-exclusively, IL-10. Recent additions to the Th2 cytokine profile also include IL-9 and IL-25. The main function of Th2 cells is to promote immune responses dominated by the antibody isotype IgE, including eosinophil and basophil immunity against extracellular infections by parasitic helminths[64].

Many studies have shown the importance of IL-4 in the differentiation of naive $CD4^+$ T cells towards the Th2 lineage[54]. However, with professional APCs such as DCs and macrophages lacking in the production of IL-4, the definitive source of exogenous IL-4 remains elusive. A number of cell types have been described as producing IL-4 and represent potential sources, including NK cells[66], mast cells, and basophils[55,67], however these cell types are unlikely to express IL-4 in the correct location and at the necessary time during Th2 polarisation. A number of studies have reported the production of low levels of IL-4 by stimulated naive $CD4^+$ T cells themselves[68,69], forming a potential autocrine feedback loop. A number of other molecules have been reported to promote Th2 polarisation other than IL-4. Prostaglandin E_2, produced by APCs, can promote the Th2 lineage by inhibiting the production of IFN-γ by activated $CD4^+$ T cells, and by inhibiting APC production of IL-12[67]. Further to this, IL-6, a cytokine produced by a wide range of cell types including APCs, has been identified to promote Th2 responses under certain circumstances[70,71]. IL-6 acts to promote the expression of IL-4, at least in mice, whereas in humans the role of IL-6 in the regulation of IL-4 expression is less clear. More recently a specialised type of epithelial cell in the intestine, called tuft cells, have been shown to induce Th2 type responses in mice via secretion of IL-25[72].

The major route of Th2 differentiation is via the action of IL-4 triggered STAT6 signalling to induce expression of *GATA3* in differentiating $CD4^+$ T cells, the master transcription factor of the Th2 lineage[73,74]. GATA3, in combination with STAT5 induced by the activation of the growth factor IL-2, induces the secretion of IL-4 by Th2 cells, as well as IL-5 and IL-13. Th2-derived IL-4 acts in an autocrine manner to maintain and promote Th2 phenotype and inhibit Th1 polarisation via STAT4 signalling and inhibition of IFN-γ production. PGE_2 and IL-6, among other factors, likely promote Th2 differentiation by the inhibition of IFN-γ and Th1 polarisation, rather than by direct activation of *GATA3* and IL-4, allowing for $CD4^+$ T cells themselves to induce their own Th2 polarisation. It is clear that Th2 differentiation

involves significantly more interaction of molecules than the IL-12 mediated Th1 induction.

Th2 cells express a unique repertoire of chemokine receptors which direct their functional potential, in the same manner as Th1 cells. These chemokine receptors include CCR3 and CCR4, which are co-expressed by eosinophils and basophils, allowing for the recruitment of all necessary cell types to the site of Th2 mediated immunity[50,64].

Th2 cells, via the secretion of the cytokines IL-4, IL-5, and IL-13, manipulate several immune subsets for the combat of extracellular parasites. B cells respond to both IL-4 and IL-13 to become activated and class-switch to produce IgE antibodies[45], while IL-5 aids the differentiation and proliferation of basophils and their precursors into matured basophils. IgE antibodies themselves trigger activation and degranulation of basophils and mast cells by cross linking cell-surface Fc receptors[75]. The strongest effect of IL-5 in humans is the activation of eosinophils, inducing terminal maturation, improving survival by delaying apoptosis, and enhancing effector functions[76]. Altogether these cytokines manipulate the immune response towards appropriate anti-parasitic immunity.

Th2 cells have received much study for their role in allergic reactions, stemming from the fact that allergic symptoms and immune pathways are similar to those seen with anti-helminth responses[77,78]. Indeed, Th2 cells, in particular a high ratio of Th2 to Th1 cells and cytokines, have been identified in a range of allergic reactions from asthma[79] to chronic dermatitis[80], characterised predominantly by infiltration of eosinophils and Th2 cells[76]. Although work on Th2 cells and associated eosinophil infiltration has largely been conducted in mouse models, Th2 involvement has also been confirmed in human studies[81]. The role of Th2 in inappropriate immunity led the way in identifying different T helper lineages involved in immune pathologies.

1.2.3 - Limitations of the Th1/Th2 Paradigm

Since the inception of the Th1/Th2 paradigm into immunology 30 years ago, it became a widely accepted phenomenon in numerous infectious models, providing a framework for studying and understanding the fundamentals of $CD4^+$ T cell biology. However, almost as soon as the paradigm was postulated, flaws in the model were reasoned[82–84], and over the past 10 years the paradigm has been amended considerably.

One initial limitation was the identification that $CD4^+$ T cells, particularly in humans but also in murine systems, would not differentiate into distinct Th1 and Th2 responses, and often a combination of both effector phenotypes could be observed. Further to this, IFN-γ and IL-4 co-expressing cells are a common occurrence in the analysis of T helper lineages, which are termed Th0 cells. Further to this, other cytokines were identified as either being produced by activated $CD4^+$ T cells, such as IL-17, or involved in the differentiation of T helper cells, such as IL-23, but were not directly related to the Th1/Th2 system at the time. Thus the paradigm has been significantly adapted and extended to include a variety of other T helper lineages.

1.2.4 - The Th17 Cell Lineage

The discovery of the Th17 cell lineage was the first major expansion of the T helper lineage paradigm, and this subset has become arguably one of the most studied lineages to date. This subset is characterised by the production of its signature cytokine IL-17A, alongside IL-17F, IL-22, and IL-21, without the production of IFN-γ and IL-4. Th17 cells are thought to primarily function in the combat of extracellular bacterial and fungal infections[85].

The identity of the new Th17 lineage was studied extensively in murine models before human counterparts were identified. The search began with the discovery of the IL-12 related cytokine IL-23, both of which share a common p40 subunit[86]; in the case of IL-23, p40 pairs with the p19 subunit. IL-23 was subsequently shown to promote IL-17 responses in murine memory T cells[87]. The first impressions of the IL-17 producing cells were that they represented Th1 cells capable of producing IL-17, due to the common features of IL-12 and IL-23 and the ability of $CD4^+$ T cells to co-express IFN-γ and IL-17 at the same time[88,89]. However, the tendency of cells to either produce IFN-γ or IL-17 alone suggested otherwise[90]. Subsequent studies identified IL-17 producing cells as a separate lineage to Th1 and Th2, and as such were termed Th17 cells. This lineage was inducible in naive $CD4^+$ T cells via combinations of IL-1β, IL-6, IL-23, and TGF-β, and expressed the *RORγt* transcription factor as their master regulator[91]. Subsequent studies identified these cells in a range of pathologies including a significant role in autoimmune models. The Th17 subset was finally identified in humans shortly afterwards[85].

Following their identification, the polarising cytokine requirements of human Th17 cells were investigated by several groups and generated controversy in the field due to a number of inconsistent studies and differences to murine Th17 cells. In comparison with the murine Th17-polarising cytokines IL-6 and TGF-β, it was

surprising that a number of studies found TGF-β actually possessed an inhibiting effect on human Th17 generation[92], while in some studies IL-6 was seemingly dispensable[93,94]. From these initial studies, IL-1β and IL-23 exhibited a prominent role in Th17 differentiation. However, a number of subsequent studies found an inability to induce Th17 cells using just these cytokines[95,96].Further work revealed that the presence of low doses of TGF-β in serum, utilised in culture media, played a prominent role in Th17 differentiation, and IL-6 played an additive role in the induction of IL-17[97,98]. As such, the consensus to date is that low dose TGF-β, alongside IL-1β and IL-6, induce the differentiation of naive $CD4^+$ T cells towards a Th17 lineage. The role of IL-23 in Th17 differentiation appears obscure, in that the IL-23 receptor is only induced upon activation by other polarising cytokines. Indeed, the optimal role of IL-23 appears to be the addition after a few days of culture[85], allowing maximal Th17 cell induction. In this way the IL-23 function in humans resembles that in mice, where it is responsible for maintaining the differentiation of Th17 cells and promoting their responses[99], as well as regulating memory Th17 responses, rather than in the direct differentiation of naive $CD4^+$ T cells. IL-21 as well appears to play an autocrine role in humans as in the mouse, produced by Th17 cells themselves to promote their own phenotype[100].

While human Th17 cells are defined by their expression of IL-17A, they also co-express IL-17F, IL-22, IL-21, IL-26 and at times IFN-γ or IL-10. This complex mix of cytokines is reflected in the polarising cytokine combinations required for Th17 differentiation, with each having a distinct effect on the effector cytokines and phenotype. IL-17F is promoted by TGF-β, IL-23, and IL-1β. IL-22 and IL-21 are promoted by IL-23, IL-21, and IL-1β, and suppressed by TGF-β. IL-10 is inhibited by IL-1β, whereas IFN-γ is promoted by IL-1β alongside IL-21, IL-6, and IL-23. Lastly, IL-26 is promoted by both TGF-β and IL-1β. As expected, most Th17 cytokines, with the exception of IFN-γ and IL-22, are suppressed by IL-12. This complex network of induction and suppression highlights Th17 cells in humans, and indeed in mice, as a heterogeneous lineage that is highly regulated by the cytokine microenvironment [92–94,97,98,100].

Human Th17 cells, like Th1 and Th2 cells, can be identified by the expression of specific chemokine receptors. CCR6 and CCR4 were both found to indicate the expression of IL-17 but not IFN-γ by $CD4^+$ cells[85], directing these 'true' Th17 cells towards inflammatory sites. Interestingly, IL-17/IFN-γ double positive cells express a distinct chemokine receptor profile, consisting of CCR6 and CXCR3, again highlighting the heterogeneity of IL-17 producing $CD4^+$ T cells. $IL\text{-}17^+$ cells can also

be identified by their expression of IL-23R. This receptor is absent on naive $CD4^+$ T cells, but is induced by the action of TGF-β and IL-1β; subsequent action of IL-23 induces upregulation of its own receptor[101].

The master transcription factor *RORC* (murine homologue *RORγt*) is expressed by human Th17 cells in a restricted manner, while co-expression of *TBX21* or *GATA3* denotes the cell's ability to co-express IFN-γ or IL-4, respectively[85]. *RORC* expression is regulated via STAT3 signalling, which can be induced directly or indirectly, by IL-6, IL-1β and TGF-β[102,103]. Several reports have shown that TGF-β alone, IL-21 alone, or the combination of IL-6 and IL-1β can induce the expression of *RORC* in human naive $CD4^+$ T cells[92,98,100]. Interestingly however, the expression of *RORC* alone does not always correlate with the expression of IL-17, as combinations of polarising cytokines are required for IL-17 production, indicating potential roles of other transcription factors.

Th17 cells may play significant roles in immunity against extracellular pathogens and fungi, due to the existence of memory $CD4^+$ T cells specific for pathogens such as *C.albicans*[85]. IL-17 functions to mediate pro-inflammatory functions; downstream effects include induction of pro-inflammatory cytokines and chemokines by acting on mesenchymal and myeloid lineage cells, induction of antimicrobial peptides by epithelial cells, and neutrophil expansion and recruitment to inflammatory sites[104]. Since their discovery, Th17 cells have been extensively studied in the pathogenesis of autoimmune diseases, and have been shown to play significant roles, which were previously attributed to Th1 cells prior to the characterisation of IL-23, including psoriasis, inflammatory bowel diseases, rheumatoid arthritis, among other diseases[93,105].

1.2.5 - The Treg Lineage

In addition to the differentiation towards effector T helper lineages mediating immunity against various pathogens, $CD4^+$ T cells also possess the ability to differentiate towards suppressive cells, known collectively as regulatory T cells (Tregs). Tregs in humans are either generated in the thymus and are referred to as natural Tregs (nTregs), or can be induced in the periphery from naive $CD4^+$ T cells, known as inducible Tregs (iTregs)[106]. Only iTregs will be discussed here.

Human Tregs were first characterised as $CD4^+CD25^+$ T cells[107], and subsequently shown to express the master transcription factor *FOXP3*[108]. Of note, CD25 and *FOXP3* are also expressed by activated human $CD4^+$ T cells at various

timepoints[109]. However, it appears the transient expression of *FOXP3* does not confer a suppressive phenotype, whereas sustained expression is an inherent feature of Tregs[110]. Further surface markers such as CD45, CD127, and CD62L have all been reported to discriminate Tregs from effector cells, and also between certain Treg subsets[111–113]. However, these markers are also expressed at varying points in the effector $CD4^+$ T cell lifecycle, and reliable identification of Tregs in humans remains problematic.

Human inducible Tregs require TCR stimulation for their function, and TGF-β is key in Treg polarisation[114]. However, the exact mechanisms by which Tregs are induced by APCs in humans *in vivo* remains elusive. Human Tregs possess several mechanisms by which immunity can be suppressed[115]. The co-inhibitory molecule CTLA-4 is constitutively expressed by Tregs, allowing for a number of mechanisms to control effector T cell responses[116]. Treg subsets are also capable of producing the immunomodulatory cytokines TGF-β and/or IL-10, which can have context-dependent suppressive functions.

Due to their suppressive ability, Tregs have therapeutic potential in autoimmunity or allergy, in that they represent a mechanism by which inappropriate immune responses can be subdued. A number of groups are therefore investigating the induction of tolerance by Tregs in autoimmune settings[117,118]. Akin to many elements of the immune system, Tregs present a 'double-edged sword'; their benefit in suppressing inappropriate immune responses to self is subverted in tumour settings, where Tregs are involved in restricting anti-tumour immunity[119].

1.2.6 - The Th22 Cell Lineage

One of the more recent lineages to have been described is that of Th22 cells. These cells are characterised by their production of IL-22, in the absence of IFN-γ, IL-17 or IL-4 secretion. These cells have also been reported to co-express TNF-α, IL-10, and IL-13. They have since been shown to facilitate tissue homeostasis, antimicrobial responses in the skin and mucosa, and tissue repair.

IL-22 was originally thought to be a Th1 cytokine, until the identification of Th17 cells, which often co-express IL-22, and the observation that both IL-17 and IL-22 possess similar functions in immunity. Indeed, murine Th17 cells consistently express both cytokines and *RORγt* appears essential for IL-22 production. However, given the identification of $CD4^+$ T cells producing IL-22 in the absence of IL-17, and roles for IL-22 in tissue repair where IL-17 is redundant, it was shown that the two cytokines are differentially regulated in humans[120]. Further to this, induction of

RORC in human $CD4^+$ T cells does not permit the production of IL-22, signifying differential regulation of RORC and IL-22 expression. This led to the identification of a population of Th22 cells in humans by several groups[121–123]. The cytokine requirements for polarisation of native $CD4^+$ T cells toward a Th22 phenotype appear to be a combination of IL-6 and TNF-α, whereas the addition of IL-1β induces the expression of IL-17 as well. IL-23 appears to play a role in promoting Th22 responses in humans, although the effects of IL-23 on IL-22 expression are much more limited compared to the promotion of IL-17. The action of TGF-β has been noted to inhibit IL-22 production.

The aryl hydrocarbon receptor transcription factor (*AHR*) has been extensively studied and shown to be fundamental in both human and murine Th22 responses, representing their master transcription factor. *AHR* is a ligand-activated transcription factor involved in xenobiotic responses, with emerging roles in immune responses. Its ligands include polycyclic aromatic hydrocarbons (PAHs) and halogenated aromatic hydrocarbons (HAHs), and the *AHR* itself is localised predominantly in the cytoplasm of cells[124]. Upon interaction with its ligands, transformation and nuclear translocation of *AHR* occurs for regulation of gene transcription. *AHR* activity features in many processes, including cell proliferation, differentiation and cytokine secretion[125]. *AHR* ligands have been shown to directly promote the expression of IL-22 in naive $CD4^+$ T cells stimulated via their TCR[126]. *AHR* activation favours IL-22 expression and partially inhibits *RORC* and IL-17 expression. In humans, IL-21 has also been reported to play a role in IL-22 induction, acting via STAT3 to control *AHR* interaction with the IL-22 promoter[127]. While much is known of the downstream effects of *AHR* activation, little is currently known about the regulation of *AHR* expression by polarising factors, particularly IL-6 and TNF-α.

The chemokine receptor repertoire of polarised human $CD4^+$ T cells has been reported to include the expression of CCR6, CCR4, and CCR10[121,123]. Expression of the cutaneous lymphocyte antigen CLA has also been reported on Th22 cells. The migratory properties of Th22 cells have been suggested to involve relocation to the skin and mucosal sites where the actions of IL-22 are most pronounced during immune responses.

IL-22 itself is a member of the IL-10 family of cytokines[128,129]. Its receptor is composed of a heterodimer of the IL-10Rβ-chain and an IL-22R chain. Expression of the IL-22 receptor is restricted to non-haematopoietic cells; immune cells such as lymphocytes and myeloid cells lack the receptor, whereas epithelial cells,

keratinocytes, hepatocytes, and other non-immune cells exhibit responsiveness to IL-22. As such, IL-22 plays a key role in the crosstalk between immune and non-immune cells. One exception to this rule is the expression of IL-22R on human monocyte-derived macrophages, though the *in vivo* relevance of this expression is uncertain. A soluble form of the receptor, known as IL-22 binding protein, is able to regulate IL-22 function by specifically binding IL-22 at higher affinities than the surface bound receptor, to limit receptor binding and signalling[130]. The cellular effects of IL-22 are mostly organ specific, with the target cells differing depending on the context. The major function of IL-22 is in the induction of epithelial immunity against extracellular pathogens, inducing the secretion of antimicrobial peptides, and also inducing migration, re-epithelialisation and proliferation of epithelial cells[128]. IL-22 also inhibits the differentiation and induces pro-inflammatory gene expression in keratinocytes[131]. Significant roles have also been highlighted in the process of wound healing[132,133,134]. Overall, IL-22 functions to maintain barrier function and aid repair of tissue damage.

Since their identification, Th22 cells have been investigated in a number of diseases. These roles have included infections such as hepatitis[135] and tuberculosis[136], autoimmune conditions such as rheumatoid arthritis[137] and psoriasis[138], and in certain cancers[139].

1.2.7 - The Tfh Cell Lineage

A further T helper lineage identified is that of T follicular helper cells (Tfh cells). These cells are characterised by the expression of the chemokine receptor CXCR5, co-stimulatory molecules ICOS and CD40L, and production of the cytokine IL-21[140,141]. Tfh cells have been identified to play fundamental roles in aiding B cell responses, a role originally attributed to Th2 cells.

Tfh cells were first identified as a subset of $CD4^+$ memory T cells which reside in the B cell areas of secondary lymphoid organs in humans[140,141]. However, 15 years later the exact differentiation requirements of Tfh cells remain unclear. A number of cytokines have been reported to induce the production of IL-21 in naive $CD4^+$ T cells in both humans and mice, including IL-6, IL-27, and IL-21 itself, via the action of STAT3[142,143]. IL-12 has also been reported to induce the expression of IL-21 in human cells[144]. These cytokines are indeed able to induce several characteristic markers of Tfh cells, including CXCR5 and ICOS, and *BCL6*. However, expression levels of these molecules are lower than those observed in Tfh cells directly isolated from germinal centres[142]. *In vitro* differentiated Tfh cells also lack the expression of

other Tfh associated molecules. For these reasons, it is believed co-stimulatory molecules may play a greater role in the induction of Tfh cells.

The transcription factor B-cell lymphoma 6 (BCL6) has been identified as the master regulator of Tfh differentiation, expressed by Tfh cells isolated from germinal centres based on CXCR5 expression[140,141]. BCL6, in contrast to the other master transcription factors, is actually a transcriptional repressor, and as such it was initially difficult to attribute the differentiation of Tfh cells to BCL6 function. However, several mechanisms of action have been suggested; BCL6 can repress other master transcription factors such as GATA3, and also suppress the expression of microRNAs involved in inhibiting the generation of Tfh cells[142]. Despite these roles, expression of BCL6 is unable to induce substantial expression of either CXCR5 or ICOS, the two main characteristic molecules of Tfh cells. In this regard other transcription factors have been suggested, including c-Maf, BATF, and IRF4.

As previously mentioned, CXCR5 is an important chemokine receptor expressed by Tfh cells in humans and mice. Upon its upregulation, CXCR5, which is also expressed by B cells, allows migration of Tfh cells into the B cell zone of secondary lymphoid organs for direct interaction with B cells.

Tfh cells exert their B cell-helping functions via the production of IL-21 and expression of co-stimulatory molecules including ICOS and CD40L. CD40L ligation with CD40 expressed on B cells induces the inhibition of apoptosis[145], which in turn allows IL-21 to enhance the differentiation of B cells, allowing for class-switching and production of all immunoglobulin isotypes[146]. The ligation of ICOS on Tfh cells with ICOSL on B cells induces the expression of IL-10 and IL-21 by Tfh cells to promote B cell activation and differentiation[147]. In this way, Tfh cells are ideally located to promote optimal B cell responses. The interest in Tfh cells has stemmed from their identification in a number of immunological disorders[148].

1.2.8 - The Th9 Cell Lineage

The latest lineage identified is that of Th9 cells, characterised by the production of IL-9, but also capable of producing IL-10, IL-17, IL-21, and IL-22[149]. Much work conducted on this subset has been performed in murine models, whereby IL-4 and TGF-β have been identified as polarising cytokines for a Th9 phenotype. These cytokines also induce Th9 polarisation in human cells[150]. A number of other factors have also been identified to promote human IL-9 production; IL-21 plays a potent role in increasing the generation of IL-9 producing cells, and type 1 IFNs induce the

expression of IL-21 by $CD4^+$ T cells, forming an indirect mechanism of Th9 promotion[151,152]. TGF-β alone is sufficient to expand memory Th9 cells.

No master transcription factor for Th9 cells has yet been identified and as such it remains unclear whether Th9 cells represent a stable lineage or a transitional phenotype. IL-4 and TGF-β each cause the upregulation of *GATA3* and *FOXP3*, respectively. Interestingly, when these cytokines were used in combination to generate Th9 cells, IL-4 was shown to inhibit *FOXP3* expression but TGF-β did not inhibit *GATA3*[152].

The physiological relevance of Th9 cells is only just beginning to be unveiled; roles have been identified in *M. tuberculosis* infection[153], parasite immunity[154] and in immunological disorders of the skin[155].

1.2.9 - T Helper Cell Plasticity

Initially it was believed that the various T helper lineages represented stable phenotypes, and that memory T helper cells would become terminally differentiated and fixed in their role. As such, upon restimulation the memory cell would secrete the cytokine repertoire it had been polarised to produce initially. Each lineage would also be responsible for the production of a set of cytokines, which were only produced by that subset. However, it has become clear that there is significant flexibility in the T helper lineages, particularly in human cells. This led to the concept of 'plasticity' within the $CD4^+$ T cell subsets[156–158]. Plasticity suggests that, although T helper lineages may become terminally differentiated and refractory to alternative pathways, a large proportion of $CD4^+$ T cells are able to shift lineages upon encounter of differing polarising conditions.

The concept of T helper plasticity has much evidence behind it in both human and murine systems. Th17 cells especially are considered plastic; they are able to express IFN-γ and shift to Th1 phenotypes, or express IL-10[159]. Indeed, Th17 cells expressing either IFN-γ or IL-10 have been found to play roles in different infections. Th1 cells and Th2 cells are rarely found separately in humans; instead, populations of both, in addition to IFN-γ/IL-4 co-expressing cells, are found routinely *in vitro* and *ex vivo*. Tregs can be converted to Th1 or Th17 cells depending on the culture conditions, and *vice versa*. Th1 cells are capable of expressing both IL-21 and IL-22. Further to this, a number of cytokines can be expressed across multiple lineages, IL-10 being the most common. Originally a Th2 cytokine, IL-10 is now acknowledged to be produced by numerous other T helper lineages.

The original concept of a master transcription factor is also changing, to the more flexible 'lineage specifying' transcription factor instead[56]. This is reinforced by the observation that many lineage transcription factors are not mutually exclusive but can be co-expressed, either transiently or stably, allowing for multiple lineage functions and plasticity between subsets. What is clear is that the variety of APCs involved in the polarisation and the molecules and cytokines expressed play a fundamental role in determining the lineages present during an immune response.

Table 1.1 - Summary of human $CD4^+$ T cell polarisation

T Helper Lineage	Polarising Cytokines	Effector Cytokines	Master Transcription Factor	Chemokine Receptor Profile	Roles in Immunity	References
Th1	IL-12 IFN-α/IFN-β IL-2, IFN-γ	IFN-γ, IL-2 TNF-β, TNF-α GM-CSF	TBX21	CXCR3 CCR5	Intracellular Pathogens, Anti-Tumour, Autoimmunity	55–62
Th2	IL-4 PGE_2, IL-6	IL-4 IL-5, IL-13, IL-10	GATA-3	CCR3 CCR4	Humoral Immunity, Parasitic Infections, Allergy	64–71,73,75–79
Th17	IL-1β, IL-6, TGF-β IL-23 IL-21	IL-17A, IL-17F, IL-21, IL-22, IL-10, IL-26	RORC	CCR6 CCR4	Extracellular Pathogens/Fungi, Autoimmunity	85–104
Treg	TGF-β	TGF-β IL-10	FOXP3	-	Suppression of Immunity	106–115,117–119
Th22	IL-6, TNF-α IL-23	IL-22 TNF-α, IL-10, IL-13	AHR	CCR4 CCR6, CCR10	Mucosal Barrier Maintenance, Wound Healing, Autoimmunity	120–139
Tfh	IL-6, IL-27, IL-21	IL-21	BCL6	CXCR5	Humoral Immunity	140–148
Th9	IL-4, TGF-β IL-21, IFN-α	IL-9 IL-10, IL-17 IL-21, IL-22	-	-	Anti-Parasitic Immunity, Skin Disorders	149–154

1.2.10 - Signal 1 in CD4$^+$ T Cell Polarisation

While polarising cytokines play a major role in CD4$^+$ T cell differentiation, a number of other factors play equally important roles. The role of signal 1 in inducing naive CD4$^+$ T cell responses has previously been discussed, however this signal plays an additional role in determining the CD4$^+$ T cell fate. It is the strength of signal 1 which affects differentiation; this can be in the form of affinity of the TCR to MHC/peptide complex, total number of TCRs triggered during activation, number and potency of APCs present, and the duration of T cell signalling[160–162].

The effects of the strength of signal 1 on CD4$^+$ T cell differentiation can easily be observed on the induction of Th1 and Th2 cells. Both high doses of antigen and higher APC:responder ratios favour IFN-γ and Th1 induction, whereas lower doses/APC:responder ratios favour Th2 induction[160,161]. In addition, more potent APCs such as DCs, are able to more efficiently promote Th1 responses over less potent APCs such as macrophages. In addition, it has recently been demonstrated that lower strength T cell activation promotes Th17 responses over Th1 responses[163], and even favours inducible Tregs[164]. However, it is difficult to determine the optimal strength of stimulation for each T cell subtype from multiple studies, and given the number of factors which determine the strength of signal 1. Overall, it appears that stronger stimulations favour Th1 polarisation, whereas weaker stimulations favour promotion of other subsets, with very weak stimulations favouring T cell differentiation towards regulatory subsets.

1.2.11 - Signal 2 in CD4$^+$ T Cell Polarisation

The role of co-stimulatory molecules has been accepted to play a significant role in the differentiation of CD4$^+$ T cells and to affect the polarisation toward certain cell lineages. Much of the evidence regarding co-stimulation-mediated polarisation is derived from murine models, with selected studies conducted in human systems. While human functions will be the main topic of the discussion in this section, due to the limited information in human systems, relation to mouse models is necessary for a more complete picture. Some of the more commonly studied co-stimulatory molecules are discussed below.

1.2.11.1 - CD80/86-CD28 Pathway

Signalling through CD28 and its interactions with CD80 and CD86 ligands has long been appreciated as a vital signal in inducing naive CD4$^+$ T cell responses. A major result of CD28 signalling is the induction of IL-2 secretion by CD4$^+$ T cells, aiding

cell survival and proliferation. A number of studies in both mouse and human systems have identified that signalling via CD28 can have polarising effects of $CD4^+$ T cells[165], promoting Th1[165], Th2[166], Th17[167,168], or even Treg differentiation[169], and modulating production of IFN-γ, IL-4, IL-17, IL-10 and IL-9, among others[170]. Indeed, the concentration of CD28 stimulating agents has been identified to play a role, with higher concentrations promoting Th1 responses and lower concentrations favouring Treg induction. The potency and importance of CD28 co-stimulation in humans has been highlighted by a clinical trial of anti-CD28 monoclonal antibody therapy, where the super-agonistic effects induced a massive cytokine storm in six healthy individuals[171]. The co-stimulatory molecules CD80/86 have been suggested to play differential roles in CD4 polarisation via interaction with CD28, with CD80 promoting a Th1 response and CD86 inducing a Th2 response in mice[172]. However, given that the majority of APCs often co-express both CD80 and CD86, the implications of these differential interactions remain unclear. The range of responses attributed to CD28 co-stimulation suggests this pathway may be related to a more general role in T cell activation than polarisation of specific T helper lineages.

1.2.11.2 - CD70-CD27 Pathway

The CD70-CD27 co-stimulatory pathway has been identified to have important functions in generating $CD4^+$ T cell responses. CD70 is expressed at varying levels on APCs, and also becomes upregulated on activated $CD4^+$ T cells[173]. CD27 is constitutively expressed on naive $CD4^+$ T cells. This co-stimulatory pathway functions to enhance T cell responses, promoting cell survival and enhancing effector functions, including having a qualitative role on $CD4^+$ T cell phenotype. The majority of studies on the function of this pathway in $CD4^+$ T cell polarisation have been conducted in mice, where CD70 ligation with CD27 has been shown to promote Th1 responses[174,175], and inhibit Th17 type responses *in vitro* and *in vivo*[176]. This interaction sensitises differentiating cells to IL-12[177], inhibits IL-17 production despite *RORγt* expression being unaffected[176], and is able to induce Th1 phenotypes in the absence of IL-12[178]. In humans, this effect has not been well studied. Limited data show that CD27 signalling can indeed promote Th1 responses or alternatively Th2, depending on culture conditions and other extrinsic factors[173,179], a phenomenon which has more recently been realised in mice[180]. Indeed, more so in human systems than in mice, it appears that CD27 signalling provides a more 'neutral' signal for T cell activation, which enhances the signals the cell receives from other sources such as polarising cytokines[179]. Nevertheless,

CD70-CD27 interaction has the capability to promote CD4 responses and potentiate $CD4^+$ T cell polarisation.

1.2.11.3 - ICOSL-ICOS Pathway

The ICOSL (CD275) - ICOS (CD278) pathway is another co-stimulatory interaction studied for its role in inducing and aiding $CD4^+$ T cell responses. ICOS is expressed at low levels or even absent on naive $CD4^+$ T cells, but becomes upregulated upon TCR ligation and CD28 activation[181]. Its counterpart, ICOSL, is expressed on both haematopoietic and non-haematopoietic cells. Studies in murine models identified roles for ICOS signalling in Tfh cell development, Th2 differentiation, and most recently in Th17 development[182]. However, limited studies in human systems have shown markedly different mechanisms of action. Firstly, in murine systems IL-4 and ICOS form a positive feedback loop, with IL-4 inducing ICOS expression by $CD4^+$ T cells, the ligation of which triggers increased IL-4 production[182]. As such, mouse Th2 cells express higher levels of ICOS than Th1 cells. Conversely in humans, Th1 cells express higher levels of ICOS, which is promoted by the actions of both IL-12 and IL-23.

Studies in humans have shown that ICOS signalling promotes the production of IFN-γ, TNF-α, IL-2, and IL-10, cytokines characteristic of a Th1 response, but not IL-4[182]. Interestingly, ICOS signalling in the absence of CD28 co-stimulation only induces low levels of cytokine production. More recently, a role for ICOS in promoting IL-17 responses has been identified[170,183], where IL-17A and IL-17F were increasingly produced by naive $CD4^+$ T cells cultured under Th17 polarising conditions. Further to this, a study of ICOS-deficient patients revealed an impaired ability to mount Th1, Th2 and Th17 type responses *in vitro*[184].

1.2.11.4 - OX40L-OX40 Pathway

OX40 (CD134) is co-stimulatory molecule of the TNF receptor superfamily, induced on $CD4^+$ T cells upon stimulation[185]. Its ligand, OX40L (CD252), is upregulated on several APC types upon activation, including DCs. Signalling via OX40 has positive effects on cell survival and proliferation, in addition to affecting the effector phenotype of $CD4^+$ T cells. Studies in mice have revealed that signalling via the OX40 co-stimulatory pathway promotes the polarisation of Th2 and Th1 cells[165], and even Th9 cells[185], and inhibits the differentiation of Th17 and Treg subsets. The role of OX40 in promoting Th2 type-responses has been reproduced in humans, with recombinant OX40L[186] or OX40L-expressing DCs[187] promoting Th2 responses, acting in synergy with IL-4. This Th2 induction appears inferior to IL-12 signalling,

which is able to override the OX40 signalling effects. Due to this polarising ability, the OX40 pathway is of interest in treating human allergy. A role for OX40 signalling in Tfh cell differentiation and function has also been reported in humans[188,189], in accordance with high expression levels of OX40 on Tfh cells.

1.2.11.5 - 4-1BBL-4-1BB Pathway

4-1BB (CD137) and its ligand 4-1BBL (CD137L) constitute another co-stimulatory pathway for the induction of T cell responses. 4-1BB is expressed on activated $CD4^+$ and $CD8^+$ T cells, upregulated upon activation via the TCR, and its ligand 4-1BBL is expressed on a variety of APCs[190]. Activation of this co-stimulatory pathway in humans and mice causes increased T cell proliferation, cytokine production, and prevention of activation-induced cell death[191]. It is also able to reverse the anergic state of $CD4^+$ T cells. Studies of this pathway in the polarisation of T cells are limited; in both humans and mice, promotion of Th1 responses can be observed[190,192,193], and in mice Th2 responses are also promoted. When $CD4^+$ T cells were analysed for their expression of 4-1BB in rheumatoid arthritis, increased levels were observed in patients over control groups, and produced increased levels of IFN-γ *in vitro*[194]. However, no data are available on the effects of 4-1BB on Th17 differentiation or other lineages in humans.

1.2.11.6 - CD40-CD40L Pathway

The CD40-CD40L co-stimulatory pathway is distinct from other co-stimulatory pathways, in that the primary direction of signalling is from the responding $CD4^+$ T cell towards the APC, in contrast to the CD86/CD28, CD70/CD27, ICOSL/ICOS, OX40L/OX40, and 4-1BBL/4-1BB pathways discussed above. CD40 is constitutively expressed by APCs and becomes further upregulated upon cell activation. CD40L (CD154) is upregulated on $CD4^+$ T cells upon TCR ligation and co-stimulation, allowing for ligation with CD40 on APCs[165]. This signalling via CD40 'licenses' the APC, enhancing expression of co-stimulatory molecules and polarising cytokines, allowing for a crosstalk between the APC and responder cell. This co-stimulatory pathway is able to promote Th1 and Th17 responses, by promoting the expression of polarising factors such as IL-12 and IL-6 by APCs[195,196].

1.2.11.7 - Limitations of Studying Co-stimulatory Pathways in Human Cells

Whilst studies of the role of co-stimulatory pathways in human $CD4^+$ T cell polarisation give valuable insight into their functions, there are several limitations.

Firstly, most co-stimulatory pathways are bidirectional in that co-stimulation promotes T cell responses but can also have significant effects on the APC as well. This concept is referred to as reverse co-stimulation. It is difficult to distinguish the direct effects on polarisation from indirect effects, such as a promotion of polarising cytokine secretion by APCs, which in turn affects $CD4^+$ T cell polarisation. In addition, most experimental studies focus on a single co-stimulatory pathway in isolation, or in combination with a limited repertoire of polarising cytokines. However, it has been extensively observed that co-stimulatory molecules display differential polarising effects depending on the cytokine context. This means that the lineages promoted by certain co-stimulatory pathways may differ in the presence of alternative stimuli. Lastly, studies of multiple co-stimulatory molecules in combination may identify different effects to the study of single molecules, in that some co-stimulatory pathways may be dominant over others. For these reasons, a number of studies focus on the role of polarising cytokines and co-stimulatory molecules in the context of individual APC subtypes.

1.2.12 - The Role of APCs in $CD4^+$ T Cell Polarisation

Given the large number of professional and non-professional APCs identified in humans and their distinct and overlapping roles in polarising $CD4^+$ T cell responses, each cell type will be discussed in turn.

Dendritic cells are the prototypic APC studied for the induction of T cell responses. A number of subtypes of DCs have been identified in humans[197], including *in vitro*-generated monocyte-derived DCs that are thought to closely resemble inflammatory DCs *in vivo*, blood-derived myeloid and plasmacytoid DCs, and skin-derived dermal DCs and Langerhans cells, among others. DC subtypes have been shown to possess a natural ability to promote certain $CD4^+$ T cell lineages over others. For example, human $CD14^+$ DCs have been shown to favour the induction of Tfh cells, aiding B cell responses, whereas Langerhans cells favour the promotion of Th2 type responses[198]. Another subset, $CD141^+$ DCs, have also been identified to promote Th2 responses[199]. Further studies have identified distinct populations of skin DCs in the polarisation of differential Th17 or Th22 populations in human $CD4^+$ T cells[200–202]. Similar DC populations with differential effects in initiating adaptive immunity can be observed in other peripheral locations, and subsets of DCs which promote Treg or Th17 differentiation have also been identified[203,204].

In addition to this natural polarising ability of different subsets, DCs display an adaptive nature, allowing for induction of different T helper lineages, depending on

their recognition of distinct pathogenic components. As discussed previously, DCs can recognise pathogens via distinct sets of pathogen recognition receptors such as TLRs, with different pathogens expressing ligands for different sets of PRRs[205]. The recognition of different ligands directs the expression of co-stimulatory molecules and polarising cytokines. In this manner, it has been shown that *E. coli* stimulation of TLR4 on DCs directs them to polarise Th1 type responses via IL-12 secretion, whereas activating DCs through TLR2 induces the production of IL-10 by DCs, favouring a Th2 response[206]. Other studies have identified peptidoglycan, another TLR2 ligand, in its ability to favour Th17 induction via the production of IL-6, IL-1β and IL-23[95]. CD40 ligation on monocyte-derived DCs has been reported to favour IL-21 induction in naive $CD4^+$ T cells[144]. Further to this, differing subsets of DCs express varying combinations of pattern recognition receptors, allowing for subset specific recognition of PRR ligands[205], and subsequent $CD4^+$ T cell polarisation.

In addition to DCs, monocytes and macrophages have both been shown to induce and polarise $CD4^+$ T cell responses. Both cellular types have been shown to induce Th1, Th2, or Th17 type responses depending on the cellular context and location[95,160,207,208]. In this way, they are able to adapt to different stimulations in the same way as DCs.

Finally, B cells are also able to stimulate $CD4^+$ T cell responses, but their effects on polarisation have not been well studied. Limited studies in humans have shown the polarisation of Th2 type responses[209,210], by direct or indirect means, and potentially in the promotion of Tfh cell responses. It is unclear if B cells may play a role in polarisation or inhibition of alternate T cell lineages.

Cells which do not constitute classical APCs can also play roles in directing $CD4^+$ T cell responses after stimulation by professional APCs. These non-classical APCs perform this role in the periphery. Basophils have been shown to promote Th2 type responses[211], whereas mast cells and mesothelial cells have been identified to promote IL-22 responses in certain contexts and disease states[212,213]. Numerous other cell types are able to influence the adaptive immune response, including neutrophils[214].

1.2.13 - $CD8^+$ T Cell Polarisation

In addition to the polarisation of $CD4^+$ T cells, $CD8^+$ T cells are also able to assume polarised states, phenotypically mirroring the T helper lineages in terms of cytokine repertoires, in addition to maintaining cytotoxic potential. Tc1 cells, characterised by IFN-γ production, are most populous in humans[215], whereas Tc2 and Tc17 cells are

minor populations occurring *in vivo*[216]. The differentiation requirements of Tc cells are similar to those required for T helper lineages. Further Tc subsets such as Tc22 cells producing IL-22 have also been identified in humans[217]. The full extent of Tc subsets in infectious and disease scenarios is just beginning to be appreciated.

1.3 - Human γδ T cells

There exist three main lymphocyte lineages in all jawed vertebrates including humans; B cells, αβ T cells, and γδ T cells[218]. Conventional αβ T cells, arguably the best studied lymphocyte subset, are defined by their expression of a TCR comprised of Vα and Vβ subunits, and form the most populous T lymphocytes in humans and other jawed vertebrates, the majority being MHC/peptide restricted. However, subsequently two more TCR chains were identified, and designated as the Vγ and Vδ chains[219–221]. Thus, γδ T cells were identified as a third subset of lymphocytes by the discovery of this 'second' TCR. In this way it became apparent that T lymphocytes could express either an αβ TCR or a γδ TCR, the latter becoming referred to as γδ T cells. Intriguingly, most of these cells appear to be not restricted by MHC/peptide complexes like their αβ T cell counterparts.

1.3.1 - The γδ TCR

One of the foundations of immunology is that the adaptive immune system, comprised primarily of T and B lymphocytes, is believed to express a multitude of different cellular receptors to allow for the recognition of all possible antigens. The process of somatic DNA recombination allows for the generation of a vast diversity in the T cell and B cell receptors expressed by the relevant cells, from a very limited number of germline-encoded gene segments[222]. For αβ TCRs, differential V, D and J segments combine to give rise to diversity, in addition to somatic mutation of such gene segments. In the same manner, the Vγ and Vδ genes are capable of the same, if not more, diversity in the generation of γδ TCRs[223,224], due to their ability to utilise multiple tandem copies of D segments. Despite this ability, only a small number of conserved γδ TCRs are observed in humans and other organisms, composed of distinct pairs of Vγ and Vδ genes, and the full diversity of these genes is never fully realised *in vivo*.

In humans, there are three main Vδ segments utilised in TCRs, denoted Vδ1, Vδ2, and Vδ3[218,225]. Other less common segments exist which have both Vδ and Vα designation, due to the position of the Vδ locus within the Vα locus. With regards to Vγ gene segments, seven are commonly incorporated into human γδ TCRs; Vγ2,

Vγ3, Vγ4, Vγ5, Vγ8, Vγ9, and Vγ11. Other Vγ segments in humans are not utilised in rearranged TCRs and appear to represent pseudogenes, in contrast to other higher primates where these segments are functional[226]. A striking pairing bias of Vγ and Vδ segments is observed in humans and other species, and specific segment combinations forming TCRs often denote differential anatomical locations and function.

1.3.2 - Human γδ T Cell Subsets

A number of distinct subsets of γδ T cells have been described in humans, and these subsets do not appear to correlate with those identified in mice, with respect to TCR structure, ligand recognition, anatomical location and functional abilities. As such, this section will discuss the more common human γδ T cell subsets only. Given the restricted nature of γδ TCRs, it has become apparent that characterising these cells based on their Vδ chain is an efficient way of distinguishing between subsets. A summary of human γδ T cell subset anatomical location is displayed in Figure 1.1.

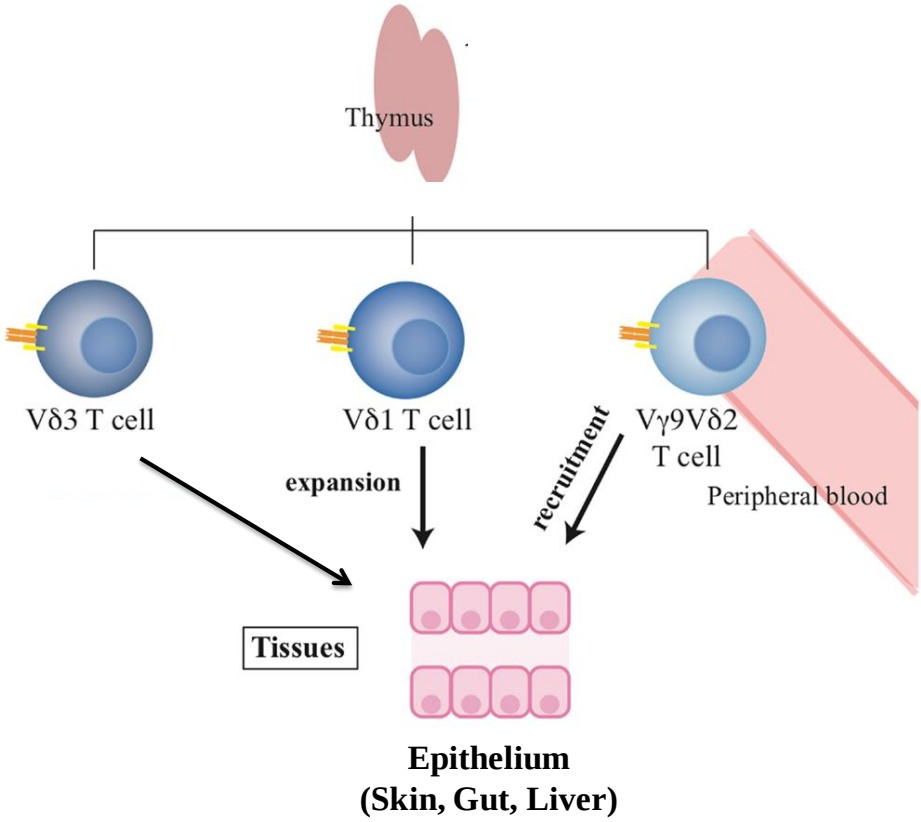

Figure 1.1 - Anatomical Locations of human γδ T Cell Subsets. Figure adapted from Rajoriya et al, 2014[227].

1.3.2.1 -Vδ1$^+$ T Cells

A major subset of human γδ T cells expresses a TCR comprised of a Vδ1 chain, paired with a number of different Vγ chains. Vδ1 T cells are a minor population of CD3$^+$ T cells in adult human peripheral blood, decreasing from approximately 50% of γδ T cells at birth to <1% of CD3$^+$ T cells in adults[228]. In contrast to the low number of circulating Vδ1 T cells, this subtype is enriched in the peripheral tissues, such as the skin and intestine[229].

Vδ1$^+$ T cells as an overall subset are capable of detecting a number of different ligands, due to the number of Vγ chains expressed in Vδ1$^+$ TCRs[218]. Only very few ligands for Vδ1$^+$ T cells have been identified, which all appear to comprise members of the MHC superfamily; distinct from the classical MHC molecules but referred to as MHC-like[230]. The MHC-like molecule CD1c, which is capable of expressing various endogenous and exogenous lipids, was the first γδ T cell ligand indentified in humans[231], recognised by a significant proportion of Vδ1$^+$ T cells. A significant number of Vδ1$^+$ cells are also reactive to CD1d molecules presenting lipids such as αGal-Cer[232], and CD1a molecules. While CD1 molecule restriction is apparent in some human Vδ1$^+$ T cells, a large proportion do not exhibit this same restriction, but instead show reactivity to tumour cells[233], CMV/HIV-infected cells[234], and certain bacterial and fungal species[235], via recognition of unknown molecules.

Functionally, Vδ1$^+$ T cells are capable of rapid secretion of cytokines upon activation, activate DCs for antigen presentation, and possess cytotoxic ability against a number of cellular targets[236]. A range of studies have explored the role of Vδ1$^+$ T cells in IL-17 production and IL-17-mediated disorders such as autoimmunity. Further to this, Vδ1$^+$ T cells have been reported to express regulatory characteristics to suppress immunity[237].

1.3.2.2 - Vδ3$^+$ T Cells

A minor cell population in humans, the majority of non-Vδ1/Vδ2 T cells express the Vδ3 chain, in combination with various Vγ chains[236,238]. Relatively little is known about this subset, which is enriched in healthy livers[239], peripheral blood of CMV infected-transplant patients[234], and HIV infection[240]. The ligand restriction of Vδ3$^+$ T cells is unclear, but reactivity to CD1d molecules has been observed albeit in an α-GalCer independent manner[238]. Rapid cytokine production including Th1, Th2, and Th17-related cytokines has been reported, as well as interaction with other cell types such as DCs. The full extent of these cells in infection and immune pathologies has yet to be identified.

1.3.2.3 - Vδ2$^+$ T Cells

The most commonly studied γδ T cells in humans express the Vδ2 TCR chain, which combines with the Vγ9 chain to form the Vγ9Vδ2 TCR[218]. This particular subset is most prominent in adult peripheral blood, comprising approximately 5% of CD3$^+$ T cells, but rapidly expand to higher numbers in certain infections. Vγ9Vδ2 T cells can also be found in peripheral tissues such as gut, liver, skin, peritoneal cavity and mucosal sites, albeit at reduced frequencies[241,242]. The Vγ9 and Vδ2 gene segments are conserved throughout primate evolution, and as such Vγ9Vδ2 T cells are found in all higher primates[243]. In contrast, this cellular subset is not present in rodents. Recent genomic analyses have indicated that other non-primate, non-rodent species such as alpacas may also possess Vγ9Vδ2 T cells, suggesting that this cell subset emerged much earlier during mammalian evolution and was eventually lost in rodents[244]. The ligand recognition and functional role of Vγ9Vδ2 T cells is discussed in detail below.

1.3.3 - Ligand Recognition by the Vγ9Vδ2 TCR

1.3.3.1 - Ligands

The identity of the ligand for the Vγ9Vδ2 TCR was first suggested by the observation that Vγ9Vδ2 T cells are able to respond to *Mycobacterium* species *in vitro*, and that their numbers are expanded in the peripheral blood of infected patients and in disease lesions[245–247]. In contrast to αβ T cells, γδ T cells were observed to respond to mycobacterial fractions which were resistant to protease action, indicating a ligand of non-peptide nature. Later studies identified that the antigen present in *Mycobacterium* preparations were small molecules comprised of carbohydrate and phosphate[248,249]. Given this identification, synthetic 'phosphoantigens' were tested which were recognised by Vγ9Vδ2 T cells, including isopentenyl pyrophosphate (IPP), which represents the first natural Vγ9Vδ2 T cell ligand identified[250,251]. However, IPP and its isomer DMAPP did not induce such pronounced expansion of Vγ9Vδ2 T cells as did mycobacterial preparations. Indeed, the concentrations of IPP and DMAPP recovered from bacterial preparations was insufficient to be attributed with the activation of Vγ9Vδ2 T cells[252]. Subsequent studies utilising genetically modified *E. coli* deficient in enzymes or components of the isoprenoid biosynthesis pathways identified that a precursor of IPP, (*E*)-4-hydroxy-3-methyl-2-butenyl pyrophosphate (HMB-PP), was a high affinity ligand for the Vγ9Vδ2 T cells, exhibiting up to 10,000x more bioactivity than its downstream products, and induced sustained TCR signalling[253].

Two pathways exist in nature for the generation of IPP, one of which relies on HMB-PP and another which is independent of this molecule. Isoprenoids are essential in metabolism, and as a group include molecules such as sterols, ubiquinones, and carotenoids[253]. These isoprenoids all derive from IPP (and DMAPP), which itself derives from two mutually exclusive biosynthesis pathways; the mevalonate pathway, present in all eukaryotes and in bacteria such as staphylococci and streptococci, and the non-mevalonate (MEP) pathway, utilised by bacteria such as *Mycobacterium tuberculosis, E. coli*, *Salmonella*, *Mycobacteria*, and *Yersinia*, as well as parasites such as *Plasmodium* and *Toxoplasma*. Bacterial species such as *Staphylococcus*, which do not utilise the MEP pathway or HMB-PP, fail to induce Vγ9Vδ2 T cell activation in the same manner.

Due to its low bioactivity, IPP at normal steady state concentrations potentially does not induce a response in Vγ9Vδ2 T cells. However, in certain scenarios such as in cancerous cells, increased expression of a rate-limiting enzyme upstream of IPP, 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, has been postulated to lead to increased accumulation of IPP in affected cells, accounting for one mechanism of Vγ9Vδ2 T cell reactivity against tumour cells[254]. Further to this, the enzyme farnesyl diphosphonate synthase (FPPS), responsible for utilising IPP units for the synthesis of higher isoprenoids, has been found to be inhibited by aminobisphosphonates such as pamidronate or zoledronate, allowing for accumulation of IPP in affected cells and potential recognition by Vγ9Vδ2 T cells[255–257].

Other ligands aside from isoprenoid metabolites have also been described in the induction of Vγ9Vδ2 T cell responses. Vγ9Vδ2 T cells express the NK receptor NKG2D alongside other NK receptors to allow detection of stress-ligands such as MICA/B and transformed cells[258].

1.3.3.2 - Presentation of Ligands

Since the discovery of the Vγ9Vδ2 T cell subset, and the realisation that these cells are not restricted by classical MHC antigen presenting molecules, the question has remained as to whether Vγ9Vδ2 T cell ligands require presentation. From the earliest studies it was clear that optimal Vγ9Vδ2 T cell activation required the presence of feeder cells or APCs[259,260], and where Vγ9Vδ2 T cells were activated alone with antigen they required cell-cell contact. This suggested the presence of a presenting molecule capable of displaying Vγ9Vδ2 T cell ligands for TCR ligation.

The recent discovery of butyrophilin 3A (BTN3A/CD277) has provided a long-sought answer to this question[236]. Initial identification of the potential role of BTN3A resulted from the ability of antibodies directed against CD277 to activate Vγ9Vδ2 T cells in a similar manner to HMB-PP and IPP[261,262]. In humans, BTN3A proteins are members of the butyrophilin family, and BTN3A itself features three family members; BTN3A1, BTN3A2, and BTN3A3, which possess extracellular domains structurally homologous to the B7 superfamily, which includes CD80 and CD86[218]. As such, BTN3A represents a group of unconventional presenting molecules for both HMB-PP and IPP, though it does not act in the same manner as other classical and non-classical antigen presenting molecules.

Two models have been proposed to describe the mechanism by which BTN3A and HMB-PP/IPP combine to stimulate Vγ9Vδ2 T cells. The first suggests BTN3A molecules are able to bind and display antigen on the cell surface, much in the same way as MHC display peptides[263], although this model is not supported by much of the evidence in the literature. The second and more accepted model, proposes that the intracellular B30.2 domain of the BTN3A1 molecule acts as a phosphoantigen sensor, and direct binding both of HMB-PP and, with a much lower affinity, of IPP to B30.2 has been observed[264,265]. Given this intracellular binding of phosphoantigen, current models propose that binding of B30.2 induces a conformational change in the BTN3A molecule itself, allowing for activation of Vγ9Vδ2 T cells[262,266]. Some groups have observed direct binding of BTN3A with the Vγ9Vδ2 TCR, whereas others report an unknown molecule may also be involved in this interaction[218]. Despite the complete process being unclear, it is certain that BTN3A molecules are essential for phosphoantigen activation of Vγ9Vδ2 T cells. BTN3A molecules themselves are expressed by a wide range of cells of haematopoietic and non-haematopoietic origin[265], providing multiple opportunities for phosphoantigen presentation and Vγ9Vδ2 T cell activation.

1.3.4 - Vγ9Vδ2 T cell Memory Subsets

Although usually described as innate immune cells, γδ T cells expressing the Vγ9Vδ2 TCR have been proposed to possess a form of immunological memory, characteristically a hallmark of adaptive immune cells. The first evidence of this was in a study of *Mycobacterium tuberculosis* infection in macaques as a model of Vγ9Vδ2 T cell responses[267,268]. Primary responses were observed to BCG in naive animals, and subsequent recall responses could be observed upon re-immunisation, as evidence by a greater magnitude and speed of response.

Following the identification of memory subsets in $CD8^+$ and $CD4^+$ αβ T cells, Vγ9Vδ2 T cell memory subsets were also identified in adult peripheral blood[269]. Instead of the conventional CD45/CCR7 memory markers to distinguish between subtypes, Vγ9Vδ2 T cells are instead divided by the expression of CD45 and CD27[270]. Naive cells exhibit a $CD45RA^+CD27^+$ phenotype, central memory cells lose CD45RA expression to become $CD45RA^-CD27^+$, effector memory cells are $CD45RA^-CD27^-$, and terminally differentiated display a $CD45RA^+CD27^-$ phenotype. These subsets respond differently to antigenic stimulation; naive cells proliferate but do not produce any cytokines and require higher antigenic concentrations to respond. Similarly, central memory cells proliferate well, albeit at lower stimulations than are required for naive cells. Effector memory cells comprise the main cytokine producing subset, whilst exhibiting reduced proliferative ability. Terminally differentiated cells appear to be potent cytolytic cells, with proliferation and cytokine production levels being low but expression of perforin and granulysin being highest in this subset. As with conventional T cells, Vγ9Vδ2 T cells progressing from naive to central memory to effector memory to terminally differentiated cells displayed progressively decreasing telomere length. Further differentiation between the effector memory subset has been identified based on the expression of FcγRIII (CD16)[271], with the $CD16^-$ subset producing high levels of cytokines and expressing low levels of killer receptors, and vice *versa* for the $CD16^+$ cells.

1.3.5 - Control of Vγ9Vδ2 T cell responses

Akin to conventional T cells, Vγ9Vδ2 T cell responses are not purely controlled by TCR ligation. Co-stimulatory molecules and cytokines have both been shown to play important roles in generating optimal Vγ9Vδ2 T cell immunity.

1.3.5.1 - Co-stimulation

While the roles of co-stimulatory molecules in induction of $CD4^+$ and $CD8^+$ T cell responses have been thoroughly investigated, the knowledge of such molecules in the induction of Vγ9Vδ2 T cell responses is much more limited. Vγ9Vδ2 T cells do indeed express a range of co-stimulatory molecules, including CD28, CD27, ICOS, and OX40, on subsets of resting and activated cells[272,273]. CD28 and CD27 are both expressed on resting Vγ9Vδ2 T cells of certain memory subsets, and CD28 in particular is downregulated upon activation. Both CD28 and CD27 co-stimulation have been shown to promote proliferation and survival of Vγ9Vδ2 T cells upon TCR ligation, whereas ICOS and OX40 have not been observed in the same role. In addition, CD27 co-stimulation promotes the production of IFN-γ by Vγ9Vδ2 T cells,

imbuing the cells with a Th1 bias[274]. With the activation of Vγ9Vδ2 T cells dependent on BTN3A molecules, which are expressed by both APCs and non-APCs, it is unclear whether co-stimulation is provided by the activating cell in all circumstances *in vivo*, and whether Vγ9Vδ2 T cells are able to respond to antigen in the absence of co-stimulation. The observation that Vγ9Vδ2 T cells upregulate CD80/86 and CD70 upon activation[272] may allow for Vγ9Vδ2 T cell populations to provide their own co-stimulatory signals via CD28 and CD27 if needed.

1.3.5.2 - Cytokines

Cytokines play a crucial role in the generation and maintenance of T cell responses in general[275]. The group of cytokines whose receptors all possess the common γ-chain, namely IL-2, IL-4, IL-7, IL-15, IL-21, and IL-9, have grown to represent a fundamental family which control the maintenance and survival of T cell populations, including Vγ9Vδ2 T cells. IL-2, produced by T cells and DCs, is a T cell growth factor which inhibits apoptosis and promotes proliferation, as well as promoting cytolytic activity. IL-4, produced by Th2 cells, mast cells and basophils, favours Th2-type immunity and antibody class switching. IL-7 is secreted by stromal cells, epithelial cells, and fibroblasts, and is crucial for development of T cells as well as their homeostatic proliferation and survival. IL-9 is produced by Th9 cells and exerts its effects via activation of epithelial cells and B cells, among others, and potentially acts as a late stage T cell growth factor. IL-15, produced by DCs, monocytes, and epithelial cells, plays roles in T cell homeostasis, survival an proliferation. Lastly, IL-21 is generated by $CD4^+$ T cell subsets and promotes B cell responses as well as acting as a T cell growth factor alongside IL-2 and IL-15.

In terms of Vγ9Vδ2 T cell responses, it has been observed that many of the common γ-chain cytokines exert differential effects on Vγ9Vδ2 T cells in terms of effector responses, and even promote the function of specific memory subsets. IL-2, utilised in the majority of Vγ9Vδ2 T cells studies, promotes Vγ9Vδ2 T cell activation, proliferation, cytokine production, and cytolytic capacity[276]. In addition, IL-2 favours the induction of a Th1-like phenotype, promoting the production of IFN-γ. The IL-2 receptor is minimally expressed on naive cells but becomes upregulated as cells progress to central memory and effector memory stages, indicating its importance in effector responses[277]. Similarly, IL-15 induces proliferation, IFN-γ production and cytotoxic molecule expression in Vγ9Vδ2 T cells[278], despite being generally regarded as a homeostatic cytokine. Further to this, IL-15 favours the proliferation and function of effector memory subsets, on which the IL-15 receptor expression is

highest[277]. Interestingly, the IL-7 receptor is predominantly expressed on naive cells and central memory cells, with lower expression on effector memory cells, and as such favours the proliferation of naive and central memory cells. In terms of functionality, IL-7 favours proliferation but is limited in its ability to promote IFN-γ secretion and cytotoxicity. Further studies have identified IL-7 as able to promote IL-17 production by Vγ9Vδ2 T cells[279]. Lastly, IL-21 treatment of Vγ9Vδ2 T cells *in vitro* induces limited proliferation of Vγ9Vδ2 T cells but also promotes the expression of Tfh-like molecules by Vγ9Vδ2 T cells[280]. As such, the common γ-chain cytokines represent one mechanism by which Vγ9Vδ2 T cell responses can be regulated.

1.3.6 - Vγ9Vδ2 T Cell Function

The discovery of the Vγ9Vδ2 TCR ligands has allowed for significant study of the roles of Vγ9Vδ2 T cells in human immunity. Vγ9Vδ2 T cells are rapidly activated upon TCR ligation and function to produce high levels of varying cytokines, exhibit cytotoxic potential, and interact with many immune cell types to exert their anti-microbial and anti-tumour effects. These rapid functions of Vγ9Vδ2 T cells in addition to their ability for immunological memory has blurred the line between the innate and adaptive response. Myeloid cells generally display innate characteristics, whereas lymphocytes display classic adaptive features; Vγ9Vδ2 T cells possess aspects of both. A summary of Vγ9Vδ2 T cell functions is displayed in Figure 1.2.

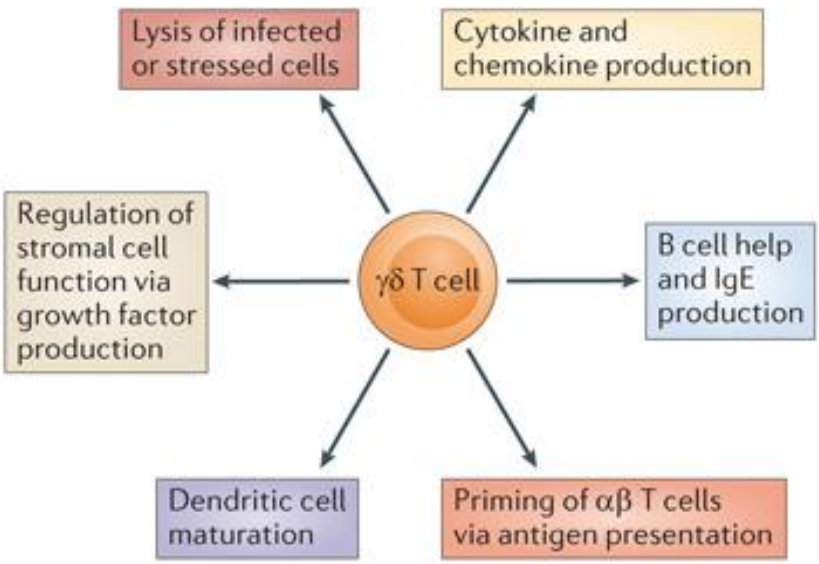

Figure 1.2 - Functions of Vγ9Vδ2 T cells. Figure adapted from Vantourout and Hayday, 2013[230].

1.3.6.1 - Cytokine production

In a similar manner to $CD4^+$ and $CD8^+$ T cells, Vγ9Vδ2 T cells exhibit plasticity with regards to their cytokine profiles produced upon activation[281]. Indeed, Vγ9Vδ2 T cells can phenotypically resemble Th1, Th2, Th17, Treg and Tfh cells under certain conditions. It is unclear whether particular subsets of Vγ9Vδ2 T cells are responsible for each effector function, or if Vγ9Vδ2 T cells are capable of true plasticity, with evidence for both possibilities in the literature. Each of these phenotypes is discussed below.

1.3.6.2 - Th1-like γδ T Cells

Vγ9Vδ2 T cells by default assume a Th1-like phenotype; upon stimulation with HMB-PP *in vitro*, significant levels of IFN-γ are produced, alongside TNF-α, LT-α, and GM-CSF[282,283]. In fact, the kinetics of IFN-γ production by Vγ9Vδ2 T cells is increased compared with that observed in $CD4^+$ and $CD8^+$ T cell responses[284], highlighting Vγ9Vδ2 T cells as an important source of Th1 cytokines. The IFN-γ producing phenotype is significantly promoted by IL-2, which also favours the production of several chemokines such as RANTES and MIP-1α[285]. This Th1-like phenotype is also promoted by the Th1 polarising cytokine, IL-12, by IL-15, and by type 1 IFN. This natural tendency for IFN-γ production is dependent on *TBX21* expression, and cells often express the chemokine receptor CXCR3 on the cell surface. Due to this functional bias towards IFN-γ production, Vγ9Vδ2 T cells characteristically mediate immunity in a similar manner to Th1 cells, as discussed previously.

1.3.6.3 - Th2-like γδ T Cells

Vγ9Vδ2 T cells are also able to assume a Th2-like phenotype, characterised by secretion of IL-4. Reflective of Th2 cell differentiation, treatment of freshly isolated Vγ9Vδ2 T cells with IL-4 allows for IL-4 production by the Vγ9Vδ2 T cells themselves[281–283]. Interestingly, production of other Th2-related cytokines IL-5 and IL-13 are not detected in Th2-like γδ T cell cultures, and instead are more readily found in cultures treated with IL-12 or IL-2[282]. IL-4 expression by Vγ9Vδ2 T cells requires the expression of *GATA3*, although expression is also detected in IL-2 treated Vγ9Vδ2 T cells[282]. In addition to IL-4 production, IL-4 treated Vγ9Vδ2 T cells upregulated expression of CD27 and the B cell maturation protein CD269, which interacts with the B cell activating factor CD257. While Vγ9Vδ2 T cells readily produce IFN-γ within 24 hours of activation, the optimal production of IL-4 appears

to require longer periods of IL-4 exposure to polarise the response and overcome the natural Th1 bias[283].

1.3.6.4 - Th17-like γδ T Cells

Given the importance of IL-17 in many diseases, cellular subsets which produce IL-17 have been of great interest. As such, the identification that Vγ9Vδ2 T cells are able to produce IL-17 has garnered much interest in this cellular subset and in γδ T cells in general. However, unlike murine γδ T cells[286], Vγ9Vδ2 T cells do not readily produce IL-17 *ex vivo*, with less than 1% IL-17$^+$ cells reported in most studies[287,288], in contrast to Th17 memory cells which can be detected in healthy adult blood. Instead, the presence of Th17 polarising cytokines in various combinations is required over a long period of culture to induce significant levels of IL-17 in Vγ9Vδ2 T cells[289,290]. Also, as previously mentioned, IL-7 has been reported to promote IL-17 production by Vγ9Vδ2 T cells derived from cord blood[279]. In adult blood, almost all IL-17$^+$ Vγ9Vδ2 T cells are also positive for IFN-γ. Despite the limited evidence for naturally occurring IL-17$^+$ Vγ9Vδ2 T cells in peripheral blood, according to one study these cells can be detected in certain peripheral tissues and disease states[291], suggesting tissue specific factors in the differentiation of Th17-like γδ T cells. IL-22 producing Vγ9Vδ2 T cell populations have been identified as well[289].

1.3.6.5 - Tfh-like γδ T Cells

Vγ9Vδ2 T cells have also been reported to exhibit T follicular helper phenotypes. The induction of this phenotype is reliant on the presence of IL-21, which supports Vγ9Vδ2 T cell proliferation but not the production of pro-inflammatory cytokines including IFN-γ and TNF-α[282]. Instead, IL-21 promotes the expression of CD40L, ICOS, and CXCR5 for migration to the B cell compartment of lymph nodes and potentiation of B cell responses[280,292]. IL-21 also maintains CD62L expression following HMB-PP stimulation[282]. Vγ9Vδ2 T cells exhibiting a follicular helper phenotype can produce CXCL13 to attract CXCR5-expressing B cells and CD4$^+$ T cells. Interestingly, and distinct from CD4$^+$ conventional Tfh cells, Tfh-like γδ T cells do not produce IL-21, but instead express IL-4 and IL-10. One report indicated that CXCR5$^+$ Vγ9Vδ2 T cells can be found in adult peripheral blood[293], although those findings could not be confirmed by our own group[241,280].

1.3.6.6 - Treg-like γδ T Cells

In addition to the various pro-inflammatory effects of Vγ9Vδ2 T cells, reports have also suggested potential regulatory characteristics. *FOXP3* is induced in Vγ9Vδ2 T

cells following HMB-PP stimulation, but full suppressive potential was dependent on the combination of IL-15 and TGF-β[294–296]. Treg-like γδ T cells are able to suppress $CD4^+$ T cell responses *in vitro*, and limited evidence *in vivo* suggests that Vγ9Vδ2 T cells may utilise their suppressive abilities in certain disease conditions[297,298].

1.3.6.7 - Cytotoxicity

In addition to the secretion of a wide range of immunomodulatory cytokines, Vγ9Vδ2 T cells are potent cytotoxic cells. Vγ9Vδ2 T cells utilise both the death ligand/receptor pathways, such as FasL/FasR to induce apoptosis, and the molecules perforin and granzyme to induce target cell lysis. Target cells include bacterial or virally infected cells and tumour cells[299–301]. The cytotoxic capacity in Vγ9Vδ2 T cells can be initiated via accumulation of IPP in transformed cells, or alternatively through receptors such as NKG2D[302], independently of TCR signalling. The expression of CD56 and/or CD16 appears to distinguish Vγ9Vδ2 T cells with cytotoxic activity[303]. Due to their functional capacity as potent anti-tumour cells, Vγ9Vδ2 T cells are of particular interest for tumour immunotherapies[256,257,304].

1.3.7 - Interactions with Other Immune Cell Subsets

Vγ9Vδ2 T cells exert a multitude of immunomodulatory effects by direct interaction with other cells of the immune system. Extensive research has shown that Vγ9Vδ2 T cells are able to interact with DCs to induce their optimal maturation. DCs typically depend on the recognition of danger signals for maturation, but this process can also be controlled by inflammatory factors such as IFN-γ and TNF-α. As Vγ9Vδ2 T cells are significant sources of these cytokines, they are able to induce maturation of DCs upon activation. Consequently, Vγ9Vδ2 T cells induce the upregulation of APC markers, co-stimulatory molecules, a switch in chemokine receptor profiles, and secretion of polarising cytokines in DC populations[236].

Vγ9Vδ2 T cells also interact with other innate immune cells. Interaction with monocytes leads to the differentiation of inflammatory DCs, alongside improving monocyte survival and activation[207]. Interaction with neutrophils leads to similar effects on neutrophil survival and effector function[305], and even induces populations of neutrophils capable of antigen uptake and presentation[306]. These interactions also provide positive effects to the Vγ9Vδ2 T cells, with both monocytes and neutrophils constituting efficient inducers of Vγ9Vδ2 T cell activation by presentation of HMB-PP via BTN3A and uptake and degradation of HMB-PP expressing organisms.

Lastly, due to their ability to assume follicular helper roles, Vγ9Vδ2 T cells are able to provide help to B cells. Via expression of CD40L, ICOS, IL-4 and IL-10, and CXCL13, Vγ9Vδ2 T cells recruit both B cells and Tfh cells and enhance generation of high affinity antibodies and class switching[236].

1.4 - γδ T Cells as APCs

1.4.1 - Professional vs. Non-professional APCs

Since their discovery, DCs have long been considered the prototype antigen presenting cell[307], potent in their ability to induce αβ T cell responses[308]. DCs, along with macrophages and B cells, have been termed 'professional APCs' of the immune system, a category which denotes the possession of all necessary factors to mount adaptive immune responses against pathogenic antigens. A wide range of other cell types, both haematopoietic and non-haematopoietic in origin, have also been reported to be capable of antigen presentation, but due to the lack of one or more aspects of professional APCs are referred to as non-professional, or atypical, APCs[309].

The ability to categorise a cell as a professional APC depends on the expression of a number of fundamental characteristics. The first and foremost is the ability to express MHC class II molecules on the cell surface, alongside a repertoire of co-stimulatory molecules. The ability to take up, process and present antigens on MHC class I and II molecules, expression of pattern recognition receptors, and the ability to alter migratory potential upon activation to home to the secondary lymphoid organs are all necessary aspects of professional APCs. Lastly, and potentially most importantly, is the ability to stimulate naive $CD4^+$ and $CD8^+$ αβ T cell responses in the process of generating adaptive immunity[309].

Dendritic cells, alongside B cells and macrophages, fulfil the relevant criteria necessary to be classified as professional APCs. Atypical APCs, however, are able to exhibit a limited range of APC characteristics, mainly the expression of MHC class II. Cells that have been reported to fill this role are numerous, and include haematopoietic cells such as mast cells[310,311], neutrophils[214,312,313], innate lymphoid cells[314–316],and even $CD4^+$ αβ T cells themselves[317,318]. Non-haematopoietic cells complement this repertoire of atypical APCs, including endothelial cells[319], epithelial cells[320] and lymph node stromal cells[321]. The main functional constraint which prevents these cells being termed professional APCs and covers most, if not all, of the atypical APCs is the lack of ability to stimulate naive αβ T cell responses. The

more likely role of these cells is in the modulation of responses already generated by one or more professional APCs, perhaps maintaining local immune responses in the peripheral tissues in a context-dependent manner.

Vγ9Vδ2 T cells are the newest addition to the list of cells with antigen presenting capabilities, which appear to possess all the necessary factors to be termed a professional APC.

1.4.2 - Identification of γδ T-APCs - Switch in Migratory Profile

The unexpected identification that human γδ T cells could act as APCs was first evidenced by the discovery that the migratory profile of Vγ9Vδ2 T cells, in terms of expression of chemokine receptors, could switch shortly after activation[241]. Circulating Vγ9Vδ2 T cells express chemokine receptors necessary for rapid recruitment to peripheral inflammatory sites, including CCR5 and CXCR3. Upon activation however, a shift in the migratory profile of Vγ9Vδ2 T cells occurs, from $CCR5^+$ (inflammatory site-homing) to $CCR7^+$ (lymph node-homing). This shift and upregulation of CCR7 is rapid, occurs within 48 hours of stimulation, and is accompanied by changes in the migratory response to relevant chemokines. The cell adhesion molecule L-selectin, or CD62L, was also observed to be upregulated on activated Vγ9Vδ2 T cells[322]. The regulation of chemokine receptors by Vγ9Vδ2T cells is in direct contrast to that exhibited by $CD4^+$ and $CD8^+$ αβ T cells. The acquisition of lymph node homing potential was reflected in the ability to identify human Vγ9Vδ2 T cells in secondary lymphoid tissues such as those from gastrointestinal sites, tonsils, and spleen. These cells could be found in both the T cell and B cell zones. Low level CCR7 expression by peripheral blood Vγ9Vδ2 T cells could also be observed in a study of healthy and immunocompromised individuals[323]. The ability of Vγ9Vδ2 T cells to acquire a lymph node-homing phenotype indicated the potential APC role of these cells in generating or being involved in adaptive immunity.

1.4.3 - Expression of APC Markers

Subsequent analysis of Vγ9Vδ2 T cells shortly after activation identified the expression of antigen presenting molecules[322]. Vγ9Vδ2 T cells from peripheral blood, and also from tonsils, substantially upregulate the expression of HLA-DR, the antigen-presenting MHC class II molecule, in combination with a wide range of classic co-stimulatory molecules. These include, but are not limited to, CD80, CD86, and CD40, and expression of these molecules is completely absent on resting Vγ9Vδ2 T cells. The repertoire of APC markers is almost indistinguishable from that

displayed by monocyte-derived DCs, and is consistent across a range of Vγ9Vδ2 T cell stimulants. Interestingly, the expression of HLA-DR is the result of *de novo* production, in contrast to cell surface relocation of preformed molecules as seen with DCs. In addition to expression of MHC class II and co-stimulatory molecules, the maturation marker CD83, usually upregulated on mature DCs, can be observed on the surface of γδ T-APCs. Several cellular adhesion molecules including CD11a, CD18, CD50, and CD54 are all expressed by γδ T-APCs, indicating the potential to form tight interactions with other immune cells. Other studies have indentified the same APC phenotype of activated Vγ9Vδ2 T cells[324,325].

1.4.4 - Uptake, Processing and Presentation of Extracellular Antigens

In addition to expressing an APC phenotype and lymph node homing receptors following activation, Vγ9Vδ2 T cells have also been shown to be able to take up, process and present antigens. The ability to take up exogenous material from the cellular microenvironment and process this material into peptides capable of being presented on MHC class I and II molecules is a hallmark of professional APCs. Vγ9Vδ2 T cells appear capable of two forms of endocytosis, the first being macropinocytosis, for the uptake of smaller particulate antigen and cellular debris[326]. Whereas DCs in an immature state exhibit high levels of endocytosis which decreases upon activation, Vγ9Vδ2 T cells appear to be less efficient in this process, showing diminished function more similar to that shown by B cells and monocytes. A second mechanism of Vγ9Vδ2 T cell antigen uptake is phagocytosis, allowing for the uptake of larger particles such as 1 μm synthetic beads or *E. coli* cells[327]. This phagocytic ability is dependent on the expression of the Fc receptor CD16. Interestingly, parallels could be drawn between phagocytosis performed by Vγ9Vδ2 T cells and mo-DCs, in that CD16 expression by resting Vγ9Vδ2 T cells is downregulated upon activation, in a similar manner to the reduction in phagocytic ability of DCs upon maturation. Vγ9Vδ2 T cells have also be shown to be able to kill and take up fragments of tumour cells for the presentation of tumour associated antigens[328]. This process was significantly improved by opsonisation of target cells with antibodies, in a mechanism the authors termed licensing.

Once an antigen has been taken up, it is processed via the proteasome and displayed on either MHC class II molecules for presentation to $CD4^+$ αβ T cells, or cross-presented for presentation on MHC class I molecules to stimulate $CD8^+$ αβ T cells. γδ T-APCs are capable of both of these pathways, and have been shown to take up and process antigens such as the complex protein mixture *Mycobacterium*

tuberculosis-purified protein derivative (PPD)[329], the influenza virus-encoded matrix protein M1, and even debris from influenza-infected cells[326], for presentation to αβ T cells, thus stimulating proliferative responses. Interestingly, γδ T-APCs were shown to outperform monocyte-derived DCs and even conventional and plasmacytoid DCs at the cross-presentation of soluble antigen, and to be as capable as DCs of presenting antigen to $CD4^+$ T cells[329].

1.4.5 - Induction of $CD8^+$ αβ T Cell Responses

The stimulation of $CD8^+$ T cells by professional APCs allows for the generation of cytotoxic T lymphocyte responses. In co-culture mixed lymphocyte reaction systems with allogeneic naive $CD8^+$ T cells, γδ T-APCs match or even exceed the magnitude of responses induced by monocyte-derived DCs[322] in identical experiments, whereas activated αβ T cells are unable to perform a similar APC role under the same conditions. The $CD8^+$ responding cells from γδ T-APC co-cultures displayed cytotoxic capability, perforin expression, IFN-γ production, and migratory reprogramming evidenced by the loss of CCR7 expression, all factors important in the generation of cytotoxic T lymphocytes (CTLs). Several other experimental systems have shown the ability of γδ T-APCs to induce $CD8^+$ T cell responses, including use of the M1 flu peptide to induce M1-specific $CD8^+$ T cell responses, where γδ T-APCs outperform their professional APC counterparts[329]. Further studies have examined the ability of Vγ9Vδ2 T cells expanded in the presence of HMB-PP or IPP, to function as professional APCs[325]. These cells maintain several of their APC characteristics over the period of culture, and efficiently induce $CD8^+$ T cell responses to PPD and M1 flu peptides. These cells are currently being examined for their potential use as cancer vaccines[330–332,304]. The ability to generate high numbers of γδ T-APCs compared with DCs, their potent APC potential, and also ability to kill tumour cells themselves, make these cells a viable alternative to DC based vaccines.

1.4.6 - Induction of $CD4^+$ αβ T Cell Responses

γδ T-APCs are also able to stimulate naive and memory $CD4^+$ T cell responses in multiple co-culture systems[322], mixed lymphocyte reactions with naive $CD4^+$ T cells, autologous APC assays with naive $CD4^+$ T cells using the bacterial superantigen TSST-1 as a surrogate antigen, and APC assays using either the single chain protein tetanus toxoid or *Mycobacterium tuberculosis* PPD. In terms of responder $CD4^+$ T cell proliferation, responses induced by γδ T-APCs are almost indistinguishable from those induced by monocyte-derived DCs, even at

APC:responder rations of 1:10^4 cells. These responses were confirmed to be dependent on cell-contact and MHC class II. As such, γδ T-APCs are potent in their ability to induce significant proliferative responses in naive and memory $CD4^+$ T cells. Further studies have found that expanded Vγ9Vδ2 T cells can efficiently stimulate $CD4^+$ T cells to PPD in the same manner[325], and in comparison with B cells showed significantly higher induction of $CD4^+$ T cell responses[324].

Upon examining the effector phenotype of naive $CD4^+$ T cells following stimulation by γδ T-APCs, strong Th1 or Th2 responses could be induced by varying the APC:responder ratios in the co-culture. High numbers of γδ T-APCs to responders favoured Th1 type responses, characterised by the intracellular production of IFN-γ, whereas lower γδ T-APC numbers promoted Th2 type responses, characterised by the intracellular expression of IL-4. The full extent of the γδ T-APC potential to polarise other T helper subsets however has yet to be investigated fully.

1.4.7 - Induction of iNKT Cell Responses

An interesting ability of γδ T-APCs is their ability to induce invariant natural killer T cell responses[333]. iNKT cells are an immunoregulatory T cell subset which play a role in initiating and facilitating anti-tumour immune responses. These cells are restricted by the CD1d antigen presenting molecule, which combines with the synthetic glycolipid α-galactosylceramide (α-GalCer). It has been shown that γδ T-APCs present α-GalCer on CD1d molecules and activate iNKT cell responses, however this expression of CD1d was not the result of *de novo* synthesis, as in the case of HLA-DR. Instead, trogocytosis, the transfer of membrane molecules between cells for presentation on their cell surface, was required for CD1d expression and α-GalCer presentation on γδ T-APCs, which was obtained from feeder cells. Indeed, trogocytosis upon Vγ9Vδ2 T cell interaction with cancer cells has also been reported[334], however this phenomenon was not predictive of functional outcomes, and due to the inherent ability of γδ T-APCs to express all relevant markers and possess distinct antigen uptake and processing pathways, this indicates that the APC phenotype and function of Vγ9Vδ2T cells is not simply the result of membrane exchange.

1.4.8 - γδ T-APC Function *in vivo*

An APC function of γδ T cells has been reported in a number of species, including cows[335], pigs[336], and mice[337], though these populations did not resemble professional APCs as human γδ T-APCs do. However, due to the lack of corresponding Vγ9Vδ2 T cells in animal models outside of higher primates, the *in*

vivo study of γδ T-APCs is restricted by access to human tissues and lymph nodes. Nevertheless, there is a small but slowly expanding body of evidence that suggests that human Vγ9Vδ2T cells are indeed able to function as APCs *in vivo*, predominantly in inflammatory scenarios. Expression of HLA-DR on Vγ9Vδ2 T cells has been reported in several inflammatory conditions[338–340], as well as in patients receiving zoledronate treatment for prostate cancer[256], breast cancer[257], and osteoporosis[255]. However, the functional relevance of this HLA-DR expression was not determined in these studies.

A study into the role of human γδ T cells in rheumatoid arthritis (RA) revealed a potential role of γδ T-APCs in the pathogenesis of disease. RA is a common, systemic autoimmune disease that predominantly affects the synovial joints. A number of studies have identified the presence and importance of Vγ9Vδ2 T cells in RA[341,342], and Vγ9Vδ2 T cells isolated from the synovial fluid of patients expressed the APC markers HLA-DR and CD86, and were capable of antigen uptake and presentation to induce $CD4^+$ T cell responses[324]. Further to this, γδ T cells isolated from the peripheral blood of gastric cancer patients were able to exhibit APC phenotypes upon stimulation with tumour cells[343]

1.4.9 - Continuing Questions

The identification that Vγ9Vδ2 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 may play a role in adaptive immune responses and interact with B and T cells in the lymph nodes. Of note, γδ T cells resemble DCs morphologically upon activation, developing numerous dendrite-like protrusions, which are absent on their resting counterparts[322]. Indeed, the fact that Vγ9Vδ2 T cells exhibit an innate-like rapid response to inflammatory stimuli, then subsequently alter their phenotype and function to migrate to the lymph nodes supports this potential novel role in directing adaptive responses. This process is likely to occur before an effective conventional T cell response, induced by DCs for example, would have time to be mounted, due to the involvement of a number of time consuming steps. Other potential advantages that γδ T-APCs may have over DCs are that γδ T cells outnumber conventional DCs in the peripheral blood and mature into fully professional APCs much more quickly. The ability to continually survey the environment for potential antigens and decide whether to actively respond or tolerate antigenic challenges appears to be restricted mainly to DCs and it does not appear that γδ T cells would be able to fill this role, due to the requirement of

activation before antigen presenting capabilities are established. Further to this, it is unlikely for the same reason than γδ T-APCs would be involved in generating tolerance to specific antigens, their main role being induction of inflammatory responses.

Despite the mounting evidence supporting the ability of human Vγ9Vδ2 T cells to function as fully competent APCs, there are a number of unanswered questions regarding their regulation and context dependent roles. It is currently uncertain in which locations γδ T cells may function as APCs *in vivo*. Limited evidence highlights the synovial fluid in RA and in certain tumours, and potential sites would include the peripheral tissues, draining lymph nodes, and mucosa, where microbial encounter is possible. It is also unclear whether Vγ9Vδ2 T cells are universally capable of APC characteristics, or whether particular subsets perform different roles, with the regulation of APC function over, for example, killing function still unclear. Other human γδ T cell subsets may also potentially feature as APCs, indicated by the observation that $V\delta1^+$ T cells also upregulate CCR7 upon activation[241]. Lastly, γδ T cells are able to stimulate naive $CD4^+$ T cell responses and polarise responding cells towards Th1 and Th2 phenotypes. However, given the recent identification of a number of alternative T helper subtypes such as Th17, Th22 and Tregs, it is unknown whether γδ T-APCs can in fact promote these subsets over others. Further to this, it is unclear whether γδ T-APCs are able to adapt to the cellular microenvironment, as DCs do, and whether any adaptation would have a 'knock-on' effect on subsequent naive $CD4^+$ T cell polarisation. What is clear is that the investigation of γδ T-APCs in humans is about 30 years behind that of DCs, providing much potential to discover the niche where γδ T cells perform professional antigen presentation *in vivo*.

1.5 - Hypothesis

Human γδ T-APCs are able to polarise naive $CD4^+$ T cell responses to different T helper lineages depending on the Vγ9Vδ2 T cell microenvironment.

1.6 - Aims

- To establish the conditions in which Vγ9Vδ2 T cells acquire an APC phenotype and relevant functional capabilities.
- To investigate the polarisation of naive and memory $CD4^+$ T cells in response to stimulation with γδ T-APCs.
- To determine which polarising molecules expressed by γδ T-APCs are important in determining the outcome of $CD4^+$ T cell polarisation.
- To relate the specific $CD4^+$ T cell subsets induced by γδ T-APCs to a specific body compartment or disease scenario.

Chapter 2 - Materials and Methods

2.1 - Cell Culture Media and Buffers

2.1.1 - Complete RPMI Medium

The cell culture medium used throughout this study, unless otherwise indicated, was RPMI-1640 medium (Invitrogen) supplemented with 10% foetal calf serum (FCS; Invitrogen), 50 mg/ml penicillin/streptomycin (Invitrogen), 2 mM L-glutamine (Invitrogen), 1% sodium pyruvate (Invitrogen) and 100 µM non-essential amino acids (NEAA; Invitrogen).

2.1.2 - Complete IMDM Medium

Where indicated, IMDM medium (Invitrogen) supplemented with 10% foetal calf serum (FCS; Invitrogen), 50 mg/ml penicillin/streptomycin (Invitrogen), 2 mM L-glutamine (Invitrogen), 1% sodium pyruvate (Invitrogen) and 100 µM non-essential amino acids (NEAA; Invitrogen) was used.

2.1.3 - Fluorescence Activated Cell Sorting (FACS) buffer

FACS buffer comprised of sterile phosphate-buffered saline (PBS) with 2% FCS (Invitrogen) and 0.02% sodium azide, passed through a 0.22 µm filter prior to use.

2.1.4 - Magnetic-activated Cell Sorting (MACS) buffer

MACS buffer comprised of sterile phosphate-buffered saline (PBS) with 2% FCS (Invitrogen) and 5 mM EDTA, passed through a 0.22 µm filter prior to use.

2.2 - Isolation of Immune Effector Cells

2.2.1 - Healthy/Patient Cohorts

Healthy volunteers were recruited locally for donations of venous blood.

Patients with a diagnosis of inflammatory bowel disease were selected based on all disease subsets, who were not currently being treated with azathioprine therapy or had alternatively been off azathioprine treatment for >6 months when samples were collected. Patients were recruited at The Royal London Hospital/Queen Mary University of London, during IBD clinics by Dr James Lindsay.

Human mucosal tissue was obtained from patients undergoing surgical resection for non-inflammatory disorders. Mucosal tissue was derived either from the terminal ileum or from the colonic mucosa.

2.2.2 - 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 and 15 mM EDTA (Fisher Scientific UK, Ltd), to prevent coagulation. Blood was subsequently separated using Lymphoprep density gradient separation media (Axis-Shield) by layering blood on top of Lymphoprep, then centrifuging at 1680 rpm (687 xg), at 18°C for 20 minutes, without brake. Mononuclear cells within the buffy coat layer were collected and washed three times with sterile PBS for the subsequent purification of immune cell subsets.

Alternatively, healthy PBMCs were isolated from blood bags supplied by the Welsh Blood Service (Velindre NHS Trust). Blood bags were diluted at 1:2 ratio with sterile PBS, and separated similarly using Lymphoprep.

PBMCs from patients diagnosed with Inflammatory Bowel Diseases were isolated in the same manner as with healthy volunteers.

2.2.2 - Purification of Vγ9Vδ2 T cells from PBMC

$V\gamma9^+$ or $V\delta2^+$ T cells (>99%) were purified from bulk PBMCs by incubation with monoclonal antibodies (mAb) directed against the Vγ9 or Vδ2 TCR chain, conjugated with PE-Cy5 (Immu360;Beckman-Coulter) or PE (B6.1; BD Biosciences) respectively, and incubated for 20 minutes at 4°C (positive selection). Cells were subsequently washed with MACS buffer and labelled with anti-PE microbeads (Miltenyi Biotec) for 20 minutes at 4°C. Dual-labelled cells were separated from PBMCs using a midi-MACS system and two LS columns (Purity after one column >95%, purity after two columns >99%). Purity of $V\gamma9^+$ or $V\delta2^+$ T cells was assessed prior to all assays to ensure minimal contamination.

2.2.3 - Purification of $CD14^+$ Monocytes from PBMC

$CD14^+$ 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 one LS column, and purity was assessed prior to use in assays (>98.7% purity).

2.2.4 - Purification of $CD4^+$ T cells from PBMC

$CD4^+$ T cells were purified from PBMC via negative selection, using the $CD4^+$ T cell Isolation Kit (Miltenyi Biotec), as per the manufacturer's instructions. Labelled PBMC populations were passed over one LS column and labelled cells were removed from $CD4^+$ T cell populations ($CD4^+$ T cell purity >95%).

In some assays, where indicated, enriched naive $CD4^+$ T cells were used. For enrichment of naive $CD4^+$ T cells ($CD4^+CD45RO^-$), total $CD4^+$ T cells isolated previously were labelled with anti-CD45RO microbeads (Miltenyi Biotec) at 4°C for 20 minutes. Following labelling, cells were passed over one LS column, and enriched naive $CD4^+$ T cells were obtained by negative selection (>95% $CD4^+CD45RA^+$).

In assays where highly purified naive $CD4^+$ T cells were used, bulk $CD4^+$ T cells were labelled with mAbs to CD4, CD45RA, and CCR7, labelled with BV421 (RPA-T4; BD Biosciences), APC (HI100; BD Biosciences), andPE-Cy7 (G043H7; BioLegend), respectively. Cells were labelled at 4°C for 20 minutes. Cells were subsequently washed and sorted based on a $CD4^+CD45RA^+CCR7^+$ phenotype using a BD FACSAria II (BD Biosciences) to high purities (>99.4%). Similarly, highly purified memory $CD4^+$ T cells, consisting of T_{CM}, T_{EM}, and T_{EMRA} cells, were sorted from the same populations based on non-naive cells ($CD45RA^-CCR7^-$) to high purities (>99%). The same method was also used to isolate naive $CD8^+$ T cells ($CD8^+CD45RA^+CCR7^+$).

2.2.5 - Isolation of Vγ9Vδ2 T cells from Intestinal Tissue Samples

Human mucosal tissue from terminal ileum and colonic mucosa were obtained from patients undergoing surgical resection. To obtain Lamina Propria Mononuclear Cells (LPMCs) from human intestinal tissue, material was collected into cold, Dutch-Modified RPMI-1640 medium (Sigma-Aldrich). Mucus and faeces were removed in calcium and magnesium-free HBSS (Sigma-Aldrich) containing 1 mM DTT (Sigma-Aldrich). Material was subsequently incubated in complete RPMI medium in 24-well plates for 3 days, to allow leukocyte egress from the mucosa. Cultures were supplemented with or without 10 nM HMB-PP, in the presence of 50 U/ml IL-2 and 20 ng/ml IL-15. LPMCs released from intestinal tissue were passed through a cell strainer[242]. Optimisation of this isolation method can be found in previous studies[344]. Work was conducted in collaboration with Dr Neil McCarthy at QMUL.

LPMCs were labelled with mAbs directed against CD3 and Vδ2, conjugated with BV421 (UCHT1; BioLegend) or PE (B6.1; BD Biosciences), respectively, at 4°C for 20 minutes. Cells were subsequently washed and sorted based on a $CD3^+Vδ2^+$ phenotype using a BD FACSAria II (BD Biosciences) to high purities (>99%).

2.3 - Functional Assays

2.3.1 - Monocyte-derived Dendritic Cell Generation

$CD14^+$ monocytes purified from PBMC were cultured for 3-4 days in complete RPMI, in the presence of 50 ng/ml GM-CSF (Miltenyi Biotec) and 50 ng/ml IL-4 (Miltenyi Biotec). At day 3/4, cultures were supplemented with fresh RPMI containing GM-CSF and IL-4. Monocyte-derived DCs were phenotyped by flow cytometry at day 5/6 and used in functional assays.

2.3.2 - Maturation of Dendritic Cells and Monocytes

Monocyte-derived DCs (immature DCs; iDCs) were cultured for 24 hours in the presence of 100 ng/ml lipopolysaccharide (LPS; Sigma Aldrich) or 1 µg/ml peptidoglycan (PGN; Sigma Aldrich). Mature DCs (mDCs) were phenotyped by flow cytometry and used in functional assays. Freshly isolated monocytes were stimulated in the same manner.

2.3.3 - Generation of γδ T-APCs

Freshly isolated $CD14^+$ monocytes were irradiated at 50 Gy, and subsequently plated into 96-well round-bottom plates as feeder cells. Freshly isolated, autologous Vγ9Vδ2 T cells were plated into wells containing monocytes at a 1:10 monocyte:Vγ9Vδ2 T cell ratio, unless otherwise stated. Cultures were treated with 10 nM or 1 nM HMB-PP (a kind gift from H. Jomaa, Giessen, Germany) alone, or in combination with one of the following cytokines; 100 U/ml IL-2 (Proleukin, Chiron), 20 ng/ml IL-15 (Miltenyi Biotec), 20 ng/ml IL-21 (Zymogenetics), 20 ng/ml IL-7 (Peprotech), 20 ng/ml IL-4 (Miltenyi Biotec). Cells were cultured for 3 days in complete RPMI. At day 3, Vγ9Vδ2 T cells were phenotyped and purity was assessed. If purity was below 99%, Vγ9Vδ2 T cells were further purified from contaminating cells using identical methods to initial cell isolation. Cells were collected and subsequently irradiated at 12 Gy, or left non-irradiated where stated in experimental protocols. Vγ9Vδ2 T cells were then utilised for assays or analysis.

2.3.4 - Cellular Proliferation Assays

For the measurement of cell proliferation, relevant cells (Vγ9Vδ2 T cells, naive $CD4^+$ T cells, etc.) 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. Cell proliferation was assessed at day 5 unless otherwise stated, by measuring CFSE dilution in the FITC channel by flow cytometry.

2.3.5 - Cellular Supernatant Generation

Cellular supernatants were generated for the measurement of secreted cytokines by ELISA. Vγ9Vδ2 T cell supernatants were obtained after the 3-day culture period of γδT-APC generation (described above). Supernatants were removed from cultures, centrifuged to ensure no cell contamination, and subsequently stored at −20°C until analysis. Similarly, DC and monocyte supernatants were generated by stimulating cells over a 3-day culture period with either 100 ng/ml LPS, 1 µg/ml PGN, or left unstimulated. Supernatants were collected in the same manner.

Polarised $CD4^+$ T cell supernatants were generated over a 24 hour culture period. Cultures were counted using a haemocytometer and re-plated at 50,000 cells per well prior to stimulation. Cells were stimulated with 10 ng/ml PMA (Sigma Aldrich) and 1 µg/ml ionomycin (Sigma Aldrich), and supernatants were collected as previously described.

To obtain supernatants from intestinal tissue cells, tissue samples from colon or terminal ileum were prepared as previously stated and plated into 24-well plates in complete RPMI. Cultures were treated with 50 U/ml IL-2 and 20 ng/ml IL-15, in the presence or absence of 10nM HMB-PP. After a culture period of 3 days, supernatants were obtained and stored at −20°C until analysis.

2.3.6 - Vγ9Vδ2 T cell Antigen Uptake

Vγ9Vδ2 T cells were stimulated in an identical manner to generation of γδT-APCs. At the relevant timepoint (1, 24, 72 and 120 hours after initial stimulation), cells were separated into triplicate wells, with two conditions incubated at 37°C and one condition incubated at 4°C for 2 hours prior to assay. FITC-conjugated BSA (1 mg/ml; Sigma Aldrich) was added to cultures at the relevant temperatures, or as control no BSA-FITC was added. After 1 hour incubation, cultures were washed three times with complete RPMI, stained with extracellular markers, and assessed for fluorescence in the FITC channel by flow cytometry.

2.3.7 - $CD4^+$ T cell Polarisation

Freshly isolated $CD4^+$ T cells were cultured in flat-bottom 96-well plates which had been pre-coated with anti-CD3 monoclonal antibodies (OKT3 functional grade, eBioscience) for >2 hours prior to $CD4^+$ T cell addition. 50,000 $CD4^+$ T cells were plated out per well in complete RPMI, unless otherwise stated. Subsequently, different combinations of cytokines and monoclonal antibodies were added to $CD4^+$ T cell cultures, as described in Table 2.1. $CD4^+$ T cells were cultured for 5 days (analysis of proliferation), or for 6 days (cytokine expression/transcription factor expression) upon which cultures were transferred to round-bottom 96-well plates and supplemented with fresh RPMI containing 50 U/ml IL-2 and 20 ng/ml IL-23 (Miltenyi Biotec), unless otherwise stated. Cells were cultured until day 9, at which timepoint cells were analysed.

For analysis of intracellular cytokines, $CD4^+$ T cell cultures were restimulated with 10 ng/ml PMA and 1 µg/ml ionomycin for 5 hours at 37°C, in complete RPMI. After 1 hour of the 5 hour incubation period, 10 µg/ml brefeldin A (BioLegend) was added to cultures, unless otherwise stated. Following the 5 hour incubation, cultures were stained for flow cytometric analysis.

For analysis of secreted cytokines, cells were counted and restimulated as previously described.

For analysis of transcription factor expression by PCR, cultures were counted and RNA was extracted from cells directly.

Table 2.1 - Polarising factors used in the polarisation of $CD4^+$ T cells

Polarising Condition	Polarising Factor	Concentration	Company
All	Anti-CD3	2.5 µg/ml (Th1 - 5 µg/ml),	eBioscience
All	Anti-CD28	1 µg/ml	eBioscience
Th1	IL-12	20 ng/ml	Miltenyi Biotec
Th1	IL-2	100 U/ml	Chiron
Th2	IL-4	20 ng/ml	Miltenyi Biotec
Th17	IL-1β	20 ng/ml	Miltenyi Biotec
Th17/Th22	IL-6	50 ng/ml	Miltenyi Biotec
Th17/Treg	TGFβ	2 ng/ml	Pharmingen
Th22	TNFα	20 ng/ml	Miltenyi Biotec
Th2/Th17/Th22/Treg	Anti-IFNγ	10 µg/ml	BioLegend
Th1/Th17/Th22/Treg	Anti-IL-4	10 µg/ml	BioLegend
-	sCD70	2 µg/ml	Gift from Jannie Borst, Netherlands Cancer Institute
-	Anti-ICOS	10 µg/ml	eBioscience

2.3.8 - Mixed Lymphocyte Reactions

γδ T-APCs were generated as previously stated and either irradiated or non-irradiated prior to assays. γδ T-APCs were plated out in 96 well round-bottom plates at either 50,000, 5000, or 500 cells per well, depending on the ratio of APC:responder. Subsequently, allogeneic $CD4^+$ T cell responders were added to γδ T-APC cultures at 50,000 cells per well. Co-cultures were incubated for either 5 days (proliferation analysis) or 9 days (cytokine/transcription factor expression). For analysis of intracellular cytokines, co-cultures were restimulated with PMA/ionomycin for 5 hours as previously stated. Cultures were then stained with fluorochrome conjugated monoclonal antibodies for analysis by flow cytometry. For analysis of cytokine secretion, cultures were counted and re-plated at 50,000 cells per well, then stimulated for 24 hours with PMA/Ionomycin as previously stated. For analysis of transcription factor expression, $CD4^+$ T cell responders were purified from surviving γδ T-APC contaminants by FACS based on CD4 expression to >99.1% purity. Following purification, RNA was extracted directly from cultures.

MLRs of $CD4^+$ T cell responders with monocytes or dendritic cells were conducted in an identical manner.

For assays where blocking of co-stimulatory molecules was conducted, γδ T-APCs were cultured with blocking monoclonal antibodies for >2 hours prior to addition of responder cells. Following this incubation, γδ T-APCs were washed three times, before $CD4^+$ T cell responder cells were added to cultures. For assays where blockade of cytokines was conducted, blocking antibodies were added directly to co-cultures at day 0 of MLR co-culture assays. Technical replicates were conducted in duplicate.

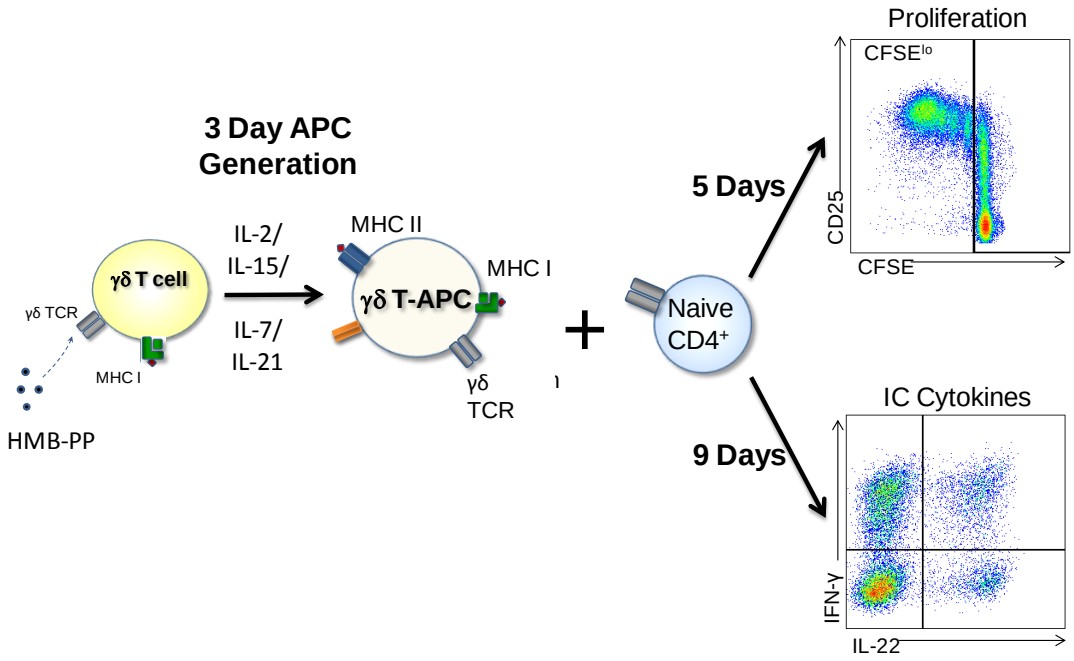

Figure 2.1 - Overall Protocol for MLR Setup.

2.3.9 - TSST-1 Assays

γδ T-APCs were generated as previously stated, and $CD4^+$ T cell populations were isolated to high purities from autologous donors. γδ T-APCs were plated into 96 well round-bottom plates at 5,000 cells per well, and either 10 ng/ml or 1 ng/ml TSST-1 (Toxin Technology) was added to γδ T-APC cultures for 1 hour. Cultures were subsequently washed three times in complete RPMI prior to addition of $CD4^+$ T cell responders at 50,000 cells per well. Co-cultures were then cultured in the same manner as MLRs for a period of 9 days. Percentages of $CD3^+CD4^+V\beta2^+$ T cells and cytokine expression were determined by flow cytometry. Technical replicates were conducted in duplicate.

2.3.10 - PPD Assays

γδ T-APCs were generated as previously stated, and $CD4^+$ T cell populations were isolated to high purities from autologous, BCG-vaccinated, healthy donors. At 48 hours into γδ T-APC generation, cells were pulsed with 1 µg/ml PPD (Sigma Aldrich) for the final 24 hours of APC generation, to allow for antigen uptake. Similarly, iDCs and monocytes were stimulated with LPS or PGN for 24 hours in combination with 1 µg/ml PPD. Following 24 hour culture with PPD, APCs were washed three times with complete RPMI. Subsequently, $CD4^+$ T cells were added to APC cultures at a 1:10 APC:responder ratio and cultured for a period of 5 or 9 days in the same

manner as MLRs described previously. Technical replicates were conducted in duplicate.

2.3.11 - Expansion of Vγ9Vδ2 T cells

Vγ9Vδ2 T cells were expanded from PBMCs of healthy donors using 1 µM zoledronate (Zometa; Novartis) for 14 days of culture, as per previously published protocols[325]. Zoledronate in complete RPMI was added to total PBMC cultures at day 0. At day 5 of culture, cells were supplemented with fresh complete RPMI containing either 100 U/ml IL-2, 20 ng/ml IL-15, 20 ng/ml IL-21, or 20 ng/ml IL-7. Similarly, cytokines in fresh RPMI were added to cultures every 2-3 days, or when media had turned acidic. After 14 days of culture, expanded Vγ9Vδ2 T cells (<90% purity) were purified from contaminating cells using an identical method to Vγ9Vδ2 T cell isolation from PBMC (>99% purity), described previously. Expanded cells were phenotyped, and subsequently either stimulated with 1 nM HMB-PP or left unstimulated for use in assays.

2.3.12 - Migration Assays

For migration assays, γδ T-APCs were generated as previously described. Transwell plates (HTS transwell 96 well permeable supports with 5 µm pores plates; Corning) were utilised for all migration assays, and 100,000 cells were used per well. Chemotaxis buffer comprised of RPMI supplemented with 5% human serum albumin and 1 M HEPES (Sigma Aldrich) was used throughout the assay. A series of 10-fold serial dilutions were prepared for CXCL10, CCL2, and CCL25, with a maximum concentration of 1 µg/ml. Alternatively, blank chemotaxis buffer was used as a negative control. Chemokines were added to lower chambers at different concentrations, and γδ T-APCs were added to upper chambers. Chemotaxis assays were run for 3 hours. Cells, which had migrated to the lower chamber, were collected and stained for phenotypic markers and assessed by flow cytometry. AccuCheck counting beads (ThermoFisher) were used to calculate the percentage of cells that had migrated. Technical replicates were conducted in duplicate.

2.4 - Flow Cytometry

2.4.1 - Staining Protocol

For all flow cytometric measurements, cells were firstly stained with Zombie Aqua Fixable Viability Kit (BioLegend) to distinguish between live and dead cells in the analysis. Live/Dead stain was diluted at 1:40 dilution, and 3 µl of diluted stain were

added to cells in 96-well, round-bottom plates. Cells were incubated with stain for 20 minutes at room temperature. Subsequently, cells were washed with FACS buffer, and treated with intravenous immunoglobulin (IvIg; Kiovig; Baxter) at a 1:1000 dilution to block Fc receptors on cells, for 15 minutes at room temperature.

For extracellular staining of antigens, Live/Dead stained cells were incubated for 20 minutes on ice, with panels of monoclonal antibodies conjugated with different fluorochromes. A complete list of all antibodies and appropriate dilutions can be found in Table 2.2. For intracellular staining of antigens, extracellularly-stained cells were incubated with Fixation Buffer (eBioscience) for 15 minutes at room temperature. Cells were subsequently incubated with Permeabilisation Buffer (eBioscience) and incubated with fluorochrome-conjugated monoclonal antibodies diluted in permeabilisation buffer, for 20 minutes at room temperature according to the manufacturer's instructions. In all cases, cells were washed with FACS buffer between each stage of staining. Cellular samples were acquired using an 8-colour BD FACSCanto II (BD Biosciences).

2.4.2 - Table of Antibodies

Table 2.2 - Antibodies used in this study

Antigen	Clone	Conjugate	Dilution	Company	Application
CD3	UCHT1	BV421	1/100	BioLegend	Flow Cytometry
CD3	SK7	FITC	1/30	BD Bioscience	Flow Cytometry
CD4	RPA-T4	APC-H7	1/80	BD Bioscience	Flow Cytometry
CD4	RPA-T4	BV421	1/100	BD Bioscience	Flow Cytometry
CD8	HIT8a	PE	1/20	BD Bioscience	Flow Cytometry
CD14	M5E2	BV421	1/40	BioLegend	Flow Cytometry
CD25	BC96	APC	1/20	eBioscience	Flow Cytometry
CD27	M-T271	FITC	1/20	BD Bioscience	Flow Cytometry
CD40	mAB89	PE	1/20	Beckman Coulter	Flow Cytometry
CD45RA	HI100	APC	1/10	BD Bioscience	Flow Cytometry
CD45RO	UCHL1	FITC	1/20	BD Bioscience	Flow Cytometry
CD70	113-16	FITC	1/20	BioLegend	Flow Cytometry
CD80	2D10.4	FITC	1/10	eBioscience	Flow Cytometry
CD83	HB15c	PE-Cy7	1/100	BD Bioscience	Flow Cytometry
CD86	IT2.2	APC	1/20	BioLegend	Flow Cytometry
CD275 (ICOSL)	2D3	PE	1/20	BioLegend	Flow Cytometry
CD275 (ICOSL)	MIH12	PE	1/20	eBioscience	Flow Cytometry
CCR2	K036C2	APC	1/20	BioLegend	Flow Cytometry
CCR4	1G1	PE-Cy7	1/20	LeukoSite	Flow Cytometry
CCR6	11A9	PE	1/20	BD Bioscience	Flow Cytometry
CCR7	G043H7	PE-Cy7	1/20	BioLegend	Flow Cytometry
CCR9	L053E8	AF647	1/30	BioLegend	Flow Cytometry
CCR10	314305	APC	1/40	R&D	Flow Cytometry
CXCR3	49801.111	FITC	1/10	R&D	Flow Cytometry
TCR Vβ2	MPB2D5	FITC	1/40	Beckman Coulter	Flow Cytometry
TCRVδ2	B6.1	PE	1/50	BD Bioscience	Flow Cytometry
TCRVγ9	Immu360	PE-Cy5	1/400	Beckman	Flow Cytometry

				Coulter	
HLA-DR	L243	APC-H7	1/40	BD Bioscience	Flow Cytometry
β7 Integrin	FIB504	APC	1/20	BioLegend	Flow Cytometry
IFN-γ	4S.B3	BV421	1/50	BioLegend	Flow Cytometry
IL-4	8D4-8	PE	1/10	BD Bioscience	Flow Cytometry
IL-9	MH9A4	AF647	1/20	BioLegend	Flow Cytometry
IL-10	JES3-9D7	PE-Cy7	1/20	BioLegend	Flow Cytometry
IL-17	64DEC17	APC	1/40	eBioscience	Flow Cytometry
IL-22	22URTI	PE-Cy7	1/40	eBioscience	Flow Cytometry
CD3	OKT3	Purified	1-5 µg/ml	eBioscience	Functional Assays
CD11a	TS1-22	Purified	10 µg/ml	Gift from Ruggero Pardi, Milan	Blocking Assays
CD18	TS1-18	Purified	10 µg/ml	Gift from Ruggero Pardi, Milan	Blocking Assays
CD28	CD28.2	Purified	1 µg/ml	eBioscience	Functional Assays
CD48	TU145	Purified	10 µg/ml	BD Bioscience	Blocking Assays
CD70	Ki-24	Purified	10 µg/ml	BD Bioscience	Blocking Assays
CD80	2D10.4	Purified	10 µg/ml	BD Bioscience	Blocking Assays
CD86	IT2.2	Purified	10 µg/ml	BD Bioscience	Blocking Assays
CD134 (OX40L)	MAB10541	Purified	10 µg/ml	R&D	Blocking Assays
CD137 (4-1BBL)	H41BB-M127	Purified	10 µg/ml	BD Bioscience	Blocking Assays
CD275 (ICOSL)	9F.8A4	Purified	10 µg/ml	BioLegend	Blocking Assays
CD278 (ICOS)	MIH12	Purified	10µg/ml	eBioscience	Functional Assays
IFN-γ	B27	Purified	10 µg/ml	BioLegend	Blocking Assays
sTNFR	-	Purified	25 mg/ml	Immunex	Blocking Assays
IL-4	8D4-8	Purified	10 µg/ml	BioLegend	Blocking Assays

IL-6	MQ2-13A5	Purified	10 µg/ml	BioLegend	Blocking Assays
IgG1	MOPC-21	Purified	10 µg/ml	BioLegend	Isotype (Functional Assays)
IgG2	MPC-11	Purified	10 µg/ml	BioLegend	Isotype (Functional Assays)
IgG3	MG3-35	Purified	10 µg/ml	BioLegend	Isotype (Functional Assays)

2.4.3 - Analysis

Unless otherwise stated, 50,000 events were collected using a BD FACS Canto II for all experiments. All analysis of raw data was performed using FlowJo software (Version 10, TreeStar Inc.), by gating on intact cells (FSC-A/SSC-A), single cells (FSC-A/FSC-H), live cells (Zombie Aqua^{-}), and expression of markers of interest. Percentages of cells were transferred to GraphPad Prism 6 software (GraphPad Software, Inc., CA, USA) for further analysis.

2.5 - ELISA

Soluble cytokines were measured in cell-free culture supernatants using ELISA kits for several cytokines. IFN-γ (BioLegend), TNF-α, IL-4, IL-5, IL-13, IL-17, IL-22, IL-10, IL-23 (all from eBioscience), IL-1β, IL-6, CXCL13, and TGF-β (all from R&D Systems) were all measured according to the manufacturers' protocols. All samples were measured in duplicate on a Dynex MRX II reader.

Analysis of ELISA data sets was conducted using Microsoft Excel, and absorbance values were calculated by subtracting values at 570 nm from those obtained at 450 nm. Concentrations of cytokines were calculated using the standard curve method.

2.6 - Real-time PCR

2.6.1 - RNA Isolation

Total RNA was extracted from cell pellets using the RNeasy Micro Kit (Qiagen) according to the manufacturer's instructions. Extracted RNA was examined

qualitatively and quantitatively using a NanoDrop ND1000 (Thermo Scientific) for RNA concentration and purity (ratios of OD at wavelengths of 230, 260, and 280 nm). RNA was subsequently divided into aliquots and stored at −80°C, or alternatively used for cDNA generation immediately.

2.6.2 - Generation of cDNA

Total RNA was used to generate cDNA, using the SuperScript VILO cDNA Synthesis Kit (ThermoFisher Scientific), according to the manufacturer's instructions. cDNA was stored at −80°C until use.

2.6.3 - Real-Time Quantitative PCR

Transcripts were quantified by real-time quantitative PCR (RT-qPCR) using a ViiA7 Real-Time PCR System (ThermoFisher Scientific). Predesigned TaqMan Gene Expression Assays and reagents were used according to the manufacturers' instructions. Probes with the following ThermoFisher Scientific assay identification numbers were utilised;

TBX21 - Hs00203436_m1,
GATA-3 - Hs00231122_m1,
RORC - Hs01076112_m1,
AHR - Hs00169233_m1,
FOXP3 - Hs01085834_m1,
BCL-6 - Hs00153368_m1,
18S RNA - Hs99999901_m1,
PPIL-2 - Hs00204962_m1

(all from ThermoFisher Scientific).

The Taqman Universal Master Mix II, no UNG (ThermoFisher Scientific) was used for PCR assays. All samples were measured in triplicate technical replicates.

2.6.4 - Analysis

The comparative ΔΔCt method was used to calculate the relative quantification and the range of confidence or samples. Data was analysed using the ExpressionSuite Software (ThermoFisher Scientific), and mRNA abundance was normalised to the amount of PPIL2 (Cyclophilin) expressed by cells, and is presented as relative expression in arbitrary units.

2.7 - 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, either the one-way ANOVA (parametric data sets), Kruskal-Wallis test (unpaired, non-parametric data sets), or Friedman test (paired, non-parametric data sets) was used. Following analysis, the Dunn's 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.

2.8 - Ethics

This study was conducted according to the principles expressed in the Declaration of Helsinki and under local ethical guidelines (Bro Taf Health Authority, Wales). The study was approved by the South East Wales Local Ethics Committee (Reference Number 04WSE04/27). All healthy blood donors provided written informed consent for the collection of samples and subsequent analysis.

For experiments conducted at QMUL Blizard Institute, ethical permissions for the study were granted by the appropriate local research ethics committees (approvals 05/ Q0405/71 from Harrow Research Ethics Committee; 10/H0704/74 from East London Research Ethics Committee 2, London, UK; P/01/023 from East London and City Health Authority Research Ethics Committee, London, UK; and 7/H0805/46 from Bromley Local Research Ethics Committee). All volunteers gave written informed consent prior to inclusion in the study[345].

Chapter 3 - Optimisation of $CD4^+$ T Cell Polarisation and Generation of Antigen Presenting Cells

3.1 - Introduction

Despite the potent APC function of Vγ9Vδ2 T cells with regard to cross-presentation of soluble antigens to $CD8^+$ T cells, the full capacity of these novel APCs to polarise distinct subsets of $CD4^+$ T helper cells is currently unknown. The ability of $CD4^+$ T cells to be polarised towards distinct effector phenotypes has been extensively studied in the literature, with numerous T helper subsets being described. Further to this, with the concept of $CD4^+$ T cell plasticity becoming more and more recognised, these T helper lineages have been shown to be capable of significant diversity in the cytokines they produce[156]. Given the large number of studies on each T helper lineage and the multitude of experimental protocols utilised by such studies, initial experiments in the present thesis focused on the optimisation of the conditions necessary for efficient $CD4^+$ T cell polarisation. By optimising these protocols, and using them to polarise each T helper subset in turn, a useful reference point would be generated for the overall study into the polarisation of $CD4^+$ T cell responses by γδ T-APCs.

In addition, different types of antigen presenting cells differ in the ability to polarise $CD4^+$ T cells towards specific lineages has been highlighted in the literature. In order to give the polarisation of $CD4^+$ T cell responses by γδ T-APCs some context and determine their efficacy at inducing different T helper lineages, identical polarisations by other antigen presenting cell subsets were necessary. For this purpose, the role of well-studied APCs such as dendritic cells and monocytes, and their characteristics, was examined.

Lastly, an efficient generation of γδ T-APCs themselves is required to study their $CD4^+$ T cell-polarising capacity. Despite the number of studies examining the ability of Vγ9Vδ2 T cells to act as antigen presenting cells, it remains unclear under which conditions these cells actually assume their novel function. The majority of studies have examined TCR stimulation in combination with IL-2 to generate the APC phenotype and function[322], while limited studies have assessed combinations of IL-2 and IL-15[325]. The family of cytokines which utilise receptors containing the common γ-chain have shown significant ability to promote Vγ9Vδ2 T cell responses[277,282]. Given the significant role of the common-γ chain cytokines in optimal γδ T cell

immunity, their potential roles in the generation of γδ T-APCs and regulation of APC function was investigated.

3.2 - Aims of Chapter

In this chapter, four experimental aims will be discussed:

- The optimisation of $CD4^+$ T cell polarisation protocols.
- The polarisation of naive $CD4^+$ T cell responses towards distinct T helper lineages.
- The generation and optimisation of dendritic cell and monocyte APC controls.
- The generation and functional comparison of γδ T-APCs produced in the presence of differing cytokine microenvironments.

3.3 - Optimisation of $CD4^+$ T cell Polarisation

As a first step in developing polarised $CD4^+$ T cell controls for subsequent APC assays, a number of factors were optimised. As previously discussed, the strength of stimulation, effect of co-stimulation, and presence of polarising cytokines are all important in determining the outcome of $CD4^+$ T cell polarisation[161]. To this end, the concentrations of stimulating agents, namely anti-CD3 and anti-CD28 antibodies, were optimised alongside other factors such as the length of the culture period and the type of culture medium. It should be noted that combinations of cytokines used for $CD4^+$ T cell polarisation, and their relevant concentrations, were not optimised here, due to the extensive study in the literature describing the most effective combinations[92,121]. For all of these experiments, 'enriched' naive $CD4^+$ T cells were used, where total $CD4^+$ T cells were isolated from healthy peripheral blood, and CD45RO expressing memory cells were subsequently depleted. As such, the purity of the resulting $CD4^+$ T cells ($CD4^+CD45RA^+$) was approximately 95% (data not shown).

In the first instance, concentrations of anti-CD3 and anti-CD28 were titrated, and the induction of $CD4^+$ T cell effector responses was determined (Figure 3.1). In combination with anti-CD3/anti-CD28 stimulation, cells were cultured in either Th1 (IL-12), Th2 (IL-4), or Th17 (IL-1β/IL-6/TGFβ) polarising cytokine conditions to promote the relevant T helper lineages, and cultured for 6 days. Following this culture period, cells were restimulated with PMA/Ionomycin to induce cytokine production, and cultured for a further 24 hours. Subsequently, culture supernatants were obtained and assessed for IFN-γ, IL-4 and IL-17 by ELISA.

Figure 3.1a displays the concentration of IFN-γ, IL-4 and IL-17 produced by cells cultured in the presence of Th1, Th2, and Th17 polarising conditions respectively, in response to increasing concentrations of anti-CD3 antibody. IFN-γ displayed a dose-dependent response, with increasing concentrations of anti-CD3 favouring increased levels of IFN-γ. This is consistent with previous studies, where stronger TCR stimulations were reported to promote Th1-type responses[160]. In contrast, IL-4 production was favoured at lower concentrations of anti-CD3, and was inhibited at concentrations of 5 µg/ml. Similarly, IL-17 production peaked at concentrations of 2.5 µg/ml anti-CD3. This too is consistent with published studies, where lower TCR signalling strengths favoured non-Th1 responses[163]. Given these results, the optimal anti-CD3 concentrations for Th1 responses was 5 µg/ml, whereas for Th2 and Th17

responses it was 2.5 µg/ml, which were consequently utilised in all following experiments.

Next, the concentration of anti-CD28 was examined (Figure 3.1b). There was little variation in the production of cytokines where anti-CD28 concentration differed, indicating a more redundant role of this pathway in $CD4^+$ T cell polarisation, at the concentrations examined. The optimal concentration of anti-CD28 appeared to be 1 µg/ml, and as such was utilised for all subsequent $CD4^+$ T cell polarisation assays.

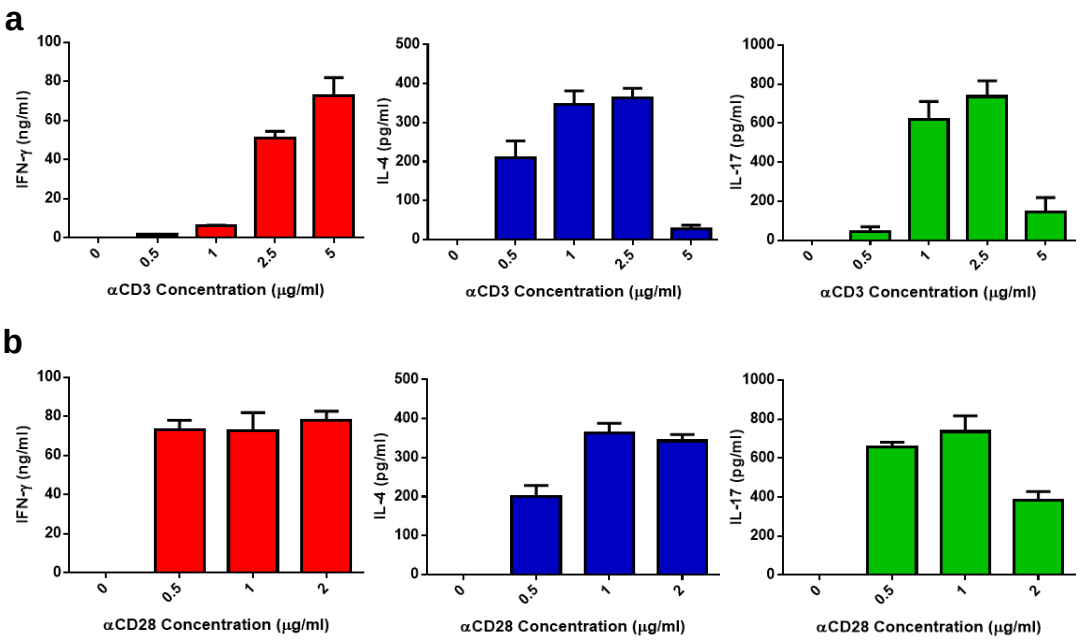

Figure 3.1 - Titration of anti-CD3 (αCD3) and anti-CD28 (αCD28) stimulating antibodies for the polarisation of CD4$^+$ T cell responses. (a) Secretion of IFN-γ, IL-4 and IL-17 by CD4$^+$ T cells cultured in the presence of Th1 (IL-12), Th2 (IL-4), or Th17 (IL-1β/IL-6/TGFβ) polarising cytokines, respectively, at varying concentrations of αCD3 antibody. All cells were stimulated with 1 μg/ml αCD28 antibody. **(b)** Secretion of IFN-γ, IL-4 and IL-17 by CD4$^+$ T cells cultured in the presence of Th1, Th2, or Th17 polarising cytokines, respectively, at varying concentrations of αCD28 antibody. Cells cultured in Th1 conditions were stimulated with 5 μg/ml αCD3, whereas Th2 and Th17 polarised cells were stimulated with 2.5 μg/ml αCD3. In all experiments, CD4$^+$ T cells were freshly isolated from peripheral blood and depleted of CD45RO expressing memory cells. Cytokine concentrations were determined in cell culture supernatants by ELISA, after 24 hour restimulation with PMA/Ionommycin. Bar charts display mean data from two healthy donors in two individual experiments. Error bars display standard deviation of samples.

With the concentrations of stimulating agents determined, different time points for analysis of intracellular cytokine production were assessed. In the literature, several different time points for CD4$^+$ T cell cytokine production have been used, from 6 to 12 days of culture after initial stimulation[92,97,98]. As such, experiments were designed to assess the optimal time point for intracellular analysis. Similarly to previous experiments, enriched naive CD4$^+$ T cells were stimulated and cultured in Th1, Th17, or Th22 (IL-6/TNFα) polarising conditions and cultured for 6-12 days. At each time point, cultures were restimulated with PMA/ionomycin and stained intracellularly for cytokines (Figure 3.2).

The observed expression of each cytokine at day 6 of culture was much lower than those seen at later time points. This may be due to the fact that $CD4^+$ T cells remained in a proliferative state at day 6, whereas at later time points cells returned to a resting state, perhaps allowing for more optimal restimulation. Nevertheless, later time points appeared optimal for assessing intracellular cytokine production, with day 9 in particular standing out across all three cytokines examined. For this reason, day 9 analysis was chosen for subsequent experiments.

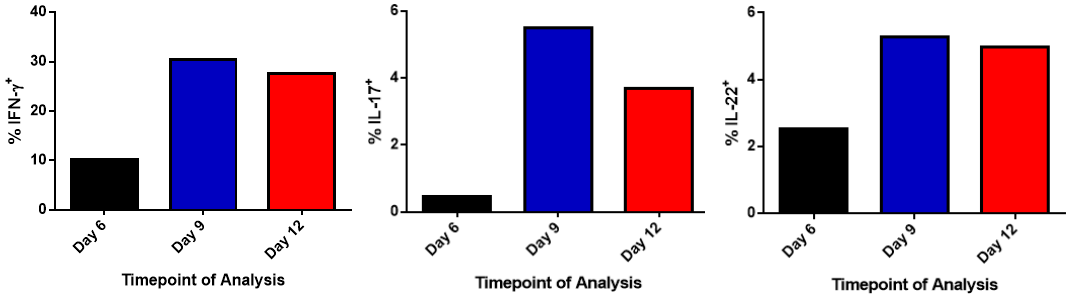

Figure 3.2 - Comparison of timepoints for intracellular analysis of CD4$^+$ T cell cytokine production. CD4$^+$ T cells were stimulated for 6-12 days in the presence of Th1 (IL-12), Th17 (IL-1β/IL-6/TGFβ), or Th22 (IL-6/TNFα) polarising cytokines, and assessed for intracellular expression of IFN-γ, IL-17, and IL-22, respectively. Cultures were restimulated with PMA/Ionomycin for 5 hours at the relevant timepoint for intracellular analysis of cytokine production by flow cytometry, and percentages of cytokine positive cells were determined. For days 9 and 12 of analysis, cultures were supplemented with IL-2/IL-23 at day 6. In all experiments, CD4$^+$ T cells were freshly isolated from peripheral blood and depleted of CD45RO expressing memory cells. Bar charts display data from one healthy donor. Data was obtained by gating on live, single, CD4$^+$ cells.

Given the late time point of analysis of CD4$^+$ T cell cytokine production, protocols which use this time point generally 'feed' cultures at approximately day 6, to improve survival of cells as they proliferate and to maintain effector phenotypes generated. For this reason, cultures were set up to investigate different combinations of cytokines in order to maintain optimal CD4$^+$ T cell polarisation readouts. IL-2 alone, or a combination of IL-2 and IL-23, were examined (Figure 3.3). IFN-γ and IL-22 were both similarly expressed across both treatments, as expected, given that IL-23 is dispensable for Th1 and Th22 responses. However, IL-23 proved to be essential for Th17 polarisation, with IL-2 alone maintaining a low percentage of IL-17$^+$ cells, whereas a combination of IL-2 and IL-23 favoured a much greater population of IL-17$^+$ cells. This is consistent with previous studies, where IL-23 was shown to maintain Th17 phenotypes and aid memory Th17 cells in their IL-17 production[85]. IL-4 production by polarised CD4$^+$ T cells was also consistent across IL-2 alone or IL-2/IL-23 treatments (data not shown). It should be noted that culture of cells with IL-23 alone did not favour the production of any cytokines examined. For these reasons, a combination of IL-2 and IL-23 was considered optimal for future experiments due to its ability to allow maximum detection of all cytokines tested.

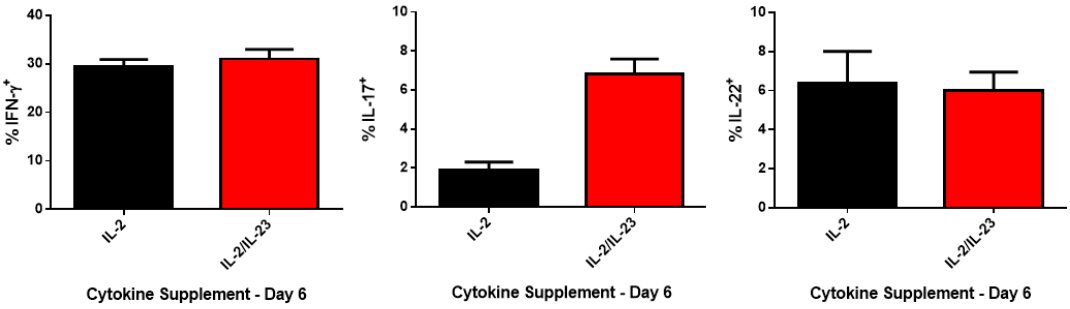

Figure 3.3 - Comparison of cytokine supplements at day 6 of CD4$^+$ T cell polarisation. CD4$^+$ T cells were stimulated for 9 days in the presence of Th1 (IL-12), Th17 (IL-1β/IL-6/TGFβ), or Th22 (IL-6/TNFα) polarising cytokines and assessed for intracellular expression of IFN-γ, IL-17, and IL-22 respectively. Cultures were supplemented with either IL-2 or a combination of IL-2 and IL-23 at day 6, and restimulated at day 9 with PMA/Ionomycin for 5 hours for intracellular analysis by flow cytometry. Resulting percentages of cytokine positive cells were determined. In all experiments, CD4$^+$ T cells were freshly isolated from peripheral blood and depleted of CD45RO-expressing memory cells. Bar charts display mean data from two healthy donors from two individual experiments. Error bars display standard deviation of samples. Data was obtained by gating on live, single, CD4$^+$ cells.

To finalise the optimisation of CD4$^+$ T cell polarisation cultures, a comparison of culture media was conducted to assess their effects on cytokine expression. The majority of studies utilise RPMI medium to study CD4$^+$ T cell polarisation, a common medium also used for Vγ9Vδ2 T cell and monocyte/dendritic cell culture. However, recently a few studies have shown the efficacy of IMDM medium to favour Th17 polarisation[346]. In order to determine which media was the best to use for future experiments, the polarisation of Th1, Th17, and Th22 lineages was examined in the presence of RPMI or IMDM media (Figure 3.4). Across all cytokines examined, percentages of cytokine-positive cells were consistent in either culture medium. The lack of efficacy of IMDM to favour Th17 differentiation in these cultures could have been due to a number of factors, not least of which the concentration of TGFβ in the fetal calf serum used to supplement the culture medium[92]. For this reason, RPMI was used for all future experiments, as it allows for examination of CD4$^+$ T cell polarisation, but is also optimal for Vγ9Vδ2 T cell culture, necessary for future assays.

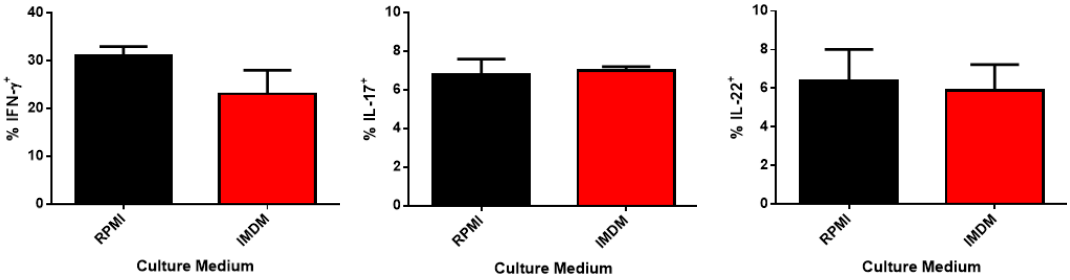

Figure 3.4 - Comparison of different culture media in the polarisation of $CD4^+$ T cells. $CD4^+$ T cells were stimulated for 9 days in the presence of Th1 (IL-12), Th17 (IL-1β/IL-6/TGFβ), or Th22 (IL-6/TNFα) polarising cytokines and assessed for intracellular expression of IFN-γ, IL-17, and IL-22 respectively. Cultures were supplemented with IL-2 and IL-23 at day 6, and restimulated at day 9 for intracellular analysis of cytokine production by flow cytometry. Resulting percentages of cytokine positive cells were determined after restimulation. $CD4^+$ T cells were freshly isolated from peripheral blood and depleted of CD45RO expressing memory cells. Bar charts display data from two healthy donors from two individual experiments. Error bars display standard deviation of samples. Data was obtained by gating on live, single, $CD4^+$ cells.

3.4 - Polarisation of Naive $CD4^+$ T cell Responses

With the optimal culture conditions determined for $CD4^+$ T cell polarisation, the next step was to investigate the ability of these conditions to polarise sorted naive $CD4^+$ T cells. Given that the optimal culture conditions in the previous section were determined using enriched naive $CD4^+$ T cell cultures, in which residual numbers of memory cells remained (up to 5% of all T cells), and the fact that highly pure naive $CD4^+$ T cells would need to be used in APC polarisation assays with γδ T-APCs, naive $CD4^+$ T cells were sorted to a high purity (>99.4% $CD4^+CD45RA^+CCR7^+$). This would confirm the optimal conditions identified for polarisation of highly pure naive $CD4^+$ T cell populations. The importance of pure cell fractions in such assays was fundamental as even minor contamination of naive $CD4^+$ T cell cultures with memory or effector cells might skew results and potentially give false readouts. In addition, pure APCs were required to be able to attribute naive $CD4^+$ T cell polarisation to each APC subset independently. Figure 3.5 displays the purity of cells isolated for all subsequent assays (Vγ9Vδ2 T cell purity >99%, monocyte purity >98.7%).

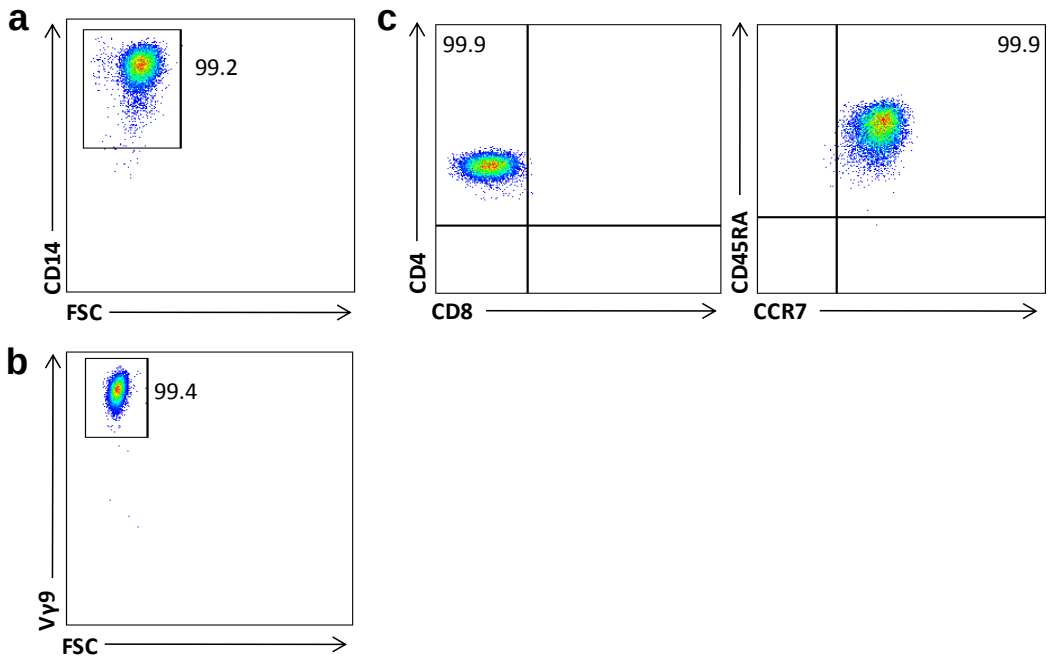

Figure 3.5 - Purity of isolated cell subsets for use in assays. (a) Representative plot displaying the purity of $CD14^+$ monocytes obtained from freshly isolated PBMC from a healthy donor, using MACS separation. Purity was assessed by flow cytometry. **(b)** Representative plot displaying the purity of TCR $V\gamma9^+$ γδ T cells obtained from freshly isolated PBMC from a healthy donor, using MACS separation. **(c)** Representative plots displaying purity of FACS sorted naive $CD4^+$ T cells obtained from freshly isolated PBMC from a healthy donor. Naive $CD4^+$ T cells were sorted based on a $CD8^-CD4^+CD45RA^+CCR7^+$ phenotype. All plots representative of >30 individual donors from individual experiments. Numbers on plots display percentages of cells. All plots are gated on live, single cells.

Having obtained highly purified naive $CD4^+$ T cells, these cells were subsequently cultured in the presence of polarising cytokines, and resulting intracellular cytokine expression determined upon restimulation. Naive $CD4^+$ T cells were cultured under Th1 (IL-12), Th2 (IL-4), Th17 (IL-1β/IL-6/TGF-β), Th22 (IL-6/TNF-α), and Treg (TGFβ) polarising conditions, and examined for the intracellular production of IFN-γ, IL-17, IL-22, IL-4, and IL-10. Figure 3.6 displays representative flow cytometry plots of cytokine expression by such polarised cells. As can be seen, IFN-γ expression by polarised naive $CD4^+$ T cells was the largest population identified, whereas IL-4, IL-17, and IL-22, were present at lower percentages in their relevant polarising conditions. This is in line with published studies, whereby IFN-γ producing cells are often the most prominent in *in vitro* experiments, compared with other T helper lineage cytokines. Under Th1 polarising conditions, minimal expression of IL-4 was detected, and *vice versa* in Th2 polarising conditions. Under Th17 polarising

conditions, significant populations of IL-17$^+$ cells were identified, with small populations of IFN-γ$^+$ and IL-22$^+$ cells identified. In contrast, under Th22 polarising conditions, small populations of IL-22 single-positive cells were observed, but significant proportions of IL-22 expressing cells were also identified as co-expressing IFNγ or IL-17.

In these experiments, only low levels of IL-10 expression were detected. Previous reports suggested that lack of protein transport inhibitor usage during intracellular staining may actually lead to higher levels of IL-10 detection[347]. This was indeed true for cultures tested in the absence of brefeldin A treatment, with frequencies of IL-10 producing cells increasing from approximately 2.5% to 4% (data not shown). However, absence of a protein transport inhibitor negates the ability to detect alternative cytokines. For this reason, intracellular cytokine stainings were carried out using the inhibitor brefeldin A, so as to allow for all cytokines to be detected, including IL-10, albeit at the cost of losing sensitivity for IL-10. Overall, these experiment demonstrated an efficient polarisation of naive CD4$^+$ T cells towards various T helper lineages in accordance with previous publications[92,121].

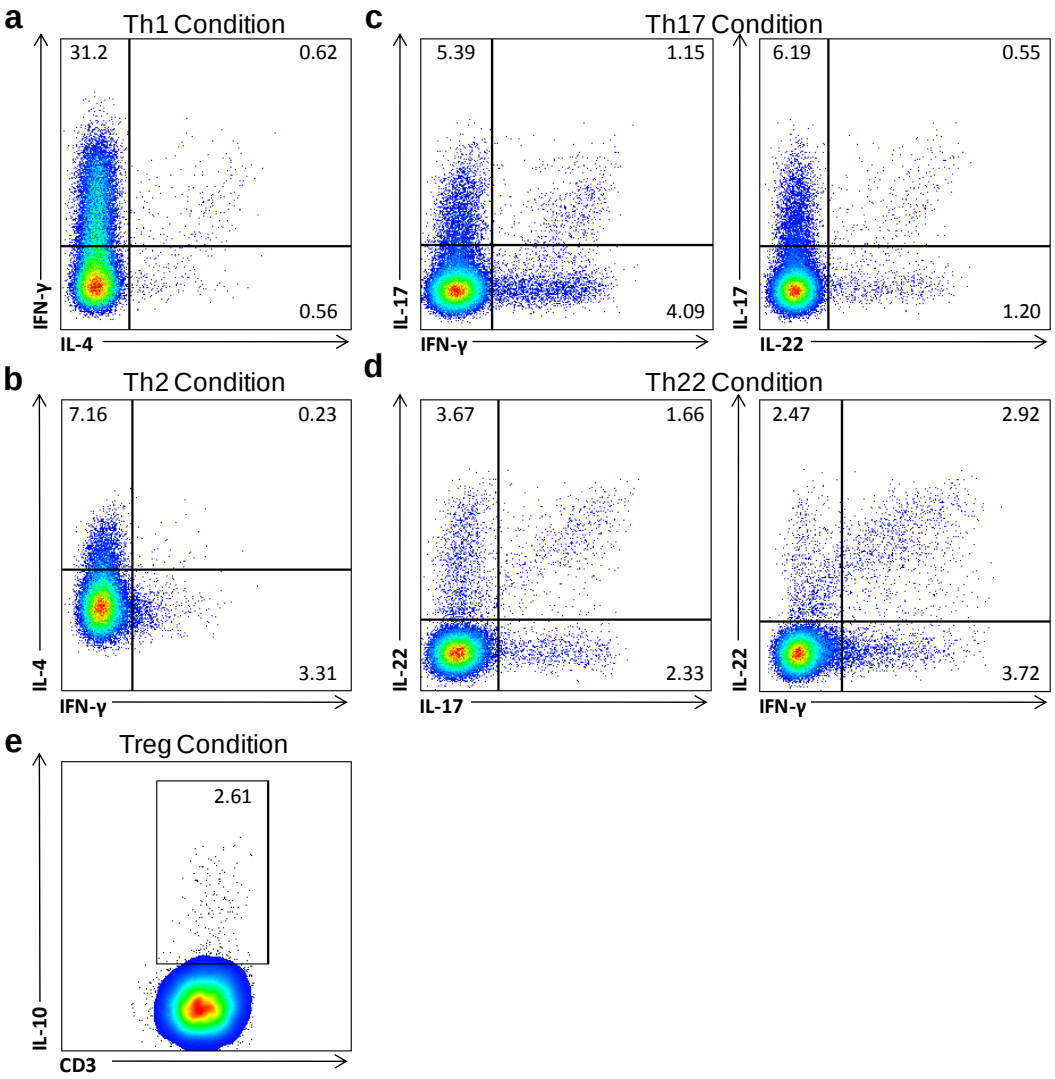

Figure 3.6 - Intracellular stainings of polarised naive CD4$^+$ T cells. Sorted naive CD4$^+$ T cells were stimulated for 9 days in the presence of T helper polarising cytokines. Cells were restimulated at day 9 for intracellular staining and analysis by flow cytometry. Cells cultured in **(a)** Th1, **(b)** Th2, **(c)** Th17, **(d)** Th22, and **(e)** Treg polarising conditions were stained for IFNγ, IL-17, IL-22, IL-4, and IL-10. Gated on live, single, CD4$^+$ cells. Representative dot plots display results for a total of 4 healthy donors from 3 individual experiments. Numbers in gates display percentages of cytokine-positive cells.

Figure 3.7 displays the overall cytokine response of naive CD4$^+$ T cells cultured in each lineage polarising condition. As expected, IFN-γ was expressed predominantly by naive CD4$^+$ T cells cultured in the presence of IL-12, to induce a Th1 phenotype. A significant level of IFN-γ expression was also observed in Th17 polarising conditions, reflective of the plasticity of human Th17 cells and their ability to co-express IFN-γ and IL-17. Th22 polarising conditions also featured heightened levels of IFN-γ expression, though this was not significant compared to unstimulated

controls. With respect to IL-17, expression levels were highest in Th17 polarising conditions, but significant levels could also be observed in Th22 polarising conditions, potentially reflecting the presence of TGF-β in the culture serum in combination with addition of IL-6[97,98]. IL-4 expression was distinctly expressed by Th2 cells, with cells cultured under other conditions expressing minimal levels.

Interestingly, Th1, Th17, and Th22-polarised cells all expressed similar levels of intracellular IL-22. Whilst commonly known as a Th17 cytokine, IL-22 can also feature strongly in Th1 type responses, explaining its presence in both of these culture conditions. The lack of increased IL-22$^+$ cells in Th22 polarising conditions is intriguing, potentially due to the fact that, while IL-6 and TNF-α have been reported to promote IL-22 production by naive CD4$^+$ T cells, these conditions have not been as extensively studied as the other polarising conditions, suggesting other factors may be important. Indeed, the original Th22 study found higher levels of IL-22 induction using certain dendritic cell subsets instead of a combination of IL-6 and TNF-α, supporting the view that other polarising factors are involved[121]. Finally, IL-10 was expressed predominantly by Treg-polarised cells but also in non-polarised cells. Of note, the overall mixed populations of cells induced under each polarising condition are reflective of the non-discrete populations observed in the human immune system as compared with murine models.

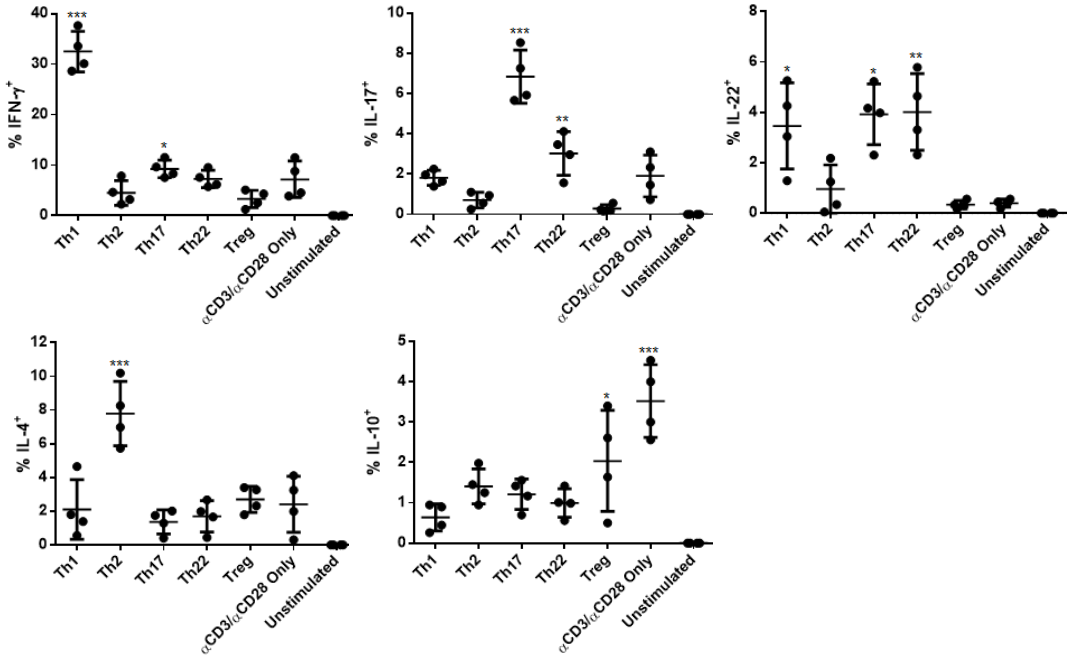

Figure 3.7 - Expression of T helper cytokines by polarised CD4$^+$ T cells. Sorted naive CD4$^+$ T cells were stimulated for 9 days in the presence of combinations of T helper polarising cytokines. Cells were restimulated at day 9 for intracellular staining and analysis by flow cytometry. Percentages of cytokine positive cells were determined by gating of live, single, CD4$^+$ cells. Data points display results from individual healthy donors. Horizontal lines display means of data sets. Error bars display standard deviation of samples. Statistical significance was determined using the Friedman Test, followed by the Dunn's multiple comparison test, *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001, ns=not significant. Significance displayed in comparison to unstimulated control.

In addition to examining the intracellular expression of cytokines in polarised CD4$^+$ T cells, the secretion of such cytokines into the culture supernatants was examined by ELISA (Figure 3.8). The production of T helper lineage cytokines showed a similar pattern of expression to that seen with intracellular expression, for example Th1 cells produced the highest concentrations of IFN-γ, whereas Th2-polarised cells produced significant levels of IL-4. In addition to the five cytokines assessed intracellularly, levels of TNF-α were also assessed in culture supernatants, and were most highly produced by Th17 cells, though this cytokine is expressed by most, if not all, T helper lineages.

Interestingly, the percentages of cytokine positive cells did not reflect exactly the levels of cytokine secreted. For example, the expression of intracellular IFN-γ by Th1 cells was approximately 3 fold higher than that observed with Th17 cells (Figure 3.7), however, the secretion of IFN-γ was only 2 fold higher by Th1 cells over Th17

(Figure 3.8). This phenomenon has been reported in the literature, whereby protein expression intracellularly and production extracellularly do not necessarily correlate exactly[348]. Despite this, overall patterns of $CD4^+$ T cell polarisation with respect to cytokine expression and production remained largely the same.

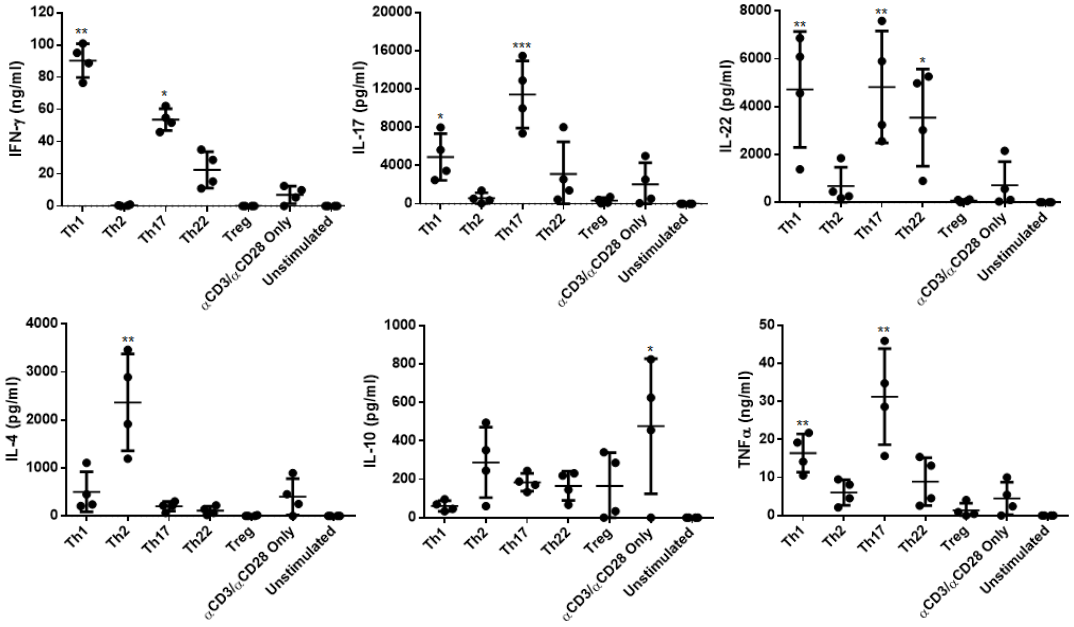

Figure 3.8 - Secretion of T helper cytokines by polarised CD4$^+$ T cells. Sorted naive CD4$^+$ T cells were stimulated for 9 days in the presence of polarising cytokine combinations. Cells were restimulated at day 9, and supernatants were collected 24 hours later for analysis by ELISA. Data points display results from individual healthy donors. Horizontal lines display means of data sets. Error bars display standard deviation of samples. Statistical significance was determined using the Friedman Test, followed by the Dunn's multiple comparison test, *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001, ns=not significant. Significance displayed in comparison to unstimulated control.

In addition to assessing cytokine production and secretion, polarised naive CD4$^+$ T cells were assessed for their expression of master transcription factors, which regulate specific phenotypes and effector functions in distinct T helper lineages[56]. Such transcription factors include the proteins *TBX21* (Th1), *GATA3* (Th2), *RORC* (Th17), *AHR* (Th22), *FOXP3* (Treg) and *BCL6* (Tfh). In identical assays to those shown in Figures 3.7 and 3.8, naive CD4$^+$ T cells were stimulated for 9 days in different polarising microenvironments. At day 9 of culture, the RNA was extracted from total, non-restimulated cell populations, and expression of each transcription factor was determined, in relation to naive CD4$^+$ T cell controls.

Figure 3.9 displays the relative gene expression levels of each transcription factor in polarised cell populations. *TBX21*, *GATA*3, and *RORC*, representing the Th1, Th2, and Th17 master transcription factors, were most highly expressed in the relevant polarising conditions. Lower expression levels of each of these transcription factors were identified in other polarising conditions, reflecting the mix of lineages induced and identified by cytokine expression. In addition, Th1 and Th17 polarised cells co-

expressed significant levels of *RORC* and *TBX21* respectively as well, highlighting the plasticity between these two lineages. Th22 cells, as expected, expressed high levels of *AHR*. However, both Th1 and Th17 polarised populations also showed significant *AHR* expression, consistent with the expression of IL-22 by the three different T cell subsets. Cells polarised in Treg-favouring conditions expressed marginally higher levels of *FOXP3* than naive $CD4^+$ T cells, though levels of expression were not as prominently affected as with other transcription factors. Treg cells should display stable *FOXP3* expression, in contrast to activated cells which show transient *FOXP3* expression[110]. Lastly, *BCL6*, representing the master transcription factor for Tfh cells[140,141], was not induced under any polarising condition. As control, $CXCR5^+$ T cells isolated directly from PBMC displayed increased *BCL6* mRNA levels compared to naive $CD4^+$ T cells.

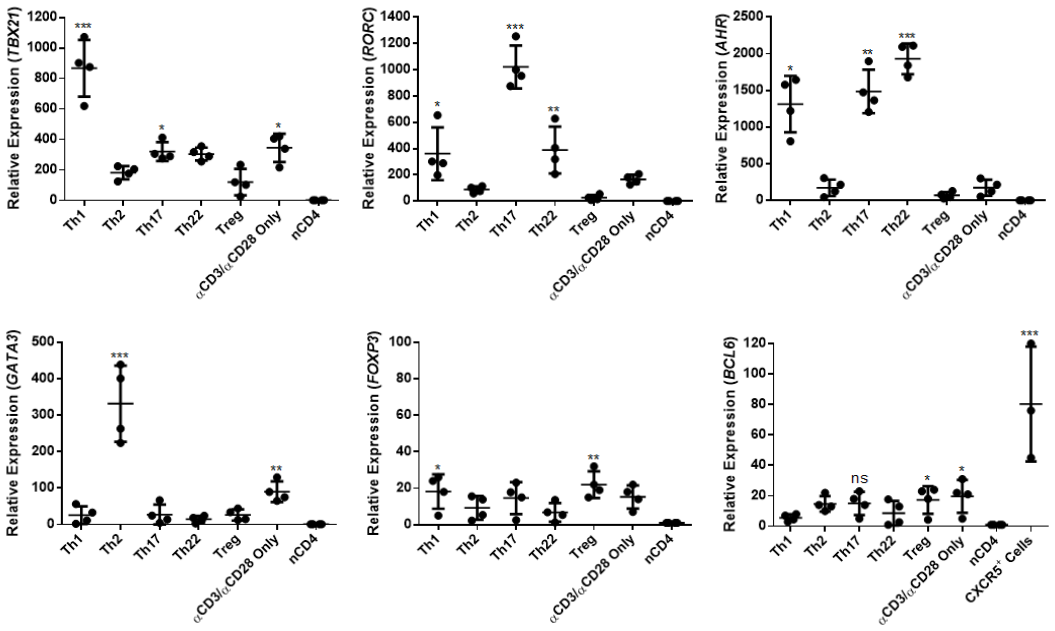

Figure 3.9 – Expression of transcription factors by polarised CD4$^+$ T cells. Sorted naive CD4$^+$ T cells were stimulated for 9 days in the presence of polarising cytokine combinations. At day 9, RNA was extracted from cultures and expression of several transcription factors was assessed by real-time PCR. As an additional control, CXCR5$^+$ cells isolated directly from PBMC were assessed for *BCL6* expression. Relative expression was determined in reference to naive CD4$^+$ T cell control. Data points display results from individual healthy donors. Horizontal lines display means of data sets. Error bars display standard deviation of samples. Statistical significance was determined using the Friedman Test, followed by the Dunn's multiple comparison test, *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001, ns=not significant. Significance displayed in comparison to unstimulated control.

In addition to measuring the functional quality of polarised cells, cell proliferation was assessed as further valuable readout for APC-induced CD4$^+$ T cell responses. Representative flow cytometry plots are shown in Figure 3.10a, displaying the dilution of CFSE and expression of CD25 by activated naive CD4$^+$ T cells. CFSE dilution, reflected by decreasing fluorescence, denotes cell proliferation, whereas CD25 is a common activation marker of T cell activation, and as such becomes expressed upon stimulation. Cells cultured under Th2 and Th17 conditions displayed high levels of proliferation at day 5 of culture, with Th1 cells and no cytokine conditions showing reduced levels of proliferation. This difference was likely due to the fact that IL-4 and IL-6, which are present in Th2 and Th17 polarising conditions respectively, function as T cell growth factors in addition to their polarising function, thereby aiding CD4$^+$ T cell proliferation. Upon co-stimulation via CD28, CD4$^+$ T cells produce IL-2 as an autologous growth factor, but this is unlikely

to be expressed at similar concentrations as IL-4 or IL-6 are added to culture. Interestingly, while cells cultured in Th1, Th17, and no cytokine conditions all upregulated CD25, Th2 cells expressed lower levels or were even negative for CD25 expression, despite proliferating at comparable levels. This indicates that Th2 cells do not utilise CD25, a component of the IL-2 receptor, and as such may rely solely on IL-4 as a growth factor. Figure 3.10b shows the comparison of $CD4^+$ T cell proliferation at days 4 and 5 of culture. Higher percentages of proliferating cells were observed at day 5 of culture than day 4, reflecting the increased amount of time for cells to proliferate. As such, day 5 analysis of proliferation was used for subsequent assays.

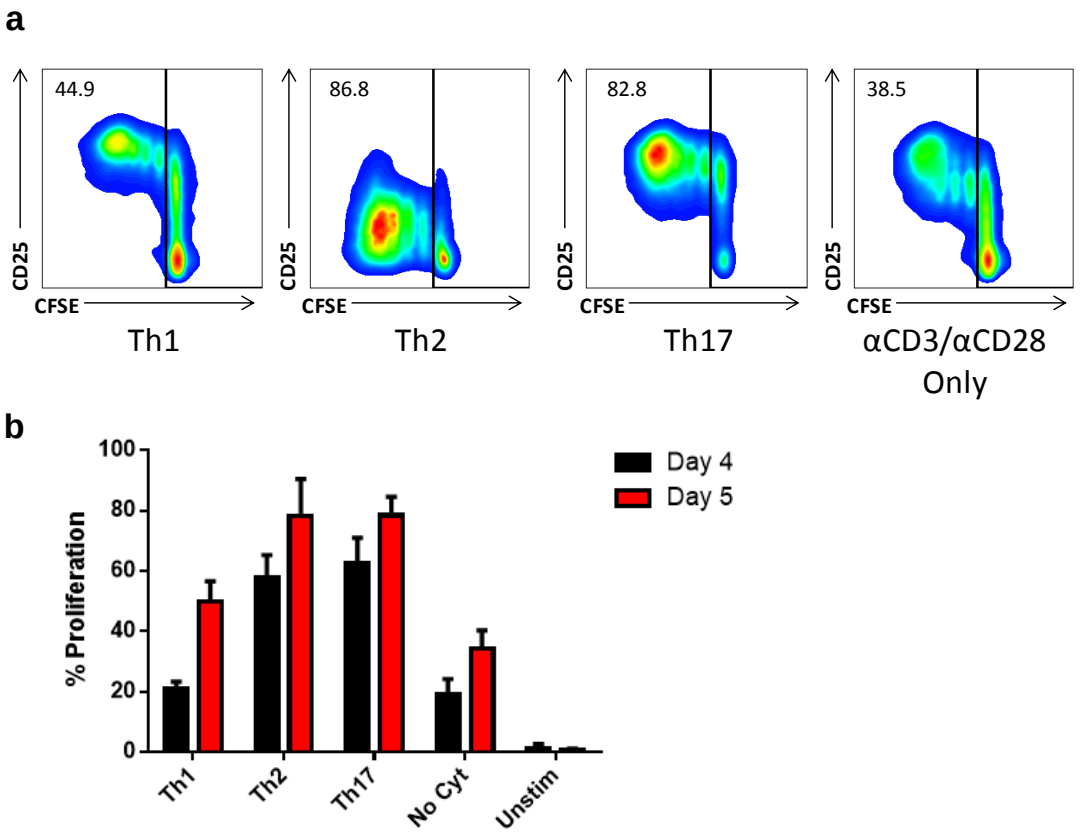

Figure 3.10 - Proliferation of polarised naive CD4$^+$ T cells. (a) Representative plots of naive CD4$^+$ T cell proliferation. Sorted naive CD4$^+$ T cells were stimulated for 5 days in the presence of combinations of T helper polarising cytokines. Proliferating cells were determined by CFSE dilution as assessed by flow cytometry, and numbers on graphs display percentages of proliferating cells. Plots are representative of two healthy donors from two individual experiments. Gated on live, single, CD4$^+$ cells. **(b)** Comparison of naive CD4$^+$ T cell proliferation at multiple timepoints. Percentages of proliferating cells were compared at days 4 and 5 for each polarising condition. Values displayed are mean values of two donors from two individual experiments. Error bars display standard deviation of samples. Gated on live, single, CD4$^+$ cells.

Taken together, by utilising highly purified naive CD4$^+$ T cells, differential polarising conditions were able to induce markedly different phenotypes in responding cells. Given the ability to detect each T helper lineage in turn, the ability to generate different APC subtypes for investigation of APC-induced CD4$^+$ T cell polarisation was next to be examined.

3.5 - Generation of APC Controls

Monocyte-derived dendritic cells are the prototypic antigen presenting cell studied in human immunology. Indeed, monocytes and DCs have been extensively shown to polarise distinct $CD4^+$ T cell responses, and are adaptable to external stimuli[206]. Such well-defined APCs polarising $CD4^+$ T cell responses under identical experimental conditions to those that were to be used with γδ T-APCs therefore represented a useful control, which ensured that data produced here were consistent with published studies.

Figure 3.11 displays the phenotype of APCs generated for future assays. Figure 3.11a shows the morphology of monocyte-derived DCs after differentiation from purified monocytes cultured with IL-4 and GM-CSF for 6 days. In addition to phenotype, cell morphology is an indication that monocytes have differentiated into DCs. As observed, monocyte-derived DCs displayed dendrite-like protrusions, indicating their efficient differentiation from monocytes. Figure 3.11b shows representative flow cytometry stainings of DCs after a 24 hour stimulation with LPS. Figure 3.11c displays the comparison of APC phenotype between monocytes and DCs before and after 24 hour stimulation with LPS. A number of APC markers were examined; HLA-DR denoting MHC class II expression, costimulatory molecules such as CD80, CD86, and CD40, the maturation marker CD83, and the monocyte marker CD14. As expected, all cells were positive for HLA-DR. The costimulatory molecules CD80 and CD86 were upregulated upon activation of cells, and were more highly expressed on DCs than on monocytes. CD40 was constitutively expressed on DCs before and after activation, while it was upregulated on stimulated monocytes. CD83 and CD14 also behaved as expected, with CD83 denoting DC maturation after stimulation with LPS, and CD14 becoming downregulated upon DC differentiation. These data show that monocytes and DCs generated *in vitro* expressed the correct repertoire of APC molecules necessary for their functions, and as such were suitable for downstream assays. Data obtained however were not statistically significant, due to the low number of replicates examined.

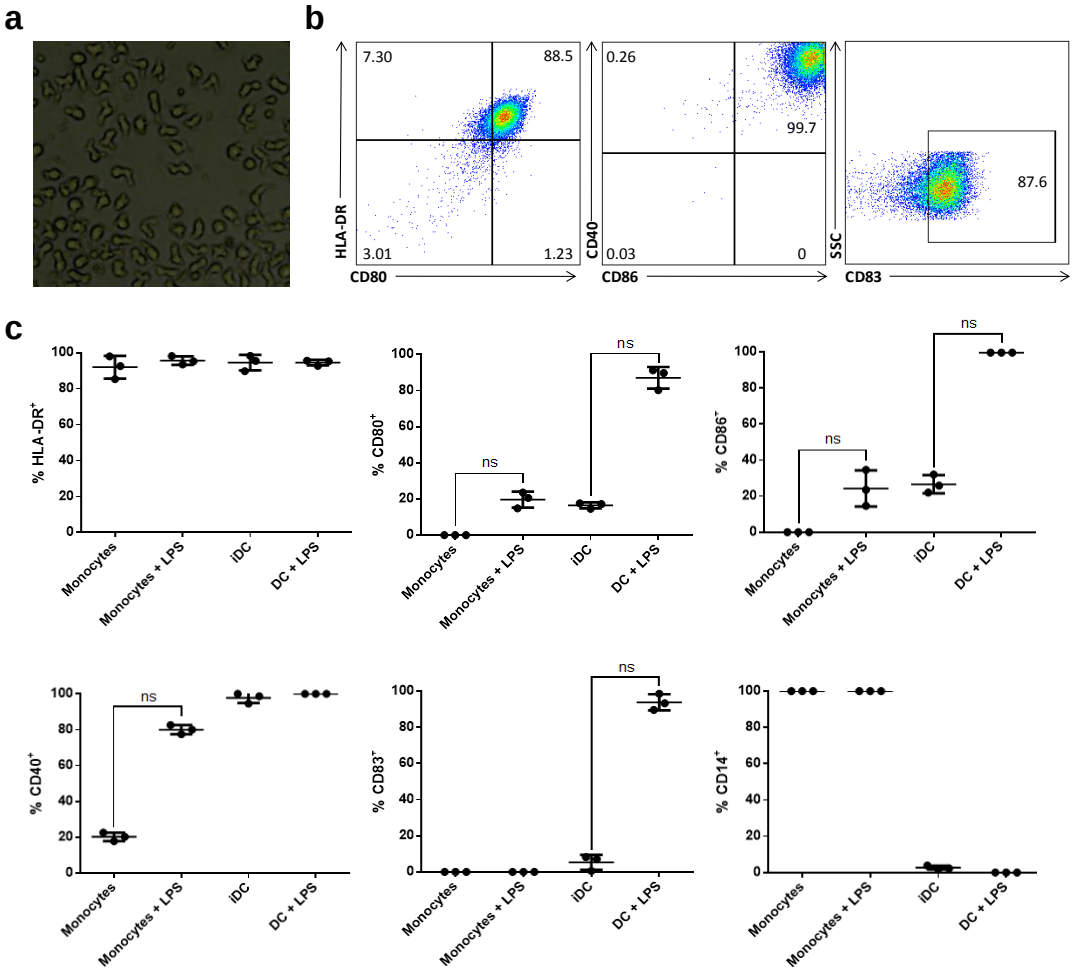

Figure 3.11 - APC phenotype of monocytes and monocyte-derived dendritic cells. (a) Cellular morphology of immature monocyte-derived dendritic cells. Freshly isolated monocytes from healthy human blood were cultured for 6 days with IL-4 and GM-CSF to generate mo-DCs. Image was taken at day 6 of cell culture. **(b)** Representative plots of APC marker expression by mature monocyte-derived DCs. Mo-DCs were generated over 6 days, and subsequently stimulated with LPS for 24 hours. APC marker expression was determined by flow cytometry. Numbers display percentages of cells. Gated on live, single cells. **(c)** Comparison of APC marker expression by unstimulated and LPS-matured monocytes and mo-DCs. Data points represent individual donors, from individual experiments. Horizontal lines display means of data sets. Error bars display standard deviation of samples. Gated on live, single cells. Statistical significance was determined using the Kruskal-Wallis test, followed by the Dunn's multiple comparison test, *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001, ns=not significant.

With APC controls generated, optimisation of naive $CD4^+$ T cell mixed lymphocyte reactions could be conducted. The concept of the mixed lymphocyte reaction relies on the fact that approximately 10% of an individual's T cells will be alloreactive, allowing them to respond to allogeneic antigen presenting cells. Monocytes and

monocyte-derived DCs were consequently cultured with allogeneic naive $CD4^+$ T cells at decreasing APC:responder cell ratios, and the proliferation of naive $CD4^+$ T cells was determined by CFSE dilution measured by flow cytometry (Figure 3.12). As expected, and reflected by their APC phenotype, LPS-matured DCs (mDCs) induced the highest levels of proliferation in naive responder cells, reaching 60% $CFSE^{lo}$ cells at an APC:responder ration of 1:1. The efficacy of APCs decreased from immature DCs (iDCs) to LPS-stimulated and unstimulated monocytes, which induced less pronounced proliferative responses than those observed with mDCs. While mDCs were able to induce $CD4^+$ T cell proliferation to APC:responder ratios as low as 1:1000, naive $CD4^+$ T cells co-cultured with monocytes required ratios of 1:10 for sufficient stimulation. With the APC:responder ratio determining the signal strength provided to the naive $CD4^+$ T cells, and the pronounced effect this signal strength has on the functional outcome, it was decided to conduct all future mixed lymphocyte reactions at multiple APC:responder ratios .

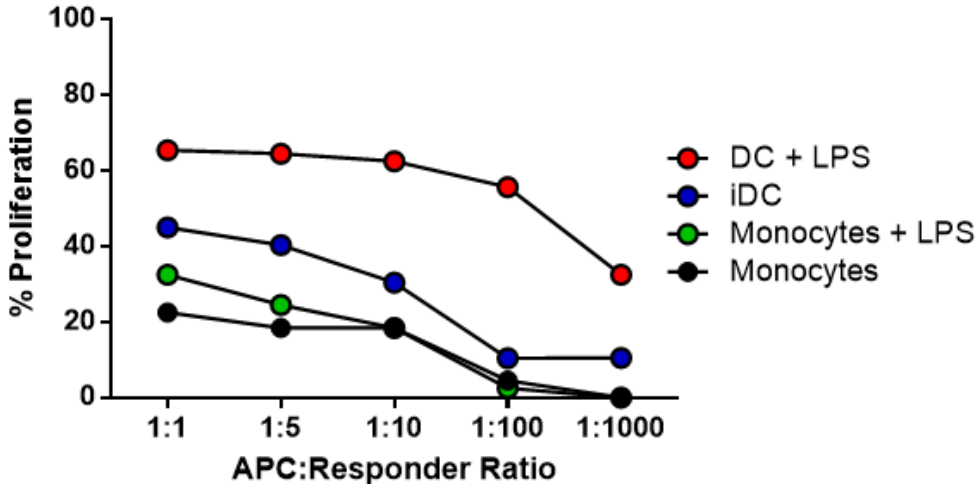

Figure 3.12 - Proliferation of naive CD4$^+$ T cells induced by co-culture with antigen presenting cells. Sorted naive CD4$^+$ T cells were cultured for 5 days with allogeneic APC types at decreasing APC:responder ratios. Proliferation of naive CD4$^+$ T cells was determined at day 5 by CFSE dilution, and percentages of CFSE^{lo} (proliferating) cells were determined. Data points display mean results of >5 healthy donors for each APC type, from >5 individual experiments.

3.6 - γδ T-APCs

With the experimental conditions for efficient CD4$^+$ T cell polarisation and generation of control APCs optimised, the generation of γδ T-APCs was the next point of focus. As previously mentioned, the common γ-chain cytokines IL-2, IL-4, IL-7, IL-15 and IL-21 regulate Vγ9Vδ2 T cells in different ways[277,282]. Given that these cytokines play distinct roles in homeostasis and inflammation[275], it is conceivable that they may be involved in regulating APC function by Vγ9Vδ2 T cells as well.

As an initial experiment to assess the responsiveness of Vγ9Vδ2 T cells to members of the common γ-chain family, isolated Vγ9Vδ2 T cells were stimulated with HMB-PP and cultured with individual cytokines in the presence of autologous monocytes serving as feeder cells, and their proliferation (Figure 3.13). Initial experiments were performed at different monocyte:Vγ9Vδ2 T cell ratios to determine the optimal culture conditions to induce Vγ9Vδ2 T cell proliferation. Monocyte:Vγ9Vδ2 T cell ratios were titrated, and the proliferation of Vγ9Vδ2 T cells in response to HMB-PP and individual common γ-chain cytokines was assessed by flow cytometry (Figure 3.13a). In these experiments, monocyte:Vγ9Vδ2 T cell ratios of 1:1 and 1:10 allowed for significant proliferation of Vγ9Vδ2 T cells, in contrast to lower ratios where proliferation was limited. As only little difference could be observed between the rate of Vγ9Vδ2 T cell proliferation at 1:1 and 1:10 ratios, the 1:10 ratio was selected for

all subsequent assays. The rationale behind this choice was that Vγ9Vδ2 T cells, once an APC function was induced, would need to be purified from co-cultures with monocyte feeder cells to use in APC assays; as such, 1:10 ratios meant fewer contaminating cells to remove in downstream applications.

In Figure 3.13b, representative histograms display proliferation of Vγ9Vδ2 T cells in response to HMB-PP and various common γ-chain cytokines. The highest levels of proliferation were observed with IL-2, IL-15, and IL-7. IL-21 was less able to aid proliferation of Vγ9Vδ2 T cells as compared to IL-2 and IL-15, whereas IL-4 showed little efficacy in supporting proliferation above the background of cells cultured with HMB-PP alone and unstimulated controls (Figure 3.13d). The individual effects of these cytokines could also be observed across decreasing concentrations of HMB-PP (Figure 3.13c), with IL-2, IL-15, and IL-7 all supporting Vγ9Vδ2 T cell proliferation at as little as 0.1 nM HMB-PP. In contrast, IL-21 was unable to maintain proliferation at this low level of stimulation, and IL-4 only supported proliferation at the highest concentration (100 nM HMB-PP). The lack of proliferation of Vγ9Vδ2 T cells in response to HMB-PP stimulation alone highlights the importance of common γ-chain cytokines in promoting Vγ9Vδ2 T cell responses. Of note, multiple concentrations of each cytokine were tested in identical assays and similar patterns of results were identified (data not shown).

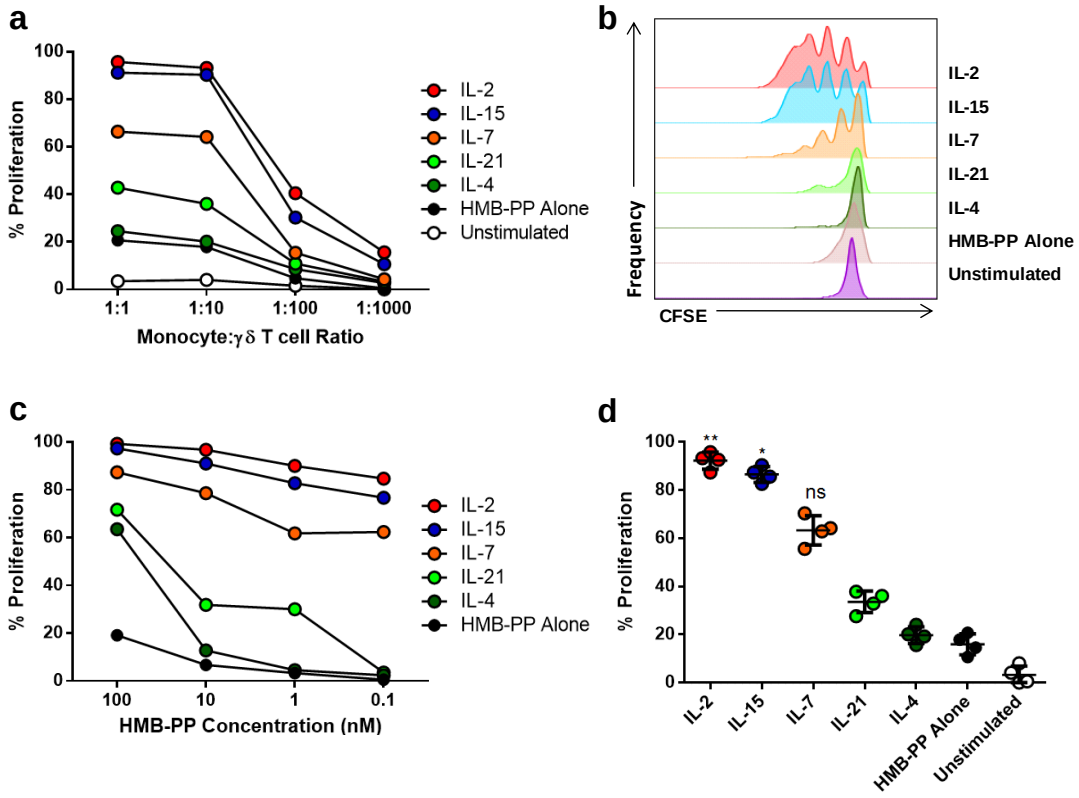

Figure 3.13 - Proliferation of Vγ9Vδ2 T cells in response to common γ-chain cytokines. **(a)** Comparison of decreasing irradiated monocyte feeder cell:Vγ9Vδ2 T cell ratios on the induction of Vγ9Vδ2 T cell proliferation. CFSE-stained Vγ9Vδ2 T cells were stimulated with 10 nM HMB-PP +/- either IL-2 (100 U/ml), IL-15 (20 ng/ml), IL-7 (20 ng/ml), IL-21 (20 ng/ml), IL-4 (20 ng/ml), or left unstimulated. Displays a representative healthy donor from 4 total donors, in 3 independent experiments. **(b)** Representative CFSE-dilution histograms of Vγ9Vδ2 T cells cultured in the presence of 1:10 ratio of irradiated monocyte:Vγ9Vδ2 T cells, in the presence of 10 nM HMB-PP and common γ-chain cytokines. Displays a representative healthy donor from 4 total donors, in 3 independent experiments. **(c)** Titration of HMB-PP concentrations and the effect on Vγ9Vδ2 T cell proliferation. Cultures were stimulated with decreasing concentrations of HMB-PP in combination with common γ-chain cytokines. Displays a representative healthy donor from 4 total donors, in 3 independent experiments. **(d)** Proliferation of Vγ9Vδ2 T cells in response to 10 nM HMB-PP in combination with different common γ-chain cytokines. Data points represent individual healthy donors, from 3 independent experiments. Percentage proliferation was determined by CFSE dilution on day 5 of culture, gated on live, single, TCR $Vγ9^+$ cells. Horizontal lines display means of data sets. Error bars represent standard deviation of samples. Statistical significance was determined using the Friedman Test, followed by the Dunn's multiple comparison test, *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001, ns=not significant. Significance displayed in comparison to unstimulated control.

With the confirmation that a number of common γ-chain cytokines support Vγ9Vδ2 T cell responses, the next step was to investigate the APC phenotype in response to these different cytokines (Figure 3.14). A time course was set up to assess expression of several APC molecules over a 5 day culture period (Figure 3.14a). The markers HLA-DR, CD86, and CD70 were absent on freshly isolated cells (day 0), and low level expression could be observed after 24 hours of stimulation with HMB-PP and IL-15. Increased expression levels were detected at day 3, and by day 5 the majority of cells expressed all three proteins. The difference in expression between days 3 and 5 was minimal, and hence it was decided to use day 3 γδ T-APCs in all future experiments.

In addition to HLA-DR, CD86 and CD70, further APC markers were assessed on Vγ9Vδ2 T cells at day 3 of culture, including the co-stimulatory proteins CD40 and CD80, the DC marker CD83 and the chemokine receptor CCR7 (Figure 3.14b). Expression of the costimulatory molecule ICOSL was also examined (Figure 3.14c), although staining for this molecule was not as distinct as for other markers. As a control, $CD19^+$ B cells, which express $ICOSL^{349}$, were stained, and revealed similar stainings via flow cytometry.

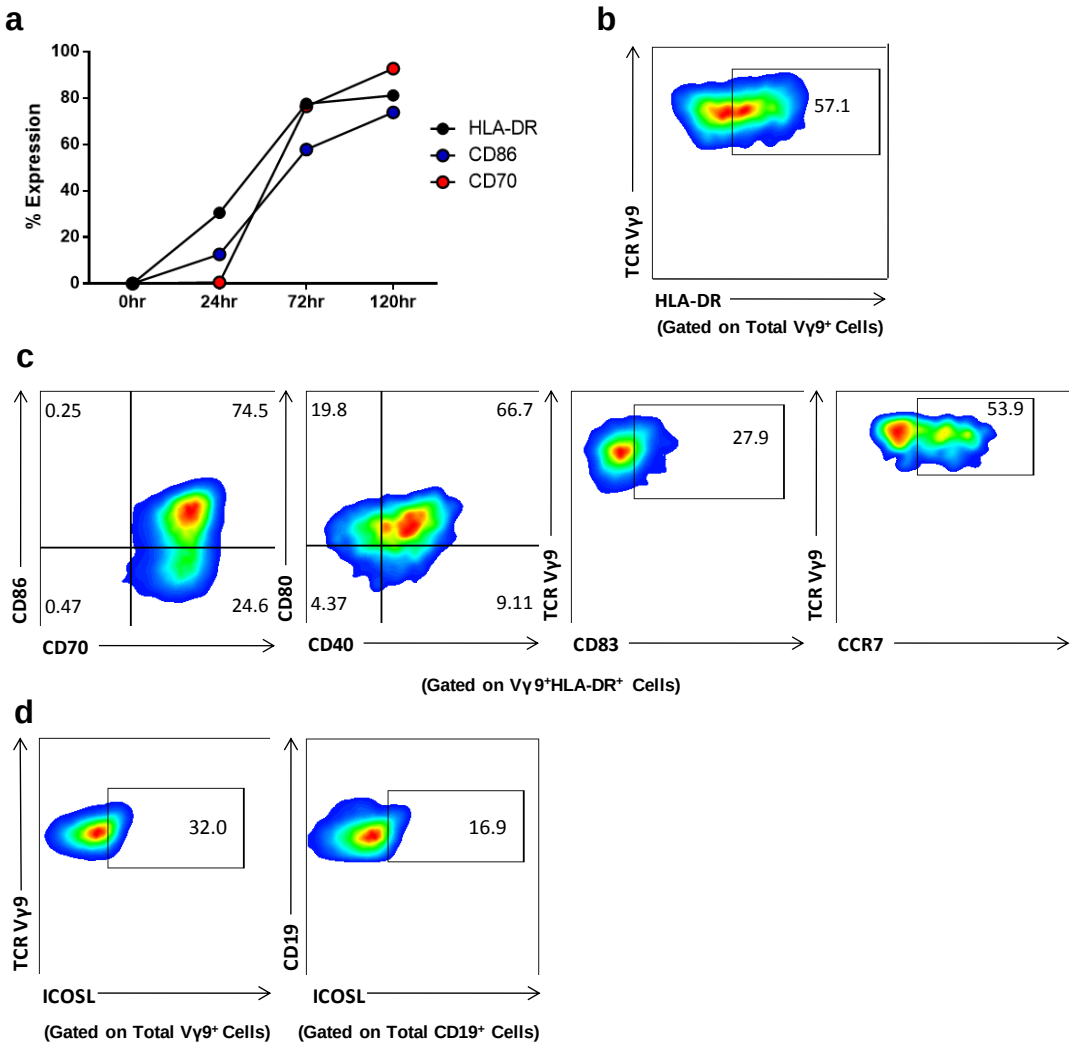

Figure 3.14 - Expression of antigen presenting cell markers by Vγ9Vδ2 T cells. (a) Time course of the expression of APC markers by Vγ9Vδ2 T cells, stimulated with 10 nM HMB-PP and IL-15. Percentage of HLA-DR positive cells were determined by gating on live, single, $Vγ9^+$ T cells. Remaining APC marker-positive cells were determined by gating on live, single, $HLA\text{-}DR^+Vγ9^+$ T cells. **(b)** Representative plots of Vγ9Vδ2 T cell HLA-DR expression at day 3 of culture. Cells were stimulated with 10 nM HMB-PP and IL-15. HLA-DR expressing cells are gated on live, single, $Vγ9^+$ T cells. **(c)** Representative plots of Vγ9Vδ2 T cell APC phenotype at day 3 of culture. Cells were stimulated with 10 nM HMB-PP and IL-15. Expression of APC markers determined by gating on $Vγ9^+$ $HLA\text{-}DR^+$ cells. Numbers inside gates represent percentage positive cells. **(d)** Representative plots showing expression of ICOSL by Vγ9Vδ2 T cells at day 3 of culture, or unstimulated $CD19^+$ B cells as a control. All figures representative of > 4 individual donors from >4 individual experiments.

Figure 3.15 displays the expression of APC markers by Vγ9Vδ2 T cells, stimulated with 10 nM HMB-PP in combination with each common γ-chain cytokine. Consistent with the robust proliferation observed, both IL-2 and IL-15 supported optimal expression of numerous APC markers, all of which were expressed to significantly

higher levels than unstimulated controls. The majority of cells cultured under these conditions showed expression of HLA-DR, CD80, CD86, CD40, and CD70, which are all necessary for induction of naive $CD4^+$ T cell responses. Expression of CD83 and ICOSL was evident on a smaller fraction of cells, but nevertheless was clearly expressed. In contrast to the robust proliferative response induced, IL-7 completely failed to support the expression of any APC marker by Vγ9Vδ2 T cells. IL-21, in a similar manner to its low level induction of proliferation, supported low level expression of APC markers such as HLA-DR and CD86, in comparison to IL-2 and IL-15. Vγ9Vδ2 T cells cultured with IL-4 failed to express APC characteristics, similarly to Vγ9Vδ2 T cells cultured in the absence of any cytokine. Of note, similar patterns of results and levels of marker expression were observed when using 1 nM HMB-PP instead of 10 nM HMB-PP (data not shown).

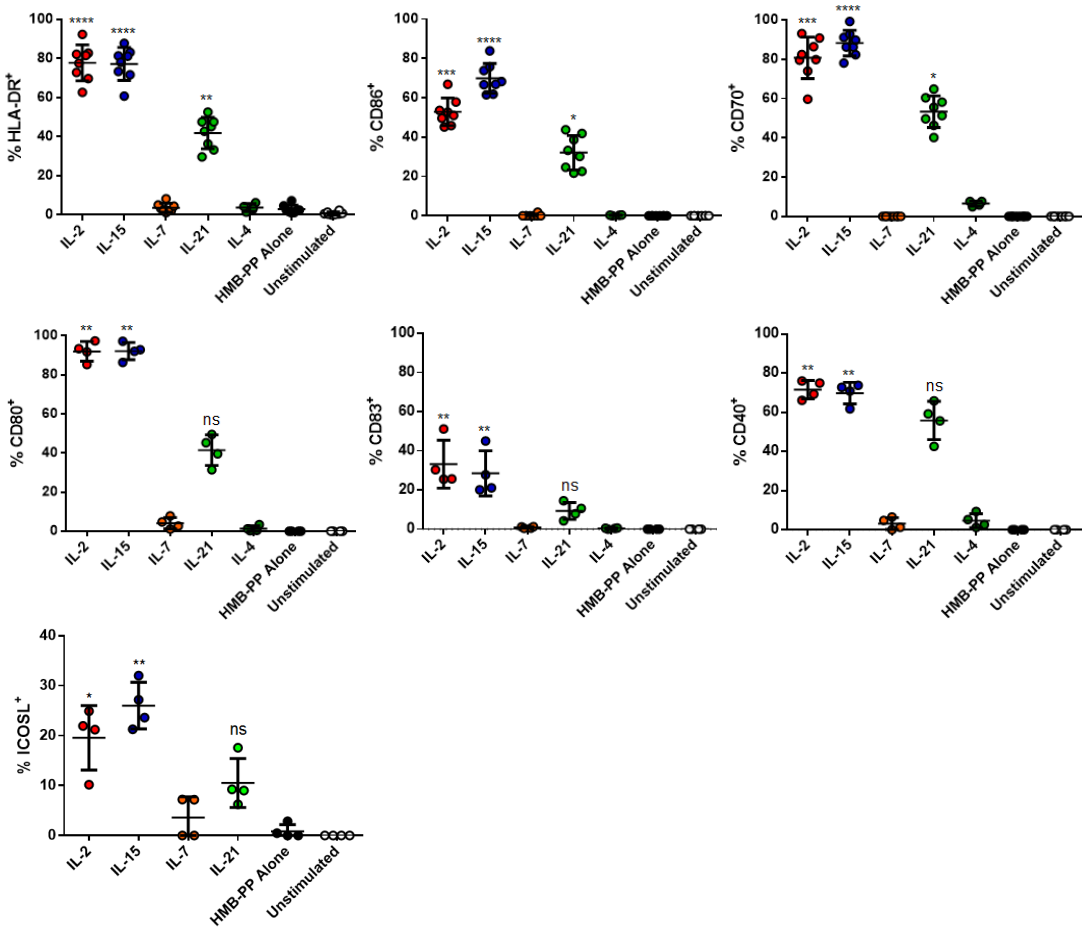

Figure 3.15 - Expression of antigen presenting cell markers by Vγ9Vδ2 T cells in response to HMB-PP and common γ-chain cytokines. Summary graphs displaying expression of APC markers by Vγ9Vδ2 T cells, stimulated with 10 nM HMB-PP in combination with different common γ-chain cytokines. Percentages of APC marker positive cells were determined at day 3 of culture by flow cytometry. Percentage of HLA-DR$^+$ cells were determined by gating on live, single, Vγ9$^+$ T cells. Remaining APC marker-positive cells were determined by gating on live, single, HLA-DR$^+$Vγ9$^+$ T cells. Data points display individual donors from individual experiments. Horizontal lines display means of data sets. Error bars display standard deviation of samples. Statistical significance was determined using the Friedman test, followed by the Dunn's multiple comparison test, *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001, ns=not significant. Significance displayed in comparison to unstimulated control.

In addition to assessing the proliferation and APC phenotype of Vγ9Vδ2 T cells, the production of cytokines was examined (Figure 3.16). In a similar manner to previous experiments, Vγ9Vδ2 T cells were cultured over 3 days, stimulated with HMB-PP and common γ-chain cytokines, and production of IFN-γ and TNF-α was determined by ELISA. Significant levels of both cytokines were produced in the presence of both IL-2 and IL-15, with reduced levels produced by IL-7 cultured cells. Interestingly,

cells cultured in the presence of IL-15 produced significantly higher levels of TNF-α as compared to those cultured with IL-2. As expected[282], IL-21 did not support the production of either cytokine, despite allowing for sufficient activation of cells in terms of proliferation and APC phenotype, and actually inhibited production of IFN-γ and to a lesser extent TNF-α as compared to HMB-PP alone controls. Other cytokines assessed, including IL-4 and IL-10, were absent in cell culture supernatants.

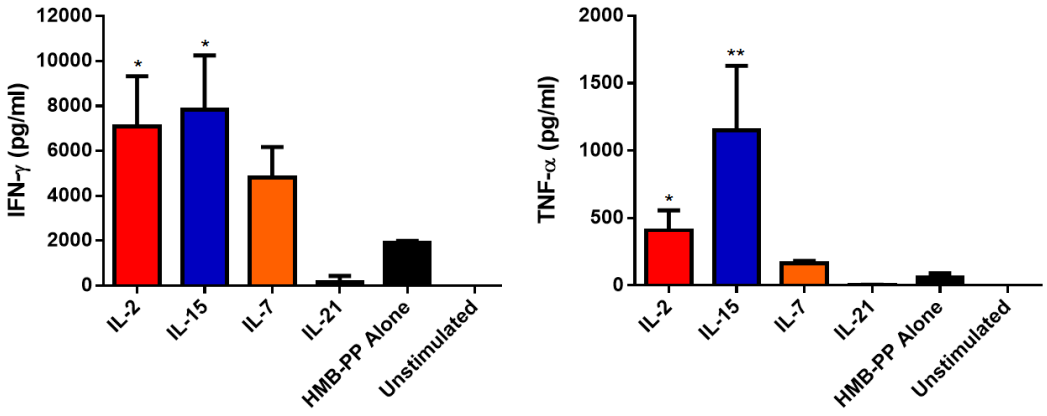

Figure 3.16 - Production of cytokines by Vγ9Vδ2 T cells in response to various common γ-chain cytokines. Vγ9Vδ2 T cells were stimulated with 10 nM HMB-PP in combination with different common γ-chain cytokines. Cytokine levels were determined in supernatants by ELISA, after 24 hours of culture. Bar charts display mean data from 4 individual donors, from 3 individual experiments. Error bars display standard deviation of samples. Statistical significance was determined using the Friedman test, followed by the Dunn's multiple comparison test, *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001. Significance displayed in comparison to unstimulated control.

The importance of cytokine production by antigen presenting cells in the polarisation of naive $CD4^+$ T cell responses has been extensively studied in the literature and discussed here. As such, an overall analysis of cytokine production by all APC types to be used in the polarisation of naive $CD4^+$ T cells was conducted (Figure 3.17). In addition to the different APC types, multiple methods of stimulation for each subset were examined. With monocyte and DC controls, LPS and PGN stimulated cells were examined for cytokine production alongside unstimulated cells. For γδ T-APCs, the four common γ-chain cytokines IL-2, IL-15, IL-7, and IL-21 were assessed, henceforth referred to as giving rise to IL-2 γδ T-APCs, those generated with IL-15 referred to as IL-15 γδ T-APCs, and so on.

A total of 11 individual cytokines were assessed by ELISA. As previously discussed, the only cytokines amongst these 11 markers produced by γδ T-APCs were IFN-γ and TNF-α. In contrast, both monocytes and DCs displayed a wide range of cytokine production, differing depending on the type of stimulation used. IL-12p70 was only produced by DCs stimulated with LPS, in contrast to IL-23 which was favoured by both DCs and monocytes stimulated with PGN. The production of IL-12p70 by LPS-matured DCs is consistent with published data, in that these cells are potent Th1 cell inducers[206]. PGN treatment of cells, in addition to inducing IL-23

production, also induced significant levels of IL-6, and IL-1β in monocytes, all cytokines directed towards Th17 cell polarisation. Indeed, PGN has previously been shown to favour Th17 cell differentiation[95]. DCs stimulated with PGN also produced high levels of IL-10, potentially allowing for Th2 cell differentiation. The cytokines IL-4, IL-17, IL-22, and IL-27, were absent in all culture supernatants examined. By analysing the cytokine profiles of different APCs, it is clear that each type of APC is likely to have the potential to polarise markedly different subsets of T helper lineages.

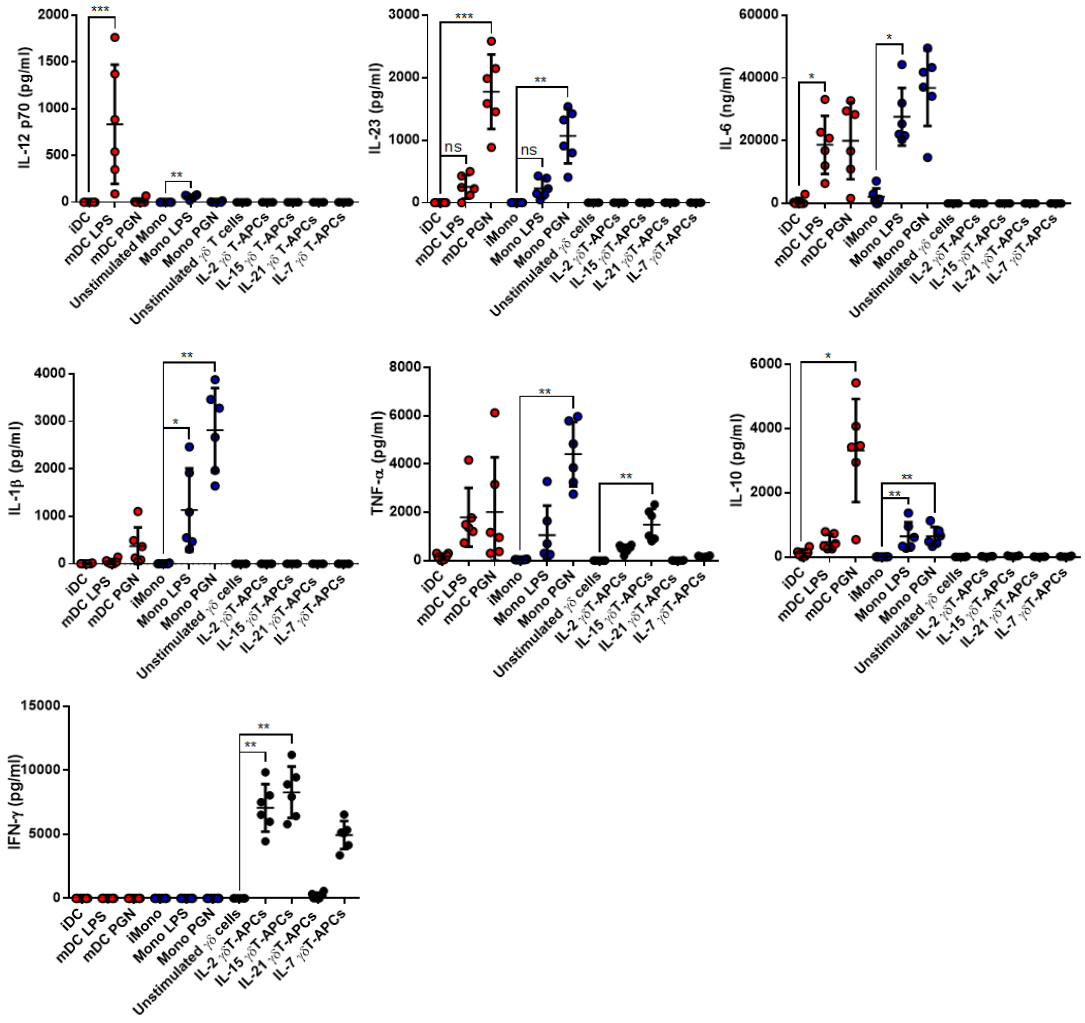

Figure 3.17 - Cytokine profiles of antigen presenting cells in response to various stimulations. Monocyte-derived dendritic cells (DCs) or monocytes (Mono) were either unstimulated (iDC/Unstimulated Mono) or stimulated for 24 hours with LPS or PGN. Vγ9Vδ2 T cells were stimulated with 10 nM HMB-PP and different common γ-chain cytokines for 24 hours. Cytokine levels were determined in culture supernatants by ELISA. Data points display individual donors from individual experiments. Horizontal lines display means of data sets. Error bars display standard deviation of samples. Statistical significance was determined using the Kruskal-Wallis test, followed by the Dunn's multiple comparison test, *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001, ns=non-significant.

One last aspect of γδ T-APCs to be examined was that of antigen uptake. Previous studies examined the process of antigen uptake by Vγ9Vδ2 T cells in detail[326], however, the control of this feature by different common γ-chain cytokines has yet to be determined. To investigate this function, FITC-labelled BSA was added to cultures for 1 hour, allowing for its uptake, and the fluorescence of cells in the FITC channel was determined by flow cytometry (Figure 3.18). BSA-FITC was added to

differentially cultured cells at various time points, either at 4°C or 37°C. As such, the fold change in MFI could be calculated between 4°C cultures, where little/no antigen would be taken up, and 37°C cultures, where antigen uptake is unhindered.

A representative histogram is displayed in Figure 3.18a, showing the clear uptake of BSA-FITC at 37°C as compared to controls. A time course was set up, to investigate the kinetics of uptake by Vγ9Vδ2 T cells after activation (Figure 3.18b). Freshly isolated cells, stimulated with HMB-PP and IL-15 for 1 hour, exhibited no difference to unstimulated cells, as expected. Progressing through the later time points, differences between stimulated and unstimulated cells became more prominent. Given that the largest differences in fold change MFI were observed at day 5, cultures of Vγ9Vδ2 T cells were set up with the different common γ-chain cytokines to assess any differences between treatments at day 5 of culture. Consistent with prior observations, both IL-2 and IL-15 treatments induced significant antigen uptake over unstimulated controls. The effect of IL-7, while allowing for an increase in antigen uptake, was not significant as compared to unstimulated cells. Given these results, it appears that increase in antigen uptake by Vγ9Vδ2 T cells correlated more with proliferative data than with APC phenotype. Indeed, there was no difference in fold change MFI between APC marker positive and negative cells in all conditions (data not shown).

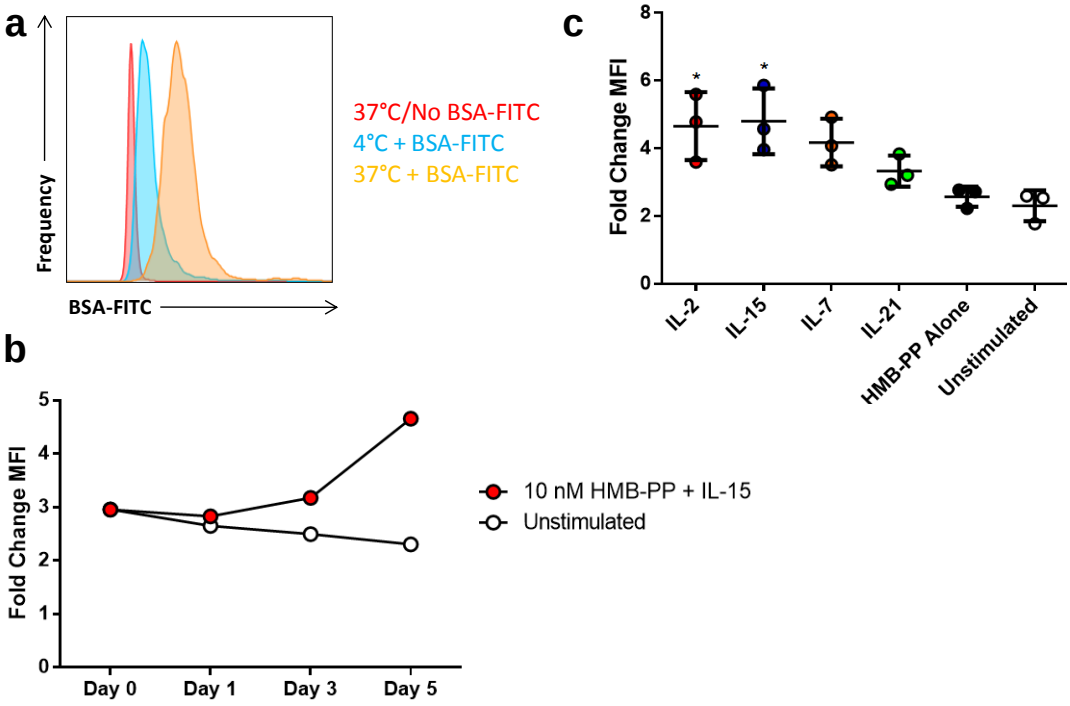

Figure 3.18 – Fluorescent antigen uptake by Vγ9Vδ2 T cells. (a) Representative histograms displaying antigen uptake by Vγ9Vδ2 T cells cultured in the presence of 10 nM HMB-PP and IL-15; red displays fluorescence in the FITC channel at 37°C with no BSA-FITC, blue at 4°C with BSA FITC, and orange at 37°C with BSA-FITC added to the culture. **(b)** Time course of fluorescent antigen uptake by unstimulated and 10 nM HMB-PP/IL-15-stimulated Vγ9Vδ2 T cells over 5 days of culture. BSA-FITC was added at each timepoint for 1 hour of culture and MFI assessed by flow cytometry. Fold change MFI calculated as the difference between MFI at 4°C and 37°C in the presence of BSA-FITC. **(c)** Fold change in MFI of Vγ9Vδ2 T cells cultured in the presence of BSA-FITC in combination with different common γ-chain cytokines at day 5 of culture. Horizontal lines display means of data sets. Error bars display standard deviation of samples. Statistical significance was determined using the Friedman test, followed by the Dunn's multiple comparison test, *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001.

3.7 - Discussion

In summary, this chapter has discussed the optimisation of appropriate controls for assays investigating the polarisation of $CD4^+$ T cells by γδ T-APCs. Using naive $CD4^+$ T cells, controls for each T helper lineage were optimised and methods for the detection of intracellular cytokines, secreted cytokines, and transcription factors were all established. In addition, dendritic cell and monocyte APC controls were generated for comparison with γδ T-APCs.

With experimental controls established, focus shifted to the regulation of APC function in Vγ9Vδ2 T cells. Data presented here show that the common γ-chain family of cytokines regulates a multitude of Vγ9Vδ2 T cell functions. IL-2 and IL-15 were able to promote 'full' functional abilities of Vγ9Vδ2 T cells, promoting their proliferation, APC phenotype, cytokine production, and increased ability to take up soluble antigen. The role of IL-21 in controlling Vγ9Vδ2 T cells appeared less potent, allowing cells to proliferate and express APC characteristics, albeit at lower levels as compared to IL-2 and IL-15. Interestingly, IL-21 also did not support production of IFN-γ and TNF-α, which has been reported previously[282]. Most surprising perhaps was the role of IL-7; this cytokine supported Vγ9Vδ2 T cell proliferation and cytokine production to similar levels as did IL-2 and IL-15, but did not support any APC marker expression from those examined. In contrast to all other cytokines, IL-4 only showed very poor efficacy in aiding any of the Vγ9Vδ2 T cell functions investigated and was thus excluded from the experiments in the following chapters.

The differences between these cytokines in regulating Vγ9Vδ2 T cell immunity, and that of T cells in general, has been described in the literature[275]. IL-2 is reported to be a pro-inflammatory cytokine, produced predominantly by T cells upon activation and co-stimulation. Given this role in immunity, it is not surprising that IL-2 would also promote APC function by Vγ9Vδ2 T cells, allowing them to promote further inflammatory immune responses. IL-15 is generally reported to be a homeostatic cytokine, though it is produced by activated DCs and monocytes, favouring cell survival and proliferation. In this chapter, IL-15 has been identified to favour an APC phenotype in Vγ9Vδ2 T cells as well. Both IL-2 and IL-15 have been examined in previous γδ T-APC studies, identified to promote this function, as well as inducing significant effector expansion and cytotoxic ability[325]. However, the role of IL-15 alone in inducing γδ T-APCs has not been presented prior to this study. IL-21 has been reported to function as a T cell growth factor, as well as favouring Vγ9Vδ2 T cell function in aiding B cell responses, inducing a Tfh cell phenotype[280,282].

Expression of co-stimulatory molecules such as CD40L and ICOS has been identified as regulated by IL-21[280], and the repertoire of molecules regulated by IL-21 has been expanded to include CD86 and CD70 (Unpublished data, Raj Bansal PhD Thesis). The reason why IL-21 supports only a sub-optimal APC phenotype as compared to IL-2 and IL-15 is unclear. This effect was also observed at higher concentrations of IL-21, suggesting the suboptimal phenotype is a result of the cytokine activity itself rather than a lower bioactivity. The role of IL-7, described as a homeostatic cytokine produced by a variety of epithelial cell types, in favouring proliferation but not APC marker expression by Vγ9Vδ2 T cells is in line with this observation, in that it supports non-inflammatory proliferation of cells, and as such does not promote an inflammatory APC phenotype.

In terms of specific effects on Vγ9Vδ2 T cells, the common γ-chain cytokines have been reported to induce responses in different memory subsets. IL-2 and IL-15 favour responses in T_{EM} cells where receptor expression is highest, whereas the IL-7 receptor is found mainly on naive and T_{CM} cells[277]. The differential effects between these cytokines could indeed be due to different memory subsets responding to each treatment, indicating T_{EM} cells may be capable of APC function whereas naive or T_{CM} cells may be limited in this functional capacity. Alternatively, longer culture periods may be required to induce APC function in naive and T_{CM} cells, which can transition towards the T_{EM} phenotype under the correct stimulatory conditions. What is clear is that APC function in Vγ9Vδ2 T cells is differentially regulated by the common γ-chain cytokines.

With respect to expression of APC markers, the majority of those examined displayed clear expression upon Vγ9Vδ2 T cell stimulation. However, staining for ICOSL, in addition to CD83, was not as defined as other markers (Figure 3.14c). Previous unpublished data (Ana de Barros, PhD thesis) described the expression of ICOSL on a proportion of Vγ9Vδ2 T cells cultured using similar protocols as described here. This subpopulation was identified as approximately 20% of Vγ9Vδ2 T cells, similar to the levels described in this study. Given that staining of $CD19^+$ B cells as a positive control displayed similar staining efficacy, and the previous data in support, it appears that Vγ9Vδ2 T cells do indeed express ICOSL at low levels after stimulation. In addition, ICOSL expression by other cellular subsets has been reported to become upregulated upon CD40 ligation[350], and as such higher expression of ICOSL by Vγ9Vδ2 T cells may also depend on cellular interaction with responder cells via the CD40 pathway. The functionality of this molecule in inducing

$CD4^+$ T cell responses, in addition to other APC markers, will be examined in subsequent assays.

In contrast to previous studies conducted examining the upregulation of APC markers by Vγ9Vδ2 T cells, the kinetics of expression presented here for HLA-DR, CD86, and CD70 (Figure 3.14a) differ from those studies, in that APC marker expression was detected at 72 hours instead of 18-24 hours. This is likely due to the culture conditions examined; previous studies utilised irradiated EBV-transfected B cells[322] at feeder cell:Vγ9Vδ2 T cell ratios of 1:3 (or ranges of 1:1-1:10 in some experiments), whereas the data presented here utilised monocyte ratios of 1:10. Given the increased number of feeder cells, and the large size of EBV-B cells compared to monocytes, it is possible these cells were more efficient at presenting HMB-PP to Vγ9Vδ2 T cells, providing a much stronger stimulation than with monocytes and allowing for more rapid expression of APC characteristics.

The regulation of antigen uptake by Vγ9Vδ2 T cells differs to other APCs such as DCs. In previous studies and again presented here, Vγ9Vδ2 T cells require activation before an increase in antigen uptake is observed. In contrast, immature DCs constantly take up and process antigen until maturation, upon which endocytic function is downregulated[326]. Given this reverse in roles, and consistent with the requirement for activation to induce APC phenotypes in Vγ9Vδ2 T cells, a likely scenario for γδ T-APC function is dependent on activation by relevant stimuli, rather than as a constant processing of antigen[322].

Given the differential regulation of APC phenotype, Vγ9Vδ2 T cells cultured in the presence of IL-2, IL-15, IL-7, or IL-21 were used for subsequent assays, assessing their ability to polarise naive $CD4^+$ T cell responses; IL-2 and IL-15 γδ T-APCs representing 'optimal' APCs, IL-21 cultured cells as 'sub-optimal', and IL-7 γδ T-APCs presenting a negative APC control, given their lack of expression of molecules necessary to induce $CD4^+$ T cell responses.

Chapter 4 - Polarisation of $CD4^+$ T Cell Responses

4.1 - Introduction

The polarisation of $CD4^+$ T cell responses is a complex process involving multiple cell types and polarising factors, which work either in collaboration to induce the required response, or antagonistically to prevent alternative phenotypes from emerging. Such factors include both cytokines and costimulatory molecules, differentially expressed by various antigen presenting cell types and under different conditions. Dendritic cells are the prototypic antigen presenting cells studied for the polarisation of naive $CD4^+$ T cell responses. These cells are capable of remarkable plasticity; able to adapt to different stimulating ligands and polarise the appropriate T helper cell lineage[206]. Alongside this plasticity, DC subsets have proven specially equipped to induce certain lineages over others, as a result of restricted expression of certain pattern recognition receptors and polarising factors[198]. In addition to DCs however, numerous other cell types have proven capable of antigen presenting function, either non-professionally such as neutrophils[306], or professionally such as B cells[210]. Alongside naive $CD4^+$ T cell polarisation, memory $CD4^+$ T cells have also been shown to exhibit plasticity in their phenotype[156], transitioning from one lineage to another upon encountering the correct conditions, and as such this process is heavily mediated by APCs.

As presented in Chapter 3, and consistent with previous published data, Vγ9Vδ2 T cells are capable of becoming antigen presenting cells. This cellular subset expresses key antigen presenting cell markers and costimulatory molecules, is capable of antigen uptake, processing and presentation, and has previously been shown to induce $CD4^+$ and $CD8^+$ T cell responses[322]. While much is known of the induction of $CD8^+$ T cells responses by γδ T-APCs, little is known of the induction of $CD4^+$ T cell responses, and the subsequent polarisation of T helper lineages. Limited data have shown that γδ T-APCs are capable of inducing distinct Th1 and Th2 responses, depending on culture conditions[322]. However, given the rapid expansion in the number of T helper lineages described in recent years, it remains unclear how γδ T-APCs may fit in with other APCs in the polarisation of $CD4^+$ T cell responses.

Vγ9Vδ2 T cells are capable of becoming antigen presenting cells under certain conditions; IL-2 and IL-15 favoured a strong APC phenotype, whereas IL-21 supported limited APC characteristics and IL-7 did not support this novel function at

all (Figure 3.15). Previous studies of γδ T-APCs have predominantly focused on the role of IL-2 to promote APC function[322,326], and as such little is known about how the cytokine microenvironment may affect Vγ9Vδ2 T cell function, with respect to APC capability and polarisation of $CD4^+$ T cell responses. Indeed, Vγ9Vδ2 T cells display marked plasticity to differential cytokines in terms of their own cytokine production[282], so it remains possible that APC function is plastic as well. As such, a central aim of this results chapter was to identify which T helper lineages are able to be induced by γδ T-APCs, and whether the cytokine microenvironment utilised for generating APCs has a knock-on effect on the subsequent T helper cell polarisation. This concept would be in line with current dendritic cell literature, where the APC has to adapt to the microenvironment and stimulation pathways, and 'make decisions' as to which lineage is most suitable to be induced[206]. Alternatively, given the DC subtype-specific nature of induction of certain $CD4^+$ T cell lineages, it may be that γδ T-APCs favour the induction of one T helper subset over others, or are only capable of inducing certain arms of the $CD4^+$ T cell immune response.

In addition to assessing the polarising capabilities of γδ T-APCs, T helper lineages induced must be examined in the context of alternative APC polarisations. Given the complex nature of $CD4^+$ T cell polarisation, and the ability of $CD4^+$ T cell subsets to co-express different T helper lineage factors, a comparison of T helper responses induced by each APC subset is useful to identify where, and under which immunological scenarios, γδ T-APCs may exhibit their APC function *in vivo*.

Once the polarising capacity of γδ T-APCs is determined, it is necessary to examine the mechanisms behind such polarisations. Due to the significant number of cytokines and costimulatory molecules required for $CD4^+$ T cell polarisation, it is unclear which factors expressed by γδ T-APCs may be important in directing $CD4^+$ T cell responses. As discussed previously, the cytokine expression profiles of γδ T-APCs compared to DCs and monocytes show distinct variations (Figure 3.17), and as such it may be unlikely that γδ T-APCs utilise the same mechanisms to direct $CD4^+$ T cell polarisation as their DC counterparts.

Whilst much of the focus in the literature and in this thesis is on the polarisation of $CD4^+$ T cell responses, the emergence of different $CD8^+$ T cell lineages parallel to $CD4^+$ T cells has identified possible functional niches where different APCs may play prominent roles in $CD8^+$ T cell polarisation[215,216]. It is unclear at present whether γδ T-APCs promote polarisation of $CD8^+$ T cell lineages.

4.2 - Aims of Chapter

The following experimental aims will be discussed in this chapter:

- Assess the ability of differentially generated γδ T-APCs to induce naive and memory $CD4^+$ T cell proliferation.
- Examine the polarisation of naive and memory $CD4^+$ T cells by γδ T-APCs, in terms of intracellular cytokine expression, cytokine secretion, transcription factor expression, and chemokine receptor expression.
- Compare T helper responses polarised by γδ T-APCs to those induced by DC and monocyte controls.
- Examine autologous polarising systems to confirm responses observed using an allogeneic, mixed lymphocyte reaction culture system.
- Assess $CD8^+$ T cell polarisation by γδ T-APCs
- Determine the polarising factors expressed by γδ T-APCs which are required for the polarisation of $CD4^+$ T cell responses.

4.3 - γδ T-APC Mediated Induction of $CD4^+$ T Cell Proliferation

As an initial step in investigating the ability of γδ T-APCs to induce $CD4^+$ T cell responses, co-cultures were established to investigate the induction of proliferation in naive and memory $CD4^+$ T cell populations. In a similar manner to those conducted with monocytes and dendritic cells (Figure 3.12), mixed lymphocyte reactions were set up utilising γδ T-APCs, and either naive $CD4^+$ T cells or memory $CD4^+$ T cells as responder cells. As discussed previously, γδ T-APCs were generated over a 3 day culture period by stimulating Vγ9Vδ2 T cells with 10 nM HMB-PP, in combination with IL-2, IL-15, IL-7, or IL-21. Once generated, γδ T-APCs were subsequently cultured at decreasing APC:responder ratios with CFSE-stained, allogeneic naive $CD4^+$ T cells for a period of 5 days, upon which proliferation, in terms of CFSE dilution, was determined by flow cytometry (Figure 4.1).

Figure 4.1 displays the proliferation induced in naive $CD4^+$ T cells by differentially generated γδ T-APCs, at decreasing APC:responder ratios. Both IL-2 and IL-15 γδ T-APCs, which exhibited the most potent APC phenotype in terms of co-stimulatory molecule expression (Figure 3.15), induced high levels of naive $CD4^+$ T cell proliferation at 1:1 APC:responder ratios. IL-2 and IL-15 γδ T-APCs both maintained this level of proliferation up to a 1:10 ratio, with further dilution of APCs resulting in reduced induction of proliferation. At a 1:1000 ratio, minimal proliferation was

observed in naive $CD4^+$ T cell populations. IL-21 γδ T-APCs exhibited a similar pattern of $CD4^+$ T cell proliferation induction, although the levels of proliferation observed were markedly lower than those induced by their IL-2 and IL-15 counterparts. This is consistent with the APC phenotype of each subset, in that IL-21 γδ T-APCs displayed significantly lower expression of costimulatory molecules necessary to induce naive $CD4^+$ T cell responses. In contrast to these responses, IL-7 γδ T-APCs were unable to induce proliferative responses in naive $CD4^+$ T cell populations, even at 1:1 cell ratios, reflective of their poor APC phenotype (Figure 3.15). In a similar manner, unstimulated Vγ9Vδ2 T cells and HMB-PP alone-stimulated cells did not induce significant proliferation in naive $CD4^+$ T cell responder populations (data not shown). As such, IL-7 γδ T-APCs represented a good negative control for subsequent assays.

By comparing these results with those obtained for monocytes and DCs in identical experiments (Figure 3.12), it can be seen that all APC subsets tested (excluding IL-7 γδ T-APCs) were able to induce naive $CD4^+$ T cell responses at a 1:10 APC:responder ratio. Decreasing this ratio to 1:100, IL-2 and IL-15 γδ T-APCs, in addition to DCs, induced nCD4 proliferation, in contrast to IL-21 γδ T-APCs and monocytes which were unable to induce responses at this ratio. The only cell capable of maintaining naive $CD4^+$ T cell proliferation at a 1:1000 ratio were DCs. Given this range of abilities to induce naive $CD4^+$ T cell responses, it was decided that assessing $CD4^+$ T cell proliferation at 1:10 APC:responder ratio would allow for examination of all APC types' ability to polarise $CD4^+$ T cell responses. The inability of γδ T-APCs to maintain naive $CD4^+$ T cell proliferation at 1:1000 ratios is in contrast to previous studies, where at lower ratios, γδ T-APCs were as efficient as DCs at inducing responses[322]. As previously discussed, the APC generation protocols differed between those studies and this one, in that perhaps γδ T-APC stimulation in this experimental system was suboptimal as compared to others, likely accounting for this reduced capacity. Nevertheless, γδ T-APCs induced similar levels of naive $CD4^+$ T cell proliferation at ratios up to 1:100 as did DCs, showing they are potent antigen presenting cells in their ability to promote $CD4^+$ T cell responses.

For these assays and subsequent ones, γδ T-APCs were treated with low dose radiation (12 Gy) to prevent significant cell proliferation whilst in culture with responder cells, minimising functional defects, as performed in previous studies[322]. Given the fact that DCs and monocytes do not proliferate in the co-culture system, whereas γδ T-APCs are still able to proliferate at day 3 when utilised in the assays,

this presented a potential inequality between APCs in the culture systems used. As such, irradiated γδ T-APCs were utilised in all future assays presented to minimise the effects that γδ T-APC proliferation may have on induction of naive $CD4^+$ T cell responses. However, where possible, the findings produced utilising irradiated γδ T-APCs were replicated using non-irradiated γδ T-APCs, and will be discussed at each stage of data presented. In comparison with the data presented in Figure 4.1 using irradiated γδ T-APCs, non-irradiated γδ T-APCs induced similar levels of proliferation, and no statistically significant differences were observed between the two populations (data not shown).

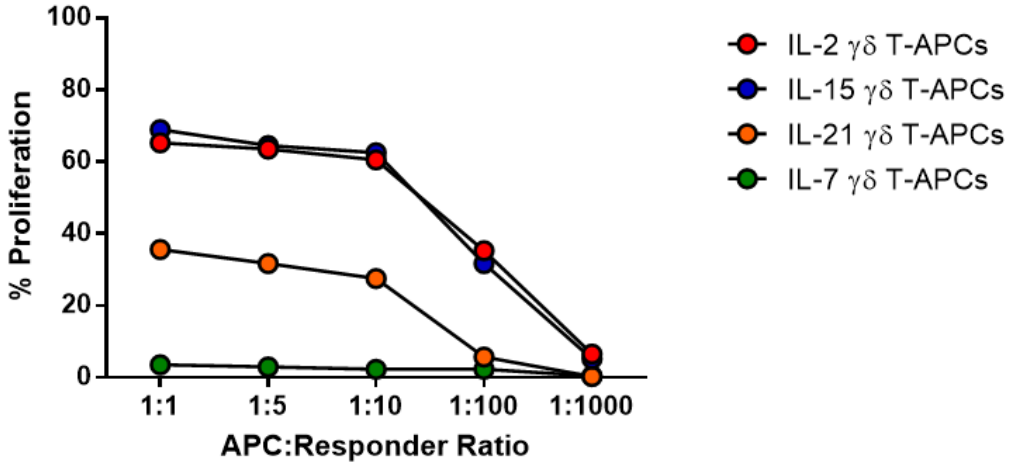

Figure 4.1 – Proliferation of naive CD4$^+$ T cells in response to decreasing γδ T-APC:responder cell ratios. Naive CD4$^+$ T cells were cultured with allogeneic γδ T-APCs at decreasing APC:responder cell ratios for a period of five days. γδ T-APCs were generated with either IL-2, IL-15, IL-21, or IL-7. Proliferation of naive CD4$^+$ T cells was determined by assessing CFSE dilution by flow cytometry, and percentages of proliferating, CFSE^{lo} cells were determined. Data displayed represent an individual healthy donor, from a total of 3 γδ T cell and CD4$^+$ T cell donors from 3 individual experiments.

Having determined a 1:10 APC:responder ratio was optimal for assessing responses induced by all APC subtypes, a comparison of proliferation induced by each APC subtype was conducted (Figure 4.2). In Figure 4.2a, representative flow cytometry histograms displaying naive CD4$^+$ proliferation induced by each γδ T-APC subtype are presented, in addition to naive CD4$^+$ T cells cultured alone for 5 days. Figure 4.2b displays a comparison of naive CD4$^+$ T cell proliferation induced by each subtype of APC, under all stimulating conditions. The levels of proliferation induced by IL-2 and IL-15 γδ T-APCs were almost identical to those promoted by LPS and PGN-treated DCs, indicating these cells possess a similar capacity to stimulate naive CD4$^+$ T cell responses in this system, with naive CD4$^+$ T cell proliferation significantly higher than that observed with naive cells cultured alone. In contrast, IL-21 γδ T-APCs induced responses more consistent with those observed when utilising immature DCs or monocytes as APCs. However, the levels of proliferation observed were not statistically significant as compared to naive CD4$^+$ T cells alone. This is likely due to the variation in proliferation; in mixed lymphocyte reactions, the reactivity of allogeneic cell populations can vary significantly depending on the combination of donors, and this variation can be seen in all conditions examined. Overall, IL-2 and IL-15 γδ T-APCs induced robust

proliferation in naive $CD4^+$ T cell populations in a similar manner to DCs, whereas IL-21 γδ T-APCs favoured low level proliferation more comparable to monocytes. IL-7 γδ T-APCs, as previously discussed, were unable to induce naive $CD4^+$ T cell proliferation.

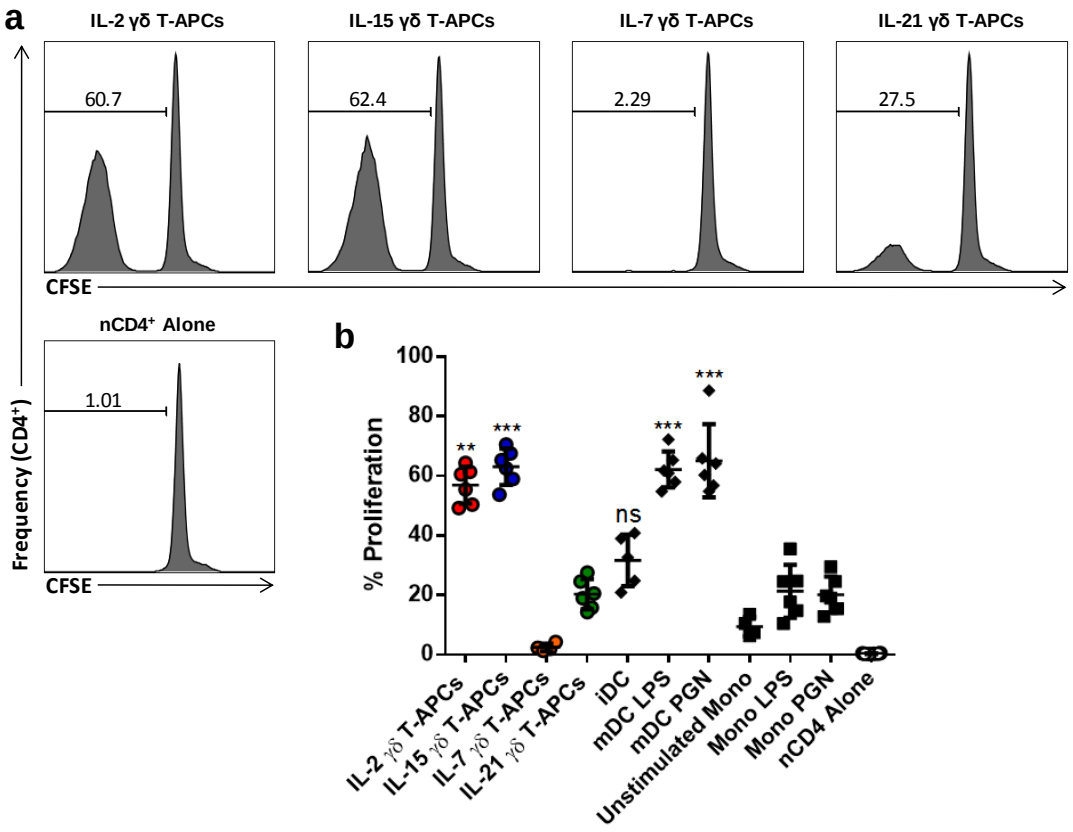

Figure 4.2 – Proliferation of naive CD4$^+$ T cells induced by different antigen presenting cells. Naive CD4$^+$ T cells were cultured with different types of APC, stimulated under differing conditions, at a 1:10 APC:responder ratio for five days. Proliferation of naive CD4$^+$ T cells was determined by assessing CFSE dilution by flow cytometry at day 5 of culture, and percentages of proliferating, CFSE^{lo} cells were determined. **(a)** Representative histograms displaying naive CD4$^+$ T cell proliferation in response to γδ T-APCs, generated under different conditions. Numbers on graphs display percentages of proliferating, CFSE^{lo} cells . **(b)** Comparison of naive CD4$^+$ T cell proliferation induced by each APC. Data points represent individual healthy APC donors from individual experiments. Horizontal lines display means of data sets. Error bars display standard deviation of samples. Statistical significance was determined using the Kruskal-Wallis test, followed by the Dunn's multiple comparison test, *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001, ns=not significant. Significance displayed in comparison to unstimulated, nCD4 alone control.

In comparison to naive CD4$^+$ T cells, memory CD4$^+$ T cells possess reduced requirements to respond to antigen, in that activation thresholds are lower and memory cells are less dependent on costimulation[46,47]. For these reasons, the induction of memory CD4$^+$ T cell proliferation by APC subtypes was also assessed, to determine if any differences could be observed compared to naive CD4$^+$ T cells (Figure 4.3). As with naive responders, IL-2 and IL-15 γδ T-APCs induced robust

memory $CD4^+$ T cell responses, promoting levels of proliferation similar to those seen with DCs. IL-21 γδ T-APCs again induced lower levels of proliferation, consistent with immature DCs and monocytes. However, as with naive $CD4^+$ T cell responses, only the high levels of proliferation induced by IL-2 and IL-15 γδ T-APCs, alongside DCs, were statistically significant. Interestingly, and potentially reflecting the reduced activation requirements of memory cells, the levels of proliferation observed with memory $CD4^+$ T cells were higher than those with naive $CD4^+$ T cells. Again, IL-7 γδ T-APCs were unable to induce any responses in memory $CD4^+$ T cell populations.

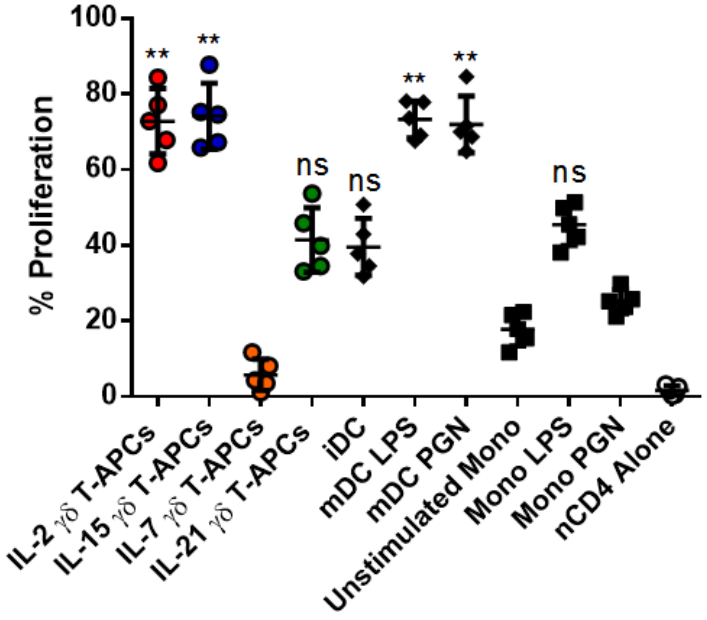

Figure 4.3 – Proliferation of memory CD4$^+$ T cells induced by different antigen presenting cells. Comparison of memory CD4$^+$ T cell proliferation induced by each type of APC. Memory CD4$^+$ T cells (CD4$^+$CD45RA$^-$) were cultured with different APCs, stimulated under differing conditions, at a 1:10 APC:responder ratio for five days. Proliferation of memory CD4$^+$ T cells was determined by assessing CFSE dilution by flow cytometry, and percentages of proliferating, CFSE^{lo} cells were determined. Data points represent individual healthy APC donors from individual experiments. Horizontal lines display means of data sets. Error bars display standard deviation of samples. Statistical significance was determined using the Kruskal-Wallis test, followed by the Dunn's multiple comparison test, *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001, ns=not significant. Significance displayed in comparison to unstimulated, nCD4 alone control.

To confirm the induction of CD4$^+$ T cell responses was indeed dependent on cell contact with γδ T-APCs, blocking antibodies against integrin molecules were added to cultures and responder cell proliferation was measured (Figure 4.4). A number of cell adhesion molecules and their integrin ligands exist to facilitate cell adhesion. Members of the β2 family of integrins are expressed exclusively by leukocytes, and are all composed of a common β chain known as CD18, in combination with one of four known α chains, referred to as CD11a/b/c/d[351,352]. CD11a/CD18, or lymphocyte function-associated antigen (LFA-1), is expressed by all leukocytes, and interacts with ICAM-1 (CD54) to facilitate cell adhesion. CD11b/CD18, or Mac-1, is primarily expressed my myeloid cells, and is also capable of ICAM-1 interaction, among other

receptors. CD11c/CD18 and CD11d/CD18 also play roles in cell adhesion, in addition to mediating phagocytosis[351].

To confirm that cell contact and adhesion between γδ T-APCs and naive $CD4^+$ T cells was essential for induction of $CD4^+$ T cell responses, γδ T-APCs were labelled with a blocking antibody to CD18, targeting all β2 integrin members, prior to co-culture with responder cells (Figure 4.4). Upon addition of this antibody, proliferation of naive $CD4^+$ T cells was completely abrogated, as compared to no blocking and isotype controls. With confirmation that β2-mediated cell adhesion was necessary for responses observed, an antibody to CD11a was added in the same manner to γδ T-APC cultures. Similarly to CD18, blocking CD11a significantly reduced proliferation of naive $CD4^+$ T cells, and no significant difference was observed between anti-CD18 and anti-CD11a treatments. These experiments indicate that LFA-1 (CD11a/CD18) interaction with ICAM-1 is the primary mechanism responsible for cell adhesion between γδ T-APCs and naive $CD4^+$ T cells, respectively.

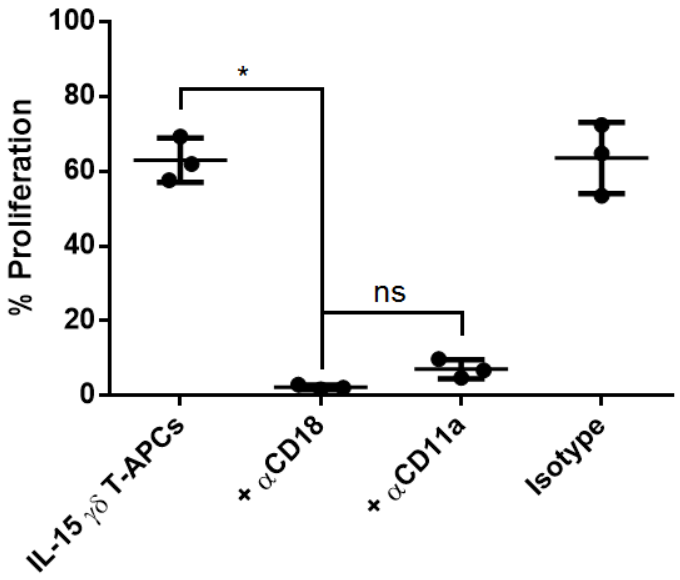

Figure 4.4 – Blockade of cell adhesion molecules in γδ T-APC:naive CD4$^+$ T cell co-cultures. Comparison of naive CD4$^+$ T cell proliferation induced by IL-15 γδ T-APCs, in the presence or absence of blocking antibodies to cell adhesion molecules. Naive CD4$^+$ T cells were cultured with allogeneic IL-15 γδ T-APCs at a 1:10 APC:responder ratio for five days. Proliferation of naive CD4$^+$ T cells was determined by assessing CFSE dilution by flow cytometry, and percentages of proliferating, CFSE^{lo} cells were determined. Data points represent individual healthy donors from individual experiments. Horizontal lines display means of data sets. Error bars display standard deviation of samples. Statistical significance was determined using the Friedman test, followed by the Dunn's multiple comparison test *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001, ns=not significant.

In summary, γδ T-APCs generated in this system are able to induce both naive and memory CD4$^+$ T cell responses, in terms of proliferation. Further to this, the mechanism by which γδ T-APCs are generated with reference to the microenvironment plays a significant role in their subsequent ability to promote CD4$^+$ T cell responses.

4.4 - Polarisation of naive CD4$^+$ T cells

With confirmation that γδ T-APCs are indeed able to induce proliferative responses in naive CD4$^+$ T cells, attention was next focused on the effector phenotype of stimulated naive CD4$^+$ T cells.

In a similar manner to previous experiments assessing CD4$^+$ T cell proliferation, γδ T-APC and naive CD4$^+$ T cell co-cultures were set up at 1:10 APC:responder ratios.

Cultures were fed at day 6 with IL-2 and IL-23 as described in Chapter 3, and incubated up until day 9,at which point cells were restimulated to induce cytokine production. Subsequently, intracellular cytokine expression was assessed by flow cytometry, and the percentages of cells expressing a number of cytokines were determined.

Figure 4.5 displays representative flow cytometry plots, showing intracellular stainings of naive $CD4^+$ T cells co-cultured with either IL-2, IL-15, or IL-21 γδ T-APCs. Upon co-culture with IL-2 γδ T-APCs, naive $CD4^+$ T cells expressed a number of different cytokines. A large percentage of $CD4^+$ T cells produced IFN-γ, in the absence of any other cytokines, and as such exhibited a classical Th1 phenotype (Figure 4.5a). In addition, a small percentage of cells also appeared to express IL-22, either alone or in combination with IFN-γ. No IL-17-expressing populations could be identified in the responder cells. With a lack of IL-17 production, the IL-22-expressing cells detected were split between IFN-γ^- (Th22) and IFN-γ^+ (Th1) lineages. In addition to these cytokines, a population of IL-4 single-positive cells was identified, representing Th2 cells (Figure 4.5b). Minimal levels of IL-9 or IL-10 producing cells were detected in IL-2 γδ T-APC co-cultures.

In comparison to IL-2 γδ T-APC mediated polarisation of naive $CD4^+$ T cells, IL-15 γδ T-APCs induced a similar repertoire of T helper cell lineages in responding cells. Populations of Th1 and Th2 cells appeared similar across both types of APC cultures, and no IL-17 producing cells were detected. However, increased populations of IL-22 expressing cells were detected when naive $CD4^+$ T cells were polarised by IL-15 γδ T-APCs, a large proportion of which were Th1 cells co-expressing IFN-γ, with a smaller subset comprising IFN-γ^- Th22 cells (Figure 4.5a).

Additionally, when naive $CD4^+$ T cells were polarised by IL-21 γδ T-APCs, similar populations were identified, with a number of exceptions. The magnitude of the Th1 response induced by IL-21 γδ T-APCs appeared to be reduced as compared with IL-2 and IL-15 counterparts, whereas IL-4 and IL-17 expressing populations were similar across all conditions. The polarisation of IL-22 producing cells was similar to that observed with IL-15 γδ T-APCs, with IL-22^+ cells split between IFN-γ^+ and IFN-γ^-. The biggest difference in IL-21 γδ T-APC-polarised naive $CD4^+$ T cell phenotype as compared to other subsets was a small but apparent population of IL-10 producing cells, which were negative for all other cytokines examined, and a small group of cells co-expressing IL-4 and IL-10 together (Figure 4.5b). These IL-10 producing populations were undetectable in IL-2 and IL-15 γδ T-APC co-cultures.

Overall, it appeared that differentially generated γδ T-APC populations were, at least partially, able to induce different magnitudes of each T helper lineage observed, while no IL-17 or IL-9 was detected across all cultures. In comparison with previously published data on naive $CD4^+$ T cell polarisation by γδ T-APCs, both Th1 and Th2 populations were identified, making these findings consistent with previous work[322]. However, the expression of IL-22 and IL-10 as cytokines induced by γδ T-APCs has not been reported before.

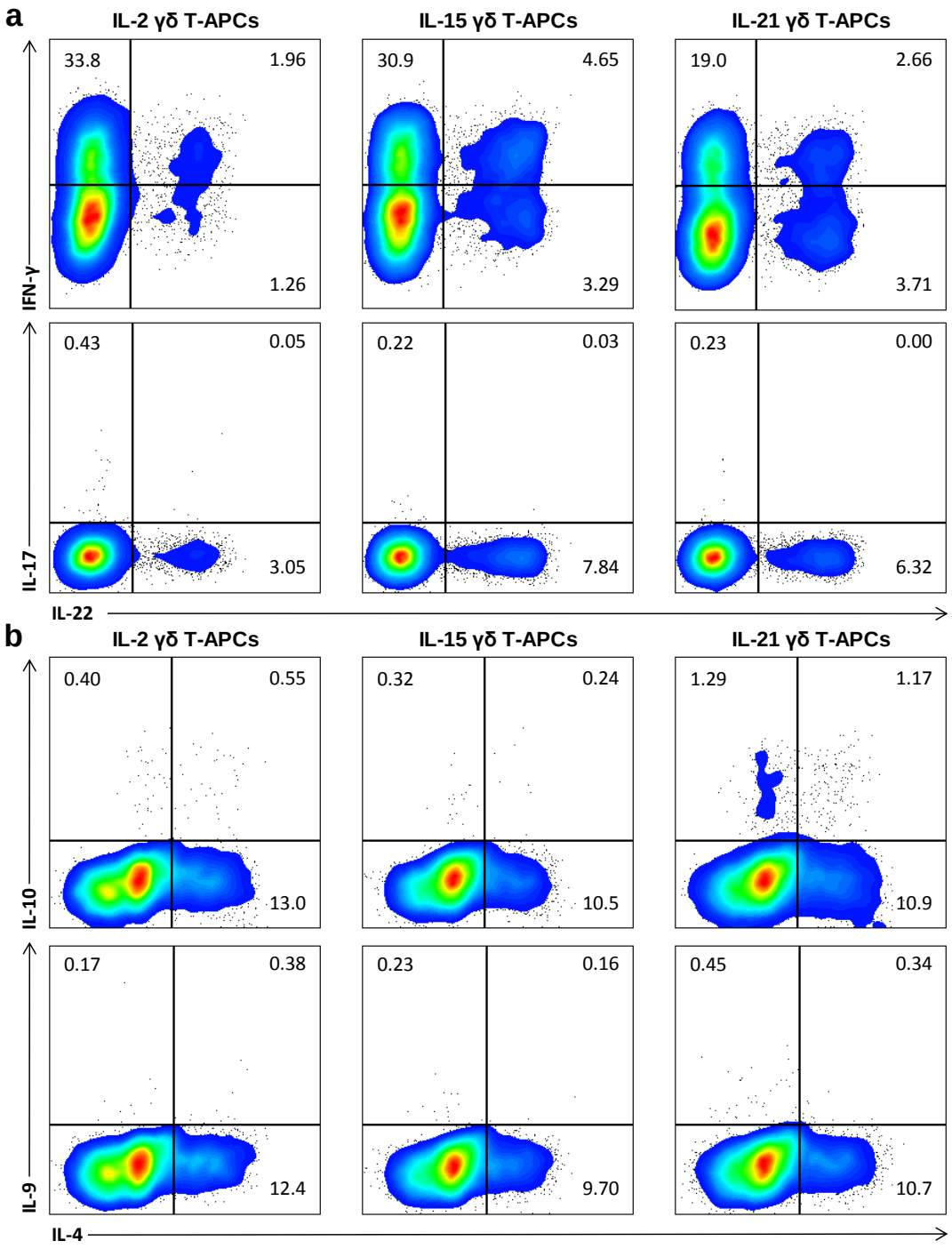

Figure 4.5 – Intracellular cytokine expression by naive CD4$^+$ T cells in response to stimulation by γδ T-APCs. Comparison of intracellular cytokine expression by naive CD4$^+$ T cells stimulated by allogeneic γδ T-APCs, generated under differing conditions, after co-culture for 9 days at 1:10 APC:responder ratios. Intracellular cytokine production was assessed by flow cytometry after cell restimulation at day 9 of culture. (a) Representative intracellular stainings displaying intracellular expression of IFN-γ, IL-17, and IL-22 by naive CD4$^+$ T cells, polarised by IL-2, IL-15, and IL-21 γδ T-APCs. (b) Representative intracellular stainings displaying intracellular expression of IL-4, IL-10, and IL-9 by naive CD4$^+$ T cells, polarised by IL-2, IL-15, and IL-21 γδ T-APCs. Plots are representative of >5 individual healthy γδ T cell donors from >5 individual experiments. Numbers on graphs display percentages of cytokine-expressing cells, gated on CD3$^+$CD4$^+$Vδ2$^-$ cells.

When dendritic cells were utilised as APCs in identical naive $CD4^+$ T cell polarisation assays, markedly different responses were observed as compared to those seen with γδ T-APCs (Figure 4.6). In terms of Th1 type responses, iDCs and PGN-treated DCs both induced similar levels of single IFN-γ^+ cells to IL-21 γδ T-APCs, whereas LPS-treated DCs induced higher percentages of Th1 cells more similar to those observed with IL-2 and IL-15 γδ T-APCs. Both iDCs and LPS-treated DCs induced low levels of IL-17 single positive cells (Th17 cells), and low levels of IL-22-producing cells. PGN-treated DCs appeared the only DC condition capable of inducing similar levels of IL-22 as compared to IL-15 and IL-21 γδ T-APCs. However, a significant proportion of these IL-22^+ cells co-expressed IL-17, and as such were characterised as Th17 cells. No DC condition examined induced levels of IL-22^+ IL-17^- cells as seen with IL-15 and IL-21 γδ T-APCs.

With respect to IL-4, IL-9 and IL-10 induction by DCs, only small populations of IL-4 single positive cells were identified, and no significant populations of IL-10 and IL-9 producing cells (data not shown). To relate these findings to published data, LPS-treated DCs have been reported to predominantly promote Th1 responses[206], as can be seen in these data (Figure 4.6). In contrast, PGN activation of APCs has been identified to promote Th17 responses over other lineages[95], reflected in these data whereby increased populations of IL-17^+ cells were detected, in contrast to the decreased levels of Th1 cells.

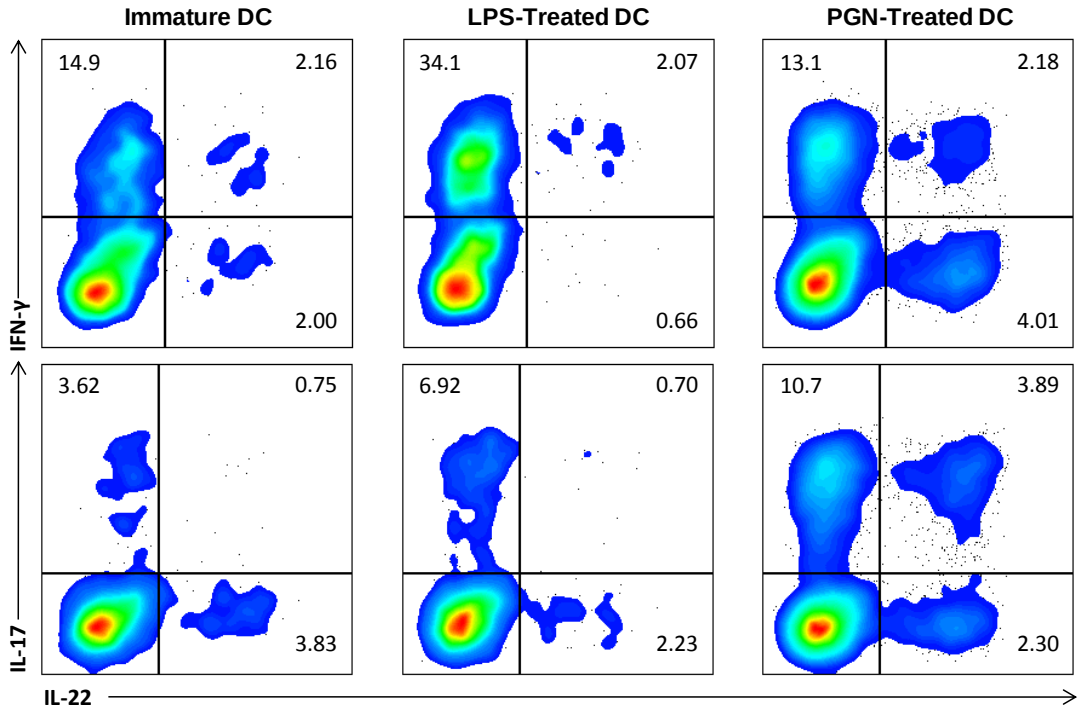

Figure 4.6 – Intracellular cytokine expression by naive CD4$^+$ T cell responses in response to stimulation by dendritic cells. Comparison of intracellular cytokine expression by naive CD4$^+$ T cells stimulated by allogeneic DCs, after co-culture for 9 days at 1:10 APC:responder ratios. DCs were cultured with LPS or PGN, or left unstimulated, for 24 hours prior to co-culture. Intracellular cytokine expression was assessed by flow cytometry after cell restimulation at day 9 of culture. Representative intracellular stainings displaying expression of IFN-γ, IL-17, and IL-22 by naive CD4$^+$ T cells, polarised by immature, LPS-treated, or PGN-treated monocyte-derived DCs. Plots are representative of >5 individual healthy DC donors from >5 individual experiments. Numbers on graphs display percentages of cytokine-expressing cells, gated on CD3$^+$CD4$^+$ cells.

In addition to DCs, monocytes were also examined for their ability to polarise distinct lineages of T helper cells (Figure 4.7). In identical experiments to DCs, monocytes were either left unstimulated or treated with LPS or PGN, and used to polarise allogeneic naive CD4$^+$ T cells over a 9 day culture period. Similar patterns of results were identified with monocyte APCs as compared with DCs. Unstimulated monocytes only induced low levels of Th1 and Th17 cells, LPS-treated monocytes favoured Th1 responses and induced little IL-17 and IL-22, and finally PGN-treated monocytes supported the polarisation of significant populations of Th17 cells. Again, no condition examined was able to promote the levels of IL-22$^+$IL-17$^-$ cells as observed with γδ T-APCs. Monocytes treated with PGN have been identified as potent Th17 inducing APCs[95], able to outperform DCs in this role despite inducing lower levels of proliferation in allogeneic naive CD4$^+$ T cell responders, as observed

in Figure 4.7. Levels of Th2 cells induced by monocytes were indistinguishable from those induced by DCs, and only low levels of IL-10 producing cells were identified. Minimal IL-9^{+} cell populations were identified (data not shown).

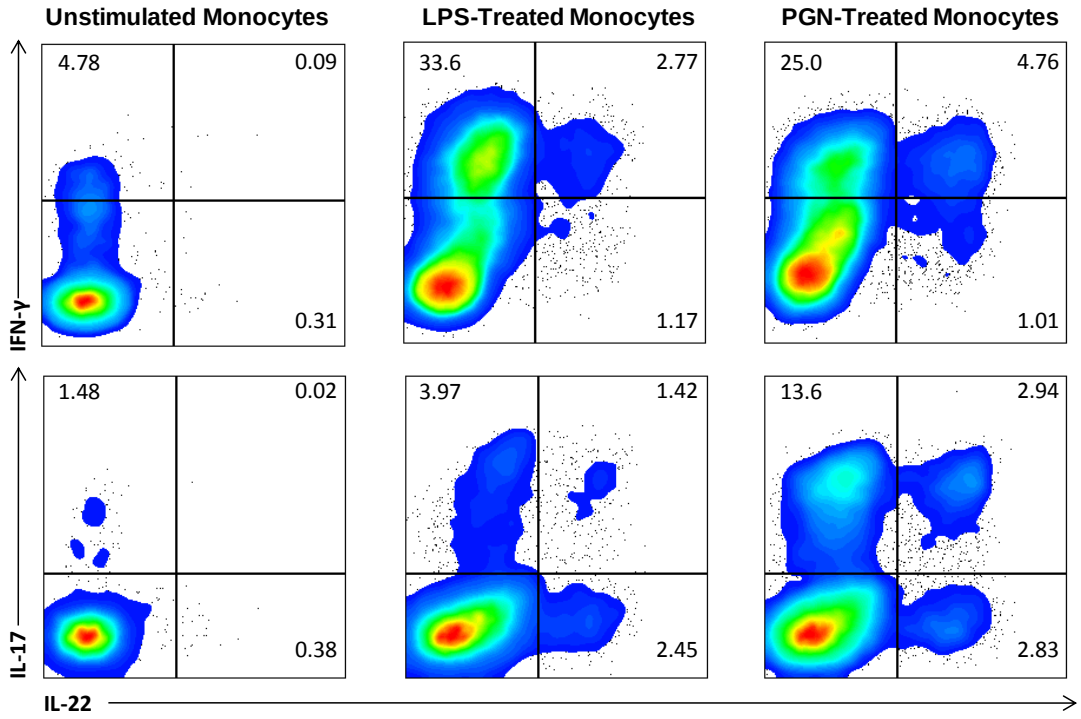

Figure 4.7 – Intracellular cytokine expression by naive CD4$^+$ T cell responses in response to stimulation by monocytes. Comparison of intracellular cytokine expression by naive CD4$^+$ T cells stimulated by allogeneic monocytes, after co-culture for 9 days at 1:10 APC:responder ratios. Monocytes were cultured with LPS or PGN, or left unstimulated, for 24 hours prior to co-culture. Intracellular cytokine expression was assessed by flow cytometry after cell restimulation at day 9 of culture. Representative intracellular stainings displaying expression of IFN-γ, IL-17, and IL-22 by naive CD4$^+$ T cells, polarised by unstimulated, LPS-treated, or PGN-treated monocytes. Plots are representative of >5 individual healthy monocyte donors from >5 individual experiments. Numbers on graphs display percentages of cytokine-expressing cells, CD3$^+$CD4$^+$ cells.

Figure 4.8 displays a summary of the percentages of cytokine positive cells in polarised naive CD4$^+$ T cell populations. With respect to IFN-γ induction, both IL-2 and IL-15 γδ T-APCs induced the highest levels in naive CD4$^+$ T cell responders, matched only by LPS-treated DCs and monocytes. In contrast, IL-21 γδ T-APCs, in a similar manner to PGN-treated monocytes and immature DCs, polarised reduced populations of IFN-γ-producing cells. The differences in IFN-γ induction observed between IL-2/IL-15 γδ T-APCs and those induced by IL-21 γδ T-APCs were statistically significant. As previously stated, IL-17 was completely absent from γδ T-APC:naive CD4$^+$ T cell co-cultures. In contrast, all monocyte and DC cultures induced at least minimal levels of IL-17, increasing from immature APCs, to LPS treated APCs, with the highest levels of IL-17 induced by PGN-treated DCs and monocytes. Despite significant variation across donors in terms of DC and

monocyte-induced Th17 polarisation, these levels of Th17 induction were significantly higher than those in γδ T-APC co-cultures.

One of the most defining effects of γδ T-APC co-culture on naive $CD4^+$ responder T cells, and that showed significant variation between Vγ9Vδ2 T cell treatments, was in the induction of IL-22. Whilst IL-2 and IL-21 γδ T-APCs induced similar percentages of $IL\text{-}22^+$ cells as did monocytes and DCs, IL-15 γδ T-APCs polarised increased populations of IL-22-expressing cells across all donors examined, the results of which were statistically significant compared to other γδ T-APC co-cultures and alternative APC controls. As previously discussed, a large proportion of these IL-22 expressing cells were IFN-γ co-producers (Th1 cells), whereas the remaining cells represented $IFN\text{-}\gamma^-$ Th22 cells. All $IL\text{-}22^+$ cells in these cultures were absent for IL-17 expression.

In terms of IL-4 induction in naive $CD4^+$ T cells, γδ T-APCs induced similar levels of positive cells, regardless of the generation protocol. Only monocytes treated with LPS and PGN were able to polarise similar levels of IL-4 producing cells. Across donors, large levels of variation in IL-4 producing cells were identified, particularly in γδ T-APC co-cultures, though there appeared to be a trend towards higher levels of IL-4 induction by γδ T-APCs than by DC controls, and to some extent monocytes. IL-9 induction in naive $CD4^+$ T cells was minimal, with marginally higher levels induced by IL-2 and IL-15 γδ T-APCs, though these levels did not exceed 2% of responding $CD4^+$ T cells. Finally, IL-10 induction by IL-21 γδ T-APCs exceeded levels induced by all other APC subtypes. The percentages of IL-10 positive cells were significantly higher that naive $CD4^+$ T cell alone cultures, but not statistically significant as compared to other conditions.

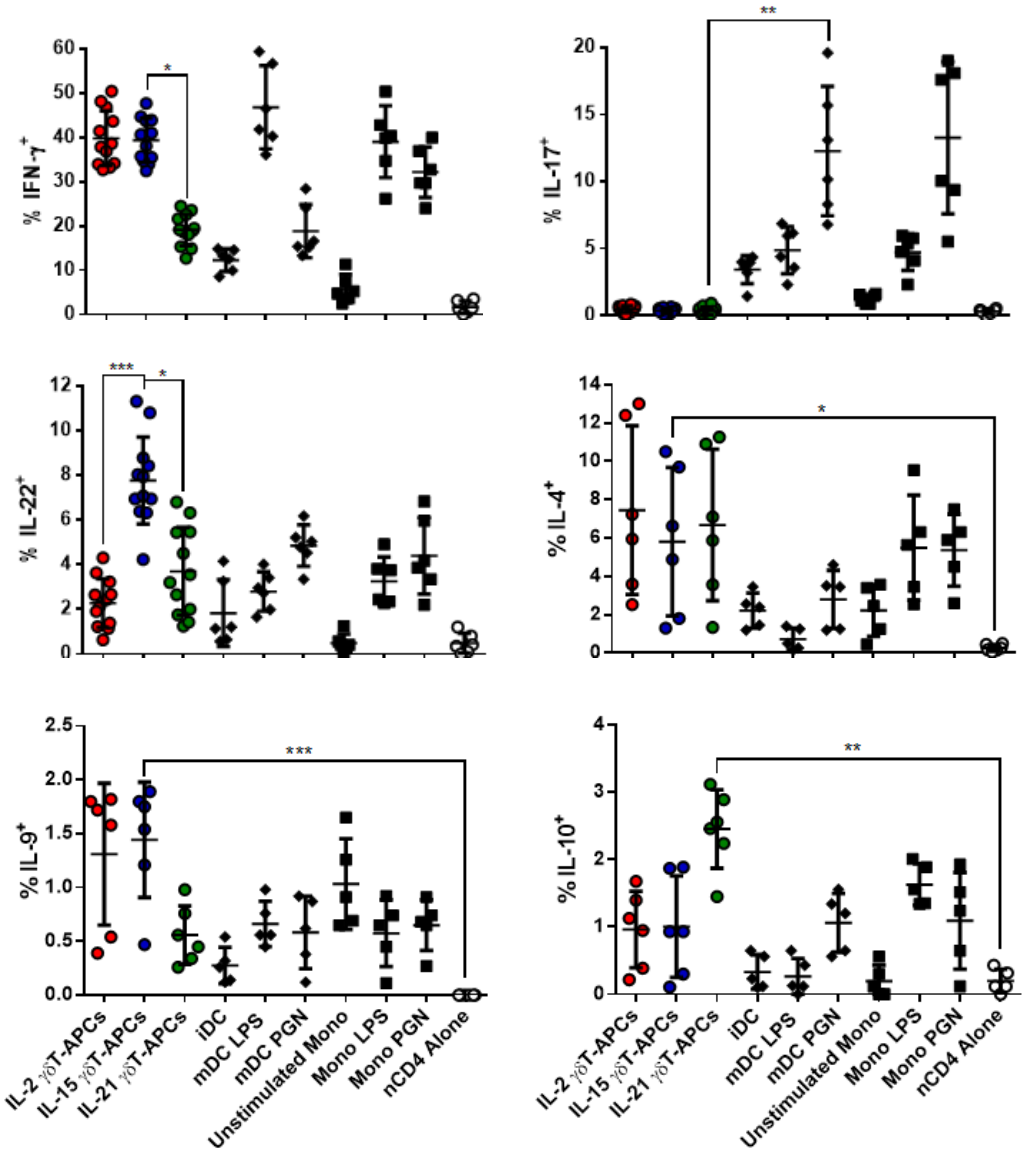

Figure 4.8 – Comparison of intracellular cytokine expression by naive CD4$^+$ T cells, polarised by different APCs. Comparison of naive CD4$^+$ T cell intracellular cytokine expression, polarised by γδ T cell, monocyte, or DC APCs, generated under different conditions. Naive CD4$^+$ T cells were cultured with relevant APC subtypes at a 1:10 APC:responder ratio for 9 days. Intracellular cytokine expression was assessed by flow cytometry after cell restimulation at day 9 of culture. Percentages of cytokine positive cells were determined by gating of live, single, CD3$^+$CD4$^+$Vγ9$^-$ cells. Data points display results from individual healthy donors. Horizontal lines display means of data sets. Error bars display standard deviation of samples. Statistical significance was determined using the Kruskal-Wallis test, followed by the Dunn's multiple comparison test, *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001. Significance displayed for most important results.

In addition to assessing intracellular cytokine expression by polarised naive $CD4^+$ T cells, the secretion of cytokines in cellular supernatants was also assessed by ELISA (Figure 4.9). Of note, given the fact that γδ T-APCs were irradiated prior to co-culture with naive $CD4^+$ T cells, the majority of APCs did not survive until day 9 of culture. As such, co-cultures mainly consisted of $CD4^+$ T cells (>97% $CD4^+$) when restimulated for analysis of cytokine secretion by ELISA (data not shown). However, small contributions of γδ T-APCs to secretion of cytokines cannot be dismissed. In addition to five of the cytokines assessed intracellularly, a further four were examined by ELISA. Similarly to intracellular expression, secretion of IFN-γ was highest amongst naive $CD4^+$ T cells stimulated with IL-2 and IL-15 γδ T-APCs, in addition to LPS treated DCs and monocytes, with IL-21 γδ T-APCs favouring lower secretion of IFN-γ by responder cells. Secretion of TNF-α by polarised naive $CD4^+$ T cells displayed a similar pattern of secretion as compared with IFN-γ. IL-17 secretion by $CD4^+$ T cells in co-culture with γδ T-APCs was undetectable, in accordance with the flow cytometric analysis. When IL-22 secretion was assessed, again IL-15 γδ T-APCs proved to be most effective in polarisation of naive responses towards expression of this cytokine, with levels significantly higher than those observed with IL-2 γδ T-APCs and PGN-treated DCs. In contrast to the intracellular data, the difference between IL-15 and IL-21 γδ T-APC induction of IL-22 secretion was not statistically significant.

The secretion of IL-4 by polarised naive $CD4^+$ T cells presented a much clearer picture than that observed with intracellular expression; IL-4 secretion was significantly higher when naive $CD4^+$ T cells were cultured with γδ T-APCs compared with alternative APC controls, though no differences were observed between different γδ T-APC conditions. In addition to IL-4, the Th2 cytokines IL-5 and IL-13 followed similar patterns of secretion to IL-4. Secretion of IL-10 by IL-21 γδ T-APC polarised responder cells was increased compared to all other conditions, and this difference was more pronounced in culture supernatant analysis than that identified by intracellular analysis. Only LPS-treated monocytes were able to induce naive $CD4^+$ T cells to produce IL-10 to levels approaching those observed with IL-21 γδ T-APCs. Finally, the chemokine CXCL13 was examined, which is involved in the recruitment of CXCR5 expressing cells and as such important in Tfh cell responses[353]. γδ T-APCs across all conditions were unable to induce this chemokine in responding cells, whereas DCs and monocytes were able to induce varying levels of CXCL13 production in naive $CD4^+$ T cell responders.

Of note, the main findings from intracellular cytokine expression and secretion were reproducible at APC:responder ratios between 1:1 and 1:100, and using non-irradiated γδ T-APCs instead of irradiated γδ T-APCs (data not shown).

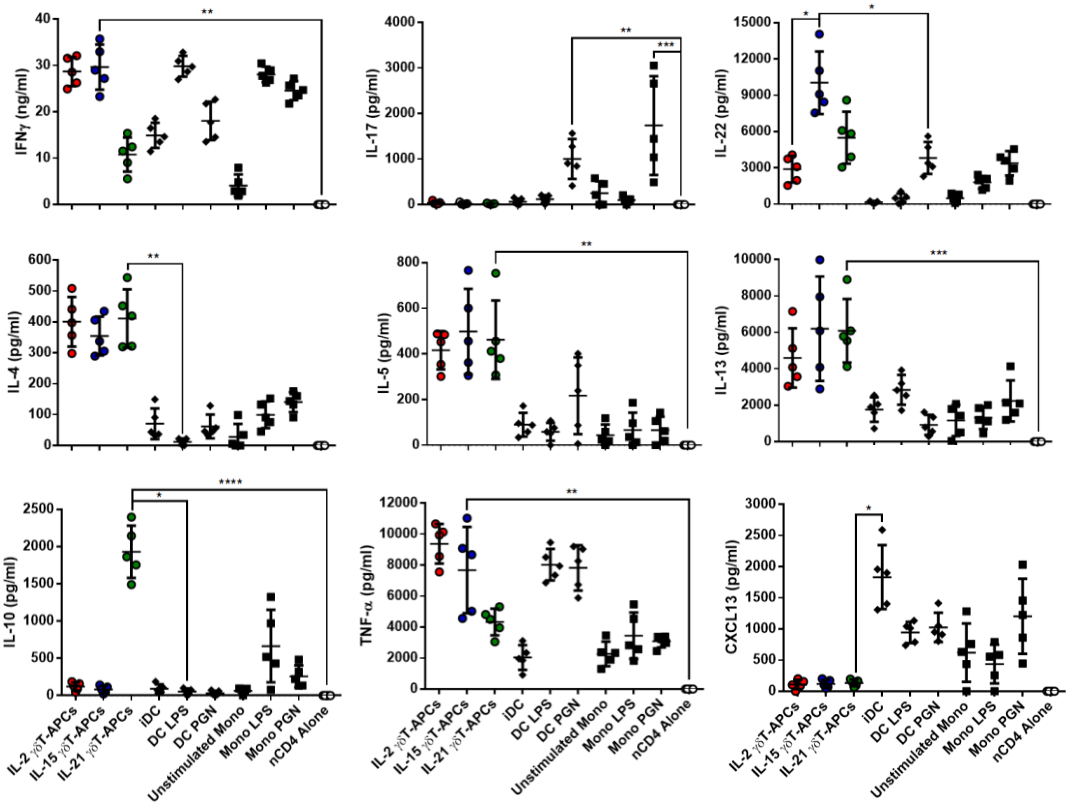

Figure 4.9 – Comparison of cytokine secretion by naive CD4$^+$ T cells, polarised by APCs. Naive CD4$^+$ T cells were cultured with γδ T cell, monocyte, or DC APCs, stimulated under different conditions, at 1:10 APC:responder ratios for 9 days. Cytokine secretion was assessed by ELISA after cell restimulation at day 9 of culture, where cells were cultured for 24 hours post-restimulation and supernatants obtained. Data points display results from individual healthy donors. Horizontal lines display means of data sets. Error bars display standard deviation of samples. Statistical significance was determined using the Kruskal-Wallis test, followed by the Dunn's multiple comparison test, *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001. Significance displayed for most important results.

Having identified a number of T helper subtypes induced in naive CD4$^+$ T cells by γδ T-APCs, expression of corresponding transcription factors induced in responder cells was examined next. In identical experiments to those previously discussed, γδ T-APCs were co-cultured with naive CD4$^+$ T cells for a period of 9 days. At day 9, CD4$^+$ T cells from co-cultures were purified from γδ T-APC contaminating cells by cell sorting and total RNA was extracted from the responder cells. Gene expression was subsequently assessed by real-time PCR. Due to the irradiation of γδ T-APCs prior to co-culture, the majority of APCs did not survive until day 9 of culture, allowing for effective purification of CD4$^+$ responder cells. As such, RNA extraction was conducted on CD4$^+$ T cell populations which were >99.1% CD4$^+$, thus minimising the contamination by γδ T-APCs.

Figure 4.10 displays the expression of transcription factors induced in naive $CD4^+$ T cells upon polarisation by γδ T-APCs generated under different conditions. Reflective of the high induction of IFN-γ, cells responding to IL-2 or IL-15 γδ T-APCs displayed increased expression of *TBX21*, in accordance with the induction of Th1 type cells. IL-21 γδ T-APCs induced lower levels of *TBX21* expression in naive $CD4^+$ T cell populations, agreeing with the cytokine analysis showing that IL-21 γδ T-APCs are less potent at inducing Th1 type responses. Consistent with the induction of IL-4 in all samples, increased expression of *GATA3* by responder cells was identified in all cultures, in comparison with naive $CD4^+$ T cell controls. The highest expression levels of the Th22-associated factor *AHR* were identified in IL-15 γδ T-APC polarised cells, with reduced expression observed in IL-2 and IL-21 γδ T-APC co-cultures. In contrast with the expression of IL-17, which was absent in all γδ T-APC co-cultures, an increased expression of *RORC*, the Th17 master transcription factor, was identified under all conditions, especially in IL-15 γδ T-APC polarised naive $CD4^+$ T cells. Minimal expression of *FOXP3* and *BCL6* were induced in γδ T-APC co-cultures, indicating an absence of Treg and Tfh cell induction under the conditions examined. In contrast to IL-10 detection, none of the transcription factors assessed displayed increased expression in IL-21 γδ T-APC co-cultures.

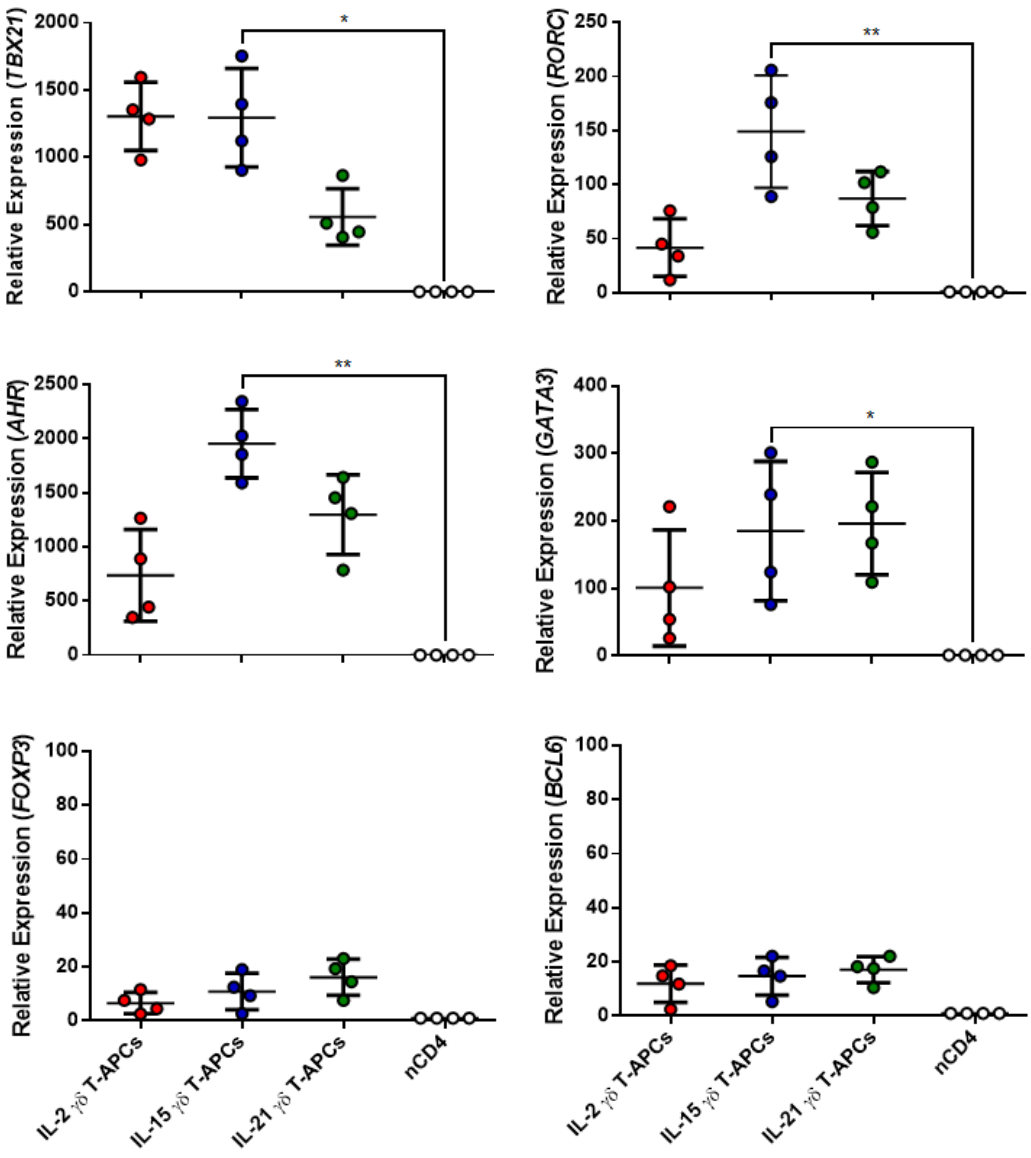

Figure 4.10 – Transcription factor expression by naive CD4$^+$ T cells, polarised by γδ T-APCs. Naive CD4$^+$ T cells were cultured with γδ T-APCs, stimulated under different conditions, at 1:10 APC:responder ratios for 9 days. Transcription factor expression was assessed at day 9 of culture by real-time PCR. Relative expression was determined in reference to naive CD4$^+$ T cell control. Data points display results from individual healthy donors. Horizontal lines display means of data sets. Error bars display standard deviation of samples. Statistical significance was determined using the Friedman test, followed by the Dunn's multiple comparison test, *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001.

In addition to characterising T helper responses by expression of cytokines and transcription factors, the major CD4$^+$ T cell lineages can be identified based on the repertoire of chemokine receptors they express. As such, responder cells after co-culture with γδ T-APCs were assessed for expression of CXCR3, CCR6, and

CCR10, which are differentially expressed by Th1, Th17, and Th22 cells respectively[92,121]. Whilst expression of each of these receptors alone would not fully characterise T helper lineages specifically, due to the fact that combinations of two or three receptors are generally used for this process, identification of each receptor would provide an indication of the lineages induced. Figure 4.11a displays representative flow cytometry stainings of each chemokine receptor, expressed by naive $CD4^+$ T cells polarised by IL-15 γδ T-APCs. Distinct populations of $CXCR3^+$ and $CXCR3^-$ cells could be identified in responder cells. However, only low levels of CCR10 and CCR6 were identified, and these populations did not display high expression of each marker. Figure 4.11b displays expression of each marker on naive $CD4^+$ T cells, polarised by γδ T-APCs generated under different conditions. Similar expression of CXCR3 was observed in IL-2 and IL-15 γδ T-APC co-cultures, with percentages of positive cells reduced in IL-21 γδ T-APC co-cultures. Consistent with Figure 4.11a, only low levels of $CCR10^+$ and $CCR6^+$ cells were identified across all co-cultures examined. Minimal expression of each chemokine receptor was identified in naive $CD4^+$ T cell populations.

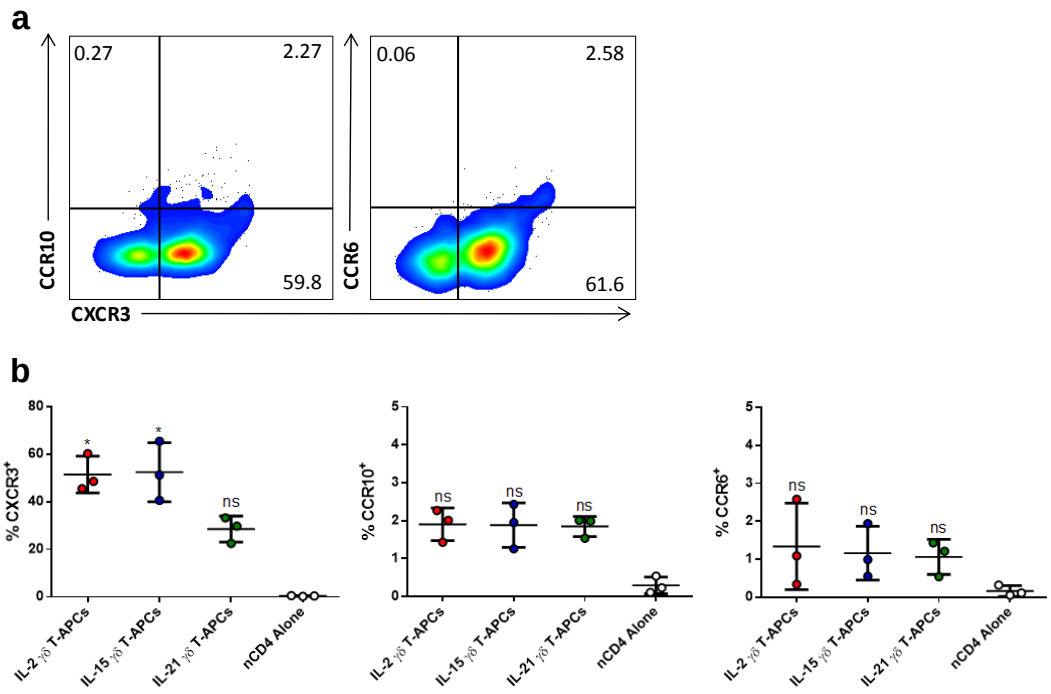

Figure 4.11 – Expression of chemokine receptors by naive CD4$^+$ T cells, polarised by γδ T-APCs. (a) Representative flow cytometry plots displaying chemokine receptor expression by naive CD4$^+$ T cells, polarised by IL-15 γδ T-APCs. Naive CD4$^+$ T cells were cultured with IL-15 γδ T-APCs for 9 days, and at day 9 were stained for chemokine receptor expression and assessed by flow cytometry. Numbers on graphs display percentages of positive cells. **(b)** Summary plots displaying chemokine receptor expression by naive CD4$^+$ T cells, polarised by differentially generated γδ T-APCs. Data points on graphs display individual γδ T cell donors. Horizontal lines display means of data sets. Error bars display standard deviation of samples. Statistical significance was determined using the Friedman test, followed by the Dunn's multiple comparison test, *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001, ns=not significant. Significance displayed in comparison to nCD4 alone control.

Whilst IL-2, IL-15 and IL-21 appeared to have significant effects on the polarising capacity of γδ T-APCs, it was unknown whether the strength of stimulation provided to γδ T-APCs during their generation may also play a role in subsequent polarisation of T helper responses. Indeed, TCR signalling strength has important roles in naive CD4$^+$ T cell polarisation[161], and as such may be important in γδ T-APC function as well. Given this possibility, γδ T-APCs generated with either 10 nM or 1 nM HMB-PP in combination with IL-15 were assessed for their abilities to polarise naive CD4$^+$ T cell responses (Figure 4.12a). Cells treated with 1 nM HMB-PP displayed upregulation of all APC markers examined, albeit at significantly reduced levels compared to cells stimulated with 10 nM HMB-PP. 1 nM HMB-PP treated γδ T-

APCs also induced lower levels of proliferation than 10 nM HMB-PP treated cells, although these differences were not statistically significant (data not shown).

Figure 4.12b displays representative flow cytometry plots of IFN-γ and IL-22 expression by naive $CD4^+$ T cells cultured in the presence of 10 nM or 1 nM HMB-PP-stimulated IL-15 γδ T-APCs, with Figure 4.12c showing a summary of the percentages of cytokine positive cells. Reduced levels of IFN-γ expression by responder cells were detected when co-cultured with 1 nM HMB-PP treated APCs compared with 10 nM, potentially reflecting a reduced level of stimulation provided by these APCs to responding cells. Levels of IL-22 expression however were not significantly affected overall. Within $IL\text{-}22^+$ cell populations, the distribution between $IFN\text{-}\gamma^+$ and $IFN\text{-}\gamma^-$ cells was altered by γδ T-APC stimulation, with more $IL\text{-}22^+$ cells co-expressing IFN-γ when polarised with 10 nM HMB-PP-treated APCs than with 1 nM HMB-PP-treated APCs. With respect to IL-4 induction by γδ T-APCs, percentages of $IL\text{-}4^+$ cells were marginally increased with 1 nM HMB-PP treated APCs as compared to 10 nM HMB-PP treated APCs, although this increase was not statistically significant (data not shown). No significant effects of HMB-PP concentrations were observed with respect to the induction of IL-10, IL-17, and IL-9 expression.

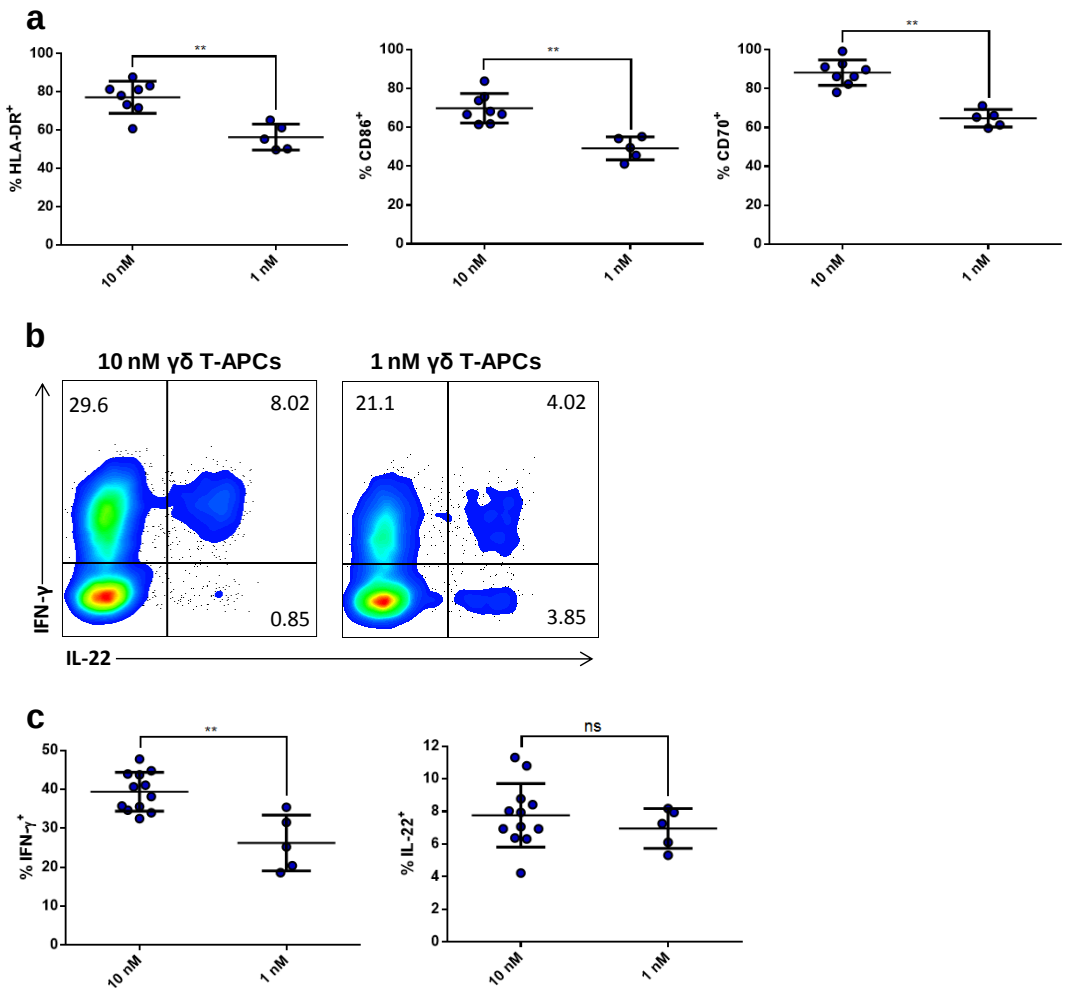

Figure 4.12 – Effect of γδ T-APC TCR stimulation strength on ability to polarise naive CD4$^+$ T cell responses. (a) Comparison of APC marker expression by γδ T-APCs stimulated with 10 nM or 1 nM HMB-PP in combination with IL-15. Percentages of APC marker positive cells were determined at day 3 of culture by flow cytometry. Percentage of HLA-DR$^+$ cells were determined by gating on live, single, Vγ9$^+$ T cells. CD86 and CD70 positive cells were determined by gating on live, single, HLA-DR$^+$Vγ9$^+$ T cells. **(b)** Representative flow cytometry plots displaying intracellular expression of IFN-γ and IL-22 by naive CD4$^+$ T cells, polarised by IL-15 γδ T-APCs generated with either 10 nM or 1 nM HMB-PP. Plots are representative of 5 individual healthy donors from 5 individual experiments. Numbers on graphs display percentages of cytokine-expressing cells. **(c)** Comparison of naive CD4$^+$ T cell expression of intracellular IFN-γ and IL-22, polarised by IL-15 γδ T-APCs generated with either 10 nM or 1 nM HMB-PP. Intracellular cytokine production was assessed by flow cytometry after cell restimulation at day 9 of culture. Percentages of cytokine positive cells were determined by gating of live, single, CD3$^+$CD4$^+$Vγ9$^-$ cells. Data points represent individual healthy γδ T cell donors from individual experiments. Horizontal lines display means of data sets. Error bars display standard deviation of samples. Statistical significance was determined using the Mann-Whitney test, *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001, ns=not significant.

4.5 - Polarisation of Memory $CD4^+$ T cells

In addition to the induction of naive $CD4^+$ T cell responses, γδ T-APCs have proven capable of robust induction of memory $CD4^+$ T cell responses. Given this potential, and the fact that memory $CD4^+$ T cells are capable of significant plasticity between T helper lineages, assays were established to examine the polarisation of memory responses by γδ T-APCs. In analogous experiments to the naive $CD4^+$ T cell assays, memory ($CD4^+CD45RA^-$) $CD4^+$ T cells were co-cultured with different APC subsets, and the cytokine expression was assessed.

Figure 4.13 displays representative flow cytometry plots of memory $CD4^+$ T cell polarisation by differentially generated γδ T-APCs. In a similar manner to naive $CD4^+$ T cell responses, six cytokines were assessed for their intracellular expression. Memory $CD4^+$ T cells stimulated with IL-2 γδ T-APCs displayed large populations of IFN-γ^+ and IL-22^+ single positive cells as well as IFN-γ^+ IL-22^+ co-producers (Figure 4.13a). In addition, small but apparent populations of IL-17^+ cells were identified, all of which were co-producing IFN-γ and a sub-population expressing IL-22 as well. Similarly to naive $CD4^+$ T cell responders, a population of Th2 cells was also identified (Figure 4.13b). Both IL-10 and IL-9 were minimally expressed in these cultures.

When memory $CD4^+$ T cells were co-cultured with IL-15 γδ T-APCs, significant populations of IFN-γ^+ and IL-17^+ cells were observed. The main difference between IL-2 and IL-15 γδ T-APCs in terms of memory cell polarisation was in the promotion of IL-22 responses; IL-15 γδ T-APCs promoted much larger populations of IL-22^+ cells than IL-2 γδ T-APCs, the majority of which were IFN-γ^+, and a smaller population representing IFN-γ^- Th22 cells. In addition, populations of Th2 cells did not appear to differ significantly between IL-2 and IL-15 γδ T-APC treatments.

The polarisation of memory $CD4^+$ T cells by IL-21 γδ T-APCs appeared to mirror the responses observed with naive $CD4^+$ T cells. Expression of IFN-γ was reduced in IL-21 γδ T-APC co-cultures compared with IL-2 and IL-15 γδ T-APCs, and IL-22 induction was more similar to IL-2 than IL-15 γδ T-APCs. Most strikingly, IL-10 single-positive cell induction in memory $CD4^+$ T cells was observed only using IL-21 γδ T-APCs as polarising cells, with no increase in IL-4 induction. Small populations of IL-9^+IL-4^- and IL-9^+IL-4^+ cells were primarily observed in IL-21 γδ T-APC co-cultures.

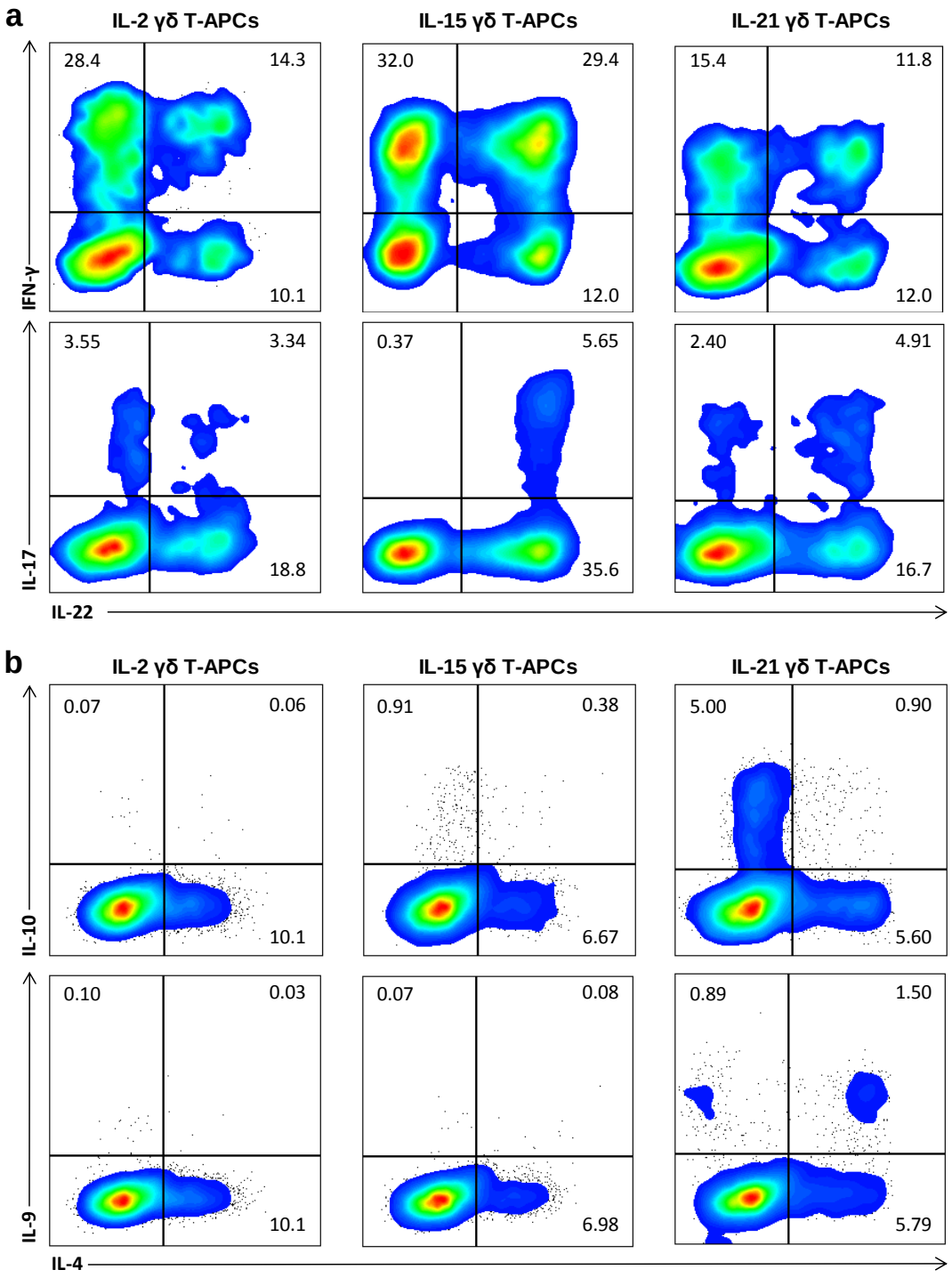

Figure 4.13 – Intracellular cytokine expression by memory CD4$^+$ T cells in response to stimulation by γδ T-APCs. Comparison of intracellular cytokine expression by memory CD4$^+$ T cells stimulated by allogeneic γδ T-APCs, generated under differing conditions, after co-culture for 9 days at 1:10 APC:responder ratios. Intracellular cytokine production was assessed by flow cytometry after cell restimulation at day 9 of culture. (a) Representative intracellular stainings displaying intracellular expression of IFN-γ, IL-17, and IL-22 by memory CD4$^+$ T cells, polarised by IL-2, IL-15, and IL-21 γδ T-APCs. (b) Representative intracellular stainings displaying intracellular expression of IL-4, IL-10, and IL-9 by memory CD4$^+$ T cells, polarised by IL-2, IL-15, and IL-21 γδ T-APCs. Plots are representative of >5 individual healthy γδ T cell donors from >5 individual experiments. Numbers on graphs display percentages of cytokine-expressing cells.

Figure 4.14 displays a summary of intracellular cytokine expression by memory $CD4^+$ T cells polarised by all APC subsets. Given that memory $CD4^+$ T cells comprise a population which has already encountered antigen *in vivo* and been polarised towards distinct T helper lineages, additional controls were established for comparison of APC-mediated polarisation. MemCD4 Day 0 conditions were measured to assess T helper phenotypes on the day of isolation, before assays were set up, by PMA/Ionomycin stimulation for 5 hours. This control allowed for identification of percentages of each lineage before specific APC-mediated polarisations had occurred. Additionally, memory $CD4^+$ T cells left unstimulated over the 9 day culture period were assessed alongside APC-polarising conditions. Lastly, memory $CD4^+$ T cells stimulated with αCD3/αCD28 antibodies were assessed for cytokine expression at day 9 of culture, to confirm any polarising effects observed utilising APCs were not simply due to memory cell expansion, but rather by active polarisation of cells.

In summary of the data presented in Figure 4.14, IFN-γ expression by memory $CD4^+$ T cells was favoured by IL-2 and IL-15 γδ T-APCs, as well as LPS-treated DCs, which matched the data observed using naive $CD4^+$ T cells. In contrast to naive $CD4^+$ T cell responses, small populations of $IL\text{-}17^+$ cells were identified when memory $CD4^+$ T cells were polarised by all γδ T-APCs. However, the magnitude of IL-17 responses was not significantly increased above the levels observed at day 0, prior to polarisation. IL-22 responses also appeared to mirror those observed with naive $CD4^+$ T cells; IL-15 γδ T-APCs favoured the highest production of IL-22, significantly increased above all other APC controls, reaching up to 40% of responding cells. IL-4 production by memory $CD4^+$ T cells was also increased among γδ T-APC polarisations as compared to DCs, whereas no significant induction of IL-9 was observed among any polarising conditions. Lastly, the promotion of IL-10 responses by IL-21 γδ T-APCs was increased above all other APC controls. The percentages of cytokine positive cells observed with memory $CD4^+$ T cells exceeded those observed with naive $CD4^+$ T cells, possibly reflecting reduced activation requirements of memory responder cells. Overall, it appeared γδ T-APCs favoured the polarisation of similar T helper lineages in memory $CD4^+$ T cells as they did in naive responder populations previously.

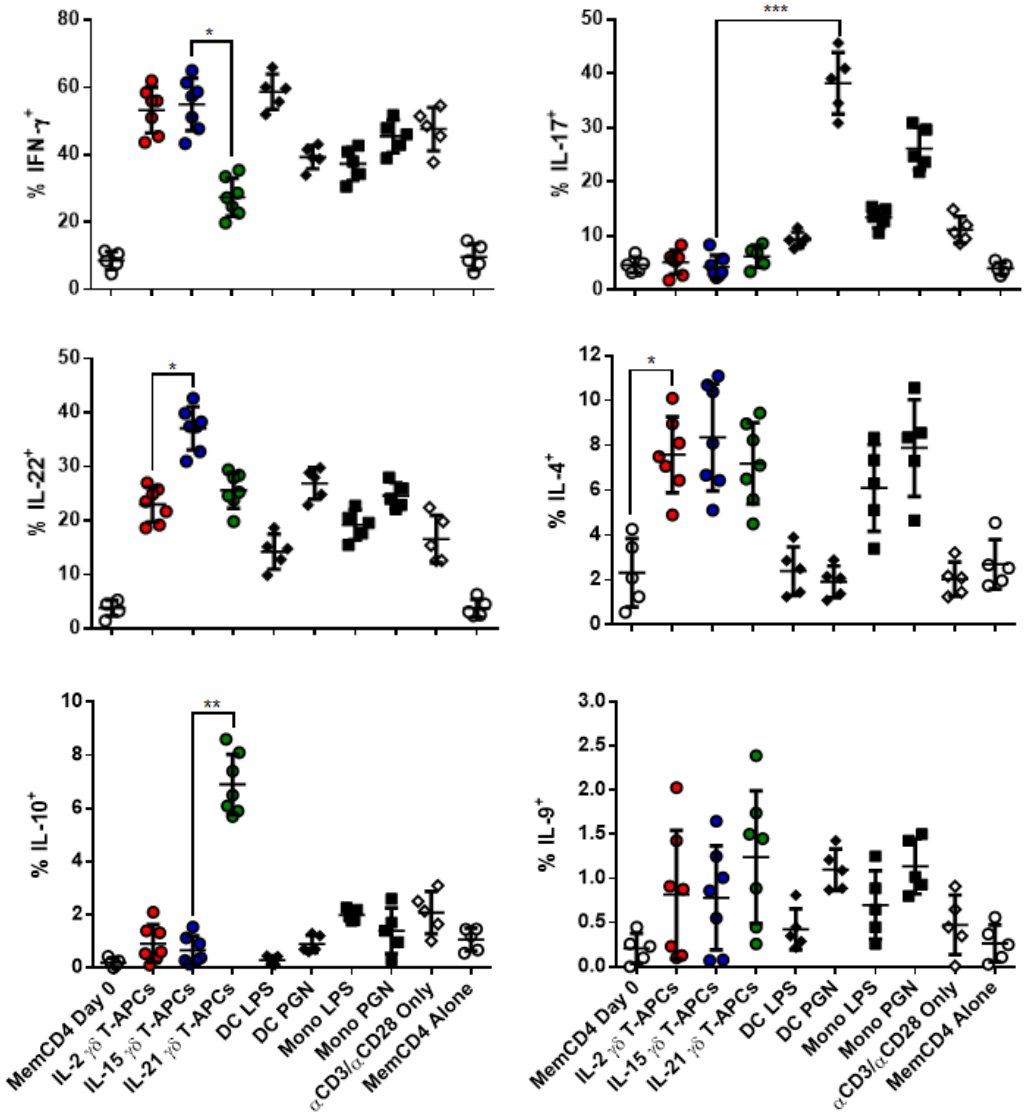

Figure 4.14 – Comparison of intracellular cytokine expression by memory CD4$^+$ T cells, polarised by different APCs. Comparison of memory CD4$^+$ T cell polarisation induced by γδ T cell, monocyte, or DC APCs, generated under different conditions. Memory CD4$^+$ T cells were cultured with relevant APC subtypes at 1:10 APC:responder ratios for 9 days. In addition, the CD4$^+$ T cell intracellular cytokine profile of freshly isolated, PMA/Ionomycin-stimulated cells (MemCD4 Day 0), and phenotype after stimulation with αCD3/αCD28 alone were determined. Intracellular cytokine production was assessed by flow cytometry after cell restimulation at day 9 of culture. Percentages of cytokine positive cells were determined by gating of live, single, CD3$^+$CD4$^+$Vγ9$^-$ cells. Data points display results from individual healthy donors. Horizontal lines display means of data sets. Error bars display standard deviation of samples. Statistical significance was determined using the Kruskal-Wallis test, followed by the Dunn's multiple comparison test, *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001. Significance displayed for most important results.

4.6 - Polarisation of Naive $CD8^+$ T Cell Responses

With the knowledge that $CD8^+$ T cells also exhibit functional plasticity in terms of cytokine production, and are able to be polarised into different effector lineages[215,216], assays were established to assess whether γδ T-APCs may contribute to this process. The $CD8^+$ T cell response to γδ T-APCs has been widely studied in terms of induction of cytotoxic activity[322,325], however the cytokine production of responding $CD8^+$ T cells, beyond IFN-γ, is unknown. As γδ T-APCs in previous assays displayed potential to promote different $CD4^+$ T cell lineages, it seemed likely these cells may also promote certain $CD8^+$ Tc lineages over others.

In identical assays to naive $CD4^+$ T cell polarisation, naive $CD8^+$ T cells ($CD8^+$ $CD45RA^+$ $CCR7^+$) were co-cultured with γδ T-APCs generated under different conditions, and responding cell proliferation and intracellular cytokine production was determined (Figure 4.15). The proliferation of naive $CD8^+$ T cells was similar to that observed with their $CD4^+$ counterparts, in that both IL-2 and IL-15 γδ T-APCs induced the highest levels of proliferation in responder cells (Figure 4.15a). IL-21 γδ T-APCs induced low levels of proliferation, and IL-7 γδ T-APCs were again unable to stimulate any response in naive $CD8^+$ T cells. When the intracellular cytokine expression of naive $CD8^+$ T cells was determined at day 9 of culture, several differences were observed compared with naive $CD4^+$ T cell polarisation. Induction of Tc1 and Tc2 cell populations were identified, with IL-2 and IL-15 γδ T-APCs favouring increased Th1 responses over IL-21 γδ T-APCs. However, cultures were completely negative for all other cytokines examined, including IL-22 and IL-10, which had been induced in naive $CD4^+$ T cell cultures. Given these data, γδ T-APCs were able to promote both Tc1 and Tc2 responses in naive $CD8^+$ T cells, but were incapable of alternative lineage induction under the conditions examined.

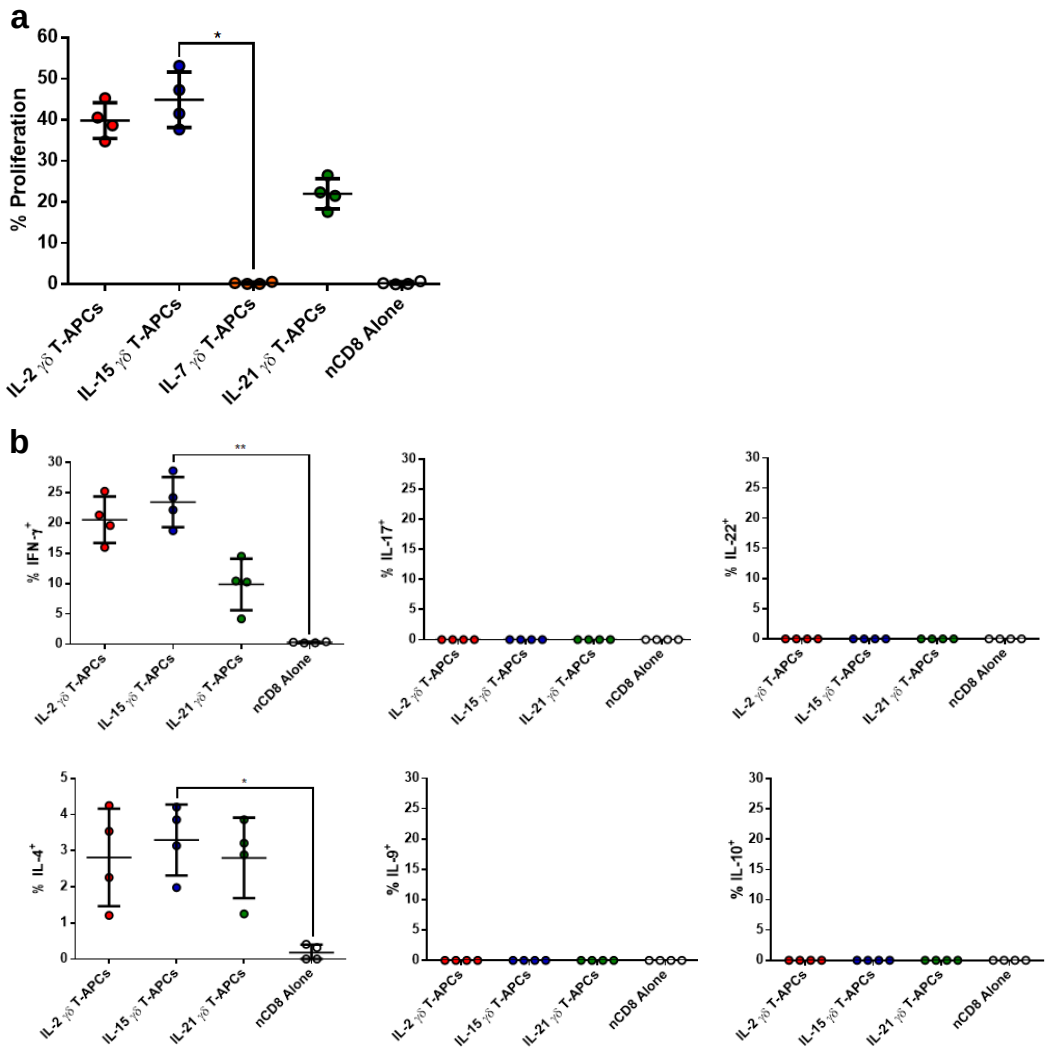

Figure 4.15 – Induction of naive CD8$^+$ T cell responses by γδ T-APCs. (a) Comparison of naive CD8$^+$ T cell proliferation induced by γδ T-APCs, generated under different conditions. Naive CD8$^+$ T cells (CD8$^+$CD45RA$^+$CCR7$^+$) were cultured with IL-2, IL-15, or IL-21 γδ T-APCs, at a 1:10 APC:responder ratio for five days. Proliferation of naive CD8$^+$ T cells was determined by assessing CFSE dilution by flow cytometry, and percentages of proliferating, CFSE^{lo} cells were determined. **(b)** Comparison of intracellular cytokine production by naive CD8$^+$ T cells, polarised by γδ T-APCs. Naive CD8$^+$ T cells were cultured with relevant APC subtypes at 1:10 APC:responder ratios for 9 days. Intracellular cytokine expression was assessed by flow cytometry after cell restimulation at day 9 of culture. Percentages of cytokine positive cells were determined by gating of live, single, CD3$^+$CD8$^+$Vγ9$^-$ cells. Data points represent individual healthy donors from individual experiments. Horizontal lines display means of data sets. Error bars display standard deviation of samples. Statistical significance was determined using the Friedman test, followed by the Dunn's multiple comparison test, *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001, ns=not significant.

4.7 - Polarisation of naive CD4$^+$ T cells by expanded γδ T-APCs

While the data presented so far have focused on the ability of freshly isolated Vγ9Vδ2 T cells to function as APCs after short term activation, several studies characterised the ability of long term activated cells, or 'expanded' Vγ9Vδ2 T cells to exhibit APC capabilities[325,326,329]. While those studies mainly examined the ability of expanded Vγ9Vδ2 T cells to take up antigen and induce CD8$^+$ T cell activation, little is known of the CD4$^+$ T cell response to expanded Vγ9Vδ2 T-APCs. Further to this, it is unclear whether the differential polarising abilities of IL-2, IL-15, and IL-21 γδ T-APCs are maintained during long term culture of APCs.

In order to investigate the ability of expanded γδ T-APCs to polarise CD4$^+$ T cell responses, long term cultures of Vγ9Vδ2 T cells were set up[325]. Vγ9Vδ2 T cells were stimulated with HMB-PP in the presence of IL-2, IL-15, IL-7, or IL-21, or in the absence of cytokines, for 14 days to expand the cells, upon which γδ T-APCs were restimulated, assessed for phenotypic markers, and used in CD4$^+$ T cell polarisation assays. Of note, expanded Vγ9Vδ2 T cells displayed minimum viability of 70% live cells at day 3, 50% at day 5, 65% at day 9, and 75% at day 14, consistent across all conditions examined. IL-2, IL-15, and IL-7 were able to maintain γδ T-APC proliferation and survival over a 14 day culture period. Similarly to 3-day generated APCs, both IL-2 and IL-15 supported expression of the APC markers HLA-DR, CD86 and CD70 after long term culture of cells (Figure 4.16a). In contrast, IL-7 did not support significant expression of these markers. Vγ9Vδ2 T cells cultured in the presence of IL-21 or in the absence of cytokines were unable to survive the 14 day culture period, with poor viability of cells by this timepoint, and as such no data is available for these conditions. In addition to assessing the expression of APC markers on expanded cells, the expression of the memory markers CD45RA and CD27 was also examined (Figure 4.16b). In the presence of IL-15, γδ T-APCs displayed a predominant CD45RA$^-$ CD27$^-$ T_{EM} phenotype. Expanded γδ T-APCs generated with IL-2 displayed an indistinguishable memory phenotype compared to IL-15 expanded cells. Highlighting the differences between IL-7 and other cytokines, IL-7 expanded cells displayed a more dominant T_{CM} phenotype, the majority of cells expressing CD27 but not CD45RA, whereas a small population comprised T_{EM} cells. These findings are in agreement with previous reports on the ability of IL-7 and IL-2/IL-15 to expand different memory subsets of Vγ9Vδ2 T cells[277].

When expanded γδ T-APCs were utilised in naive CD4$^+$ T cell polarisation assays, the results reflected those observed with 3-day generated γδ T-APCs. In terms of

the induction of naive $CD4^+$ T cell proliferation, IL-2 and IL-15 expanded γδ T-APCs induced high levels of proliferation, whereas IL-7 expanded γδ T-APCs were unable to induce proliferative responses (Figure 4.16c). The levels of proliferation induced by IL-2 and IL-15 expanded γδ T-APCs were marginally lower than those observed previously with 3-day generated γδ T-APCs. When intracellular cytokine expression was assessed, significant induction of IFN-γ^+ and IL-22^+ cells were identified, in the absence of IL-17 expressing cells. Both IL-2 and IL-15 expanded γδ T-APCs induced similar levels of IFN-γ, whereas IL-22 induction by IL-15-expanded γδ T-APCs exceeded that induced by IL-2-expanded γδ T-APCs. In terms of IL-4, low levels of Th2 cells were apparent in both culture conditions, and only minimal IL-10 or IL-9 expression was found (data not shown). Given these findings, it appears expanded γδ T-APCs do indeed maintain their ability to induce and polarise naive $CD4^+$ T cell responses.

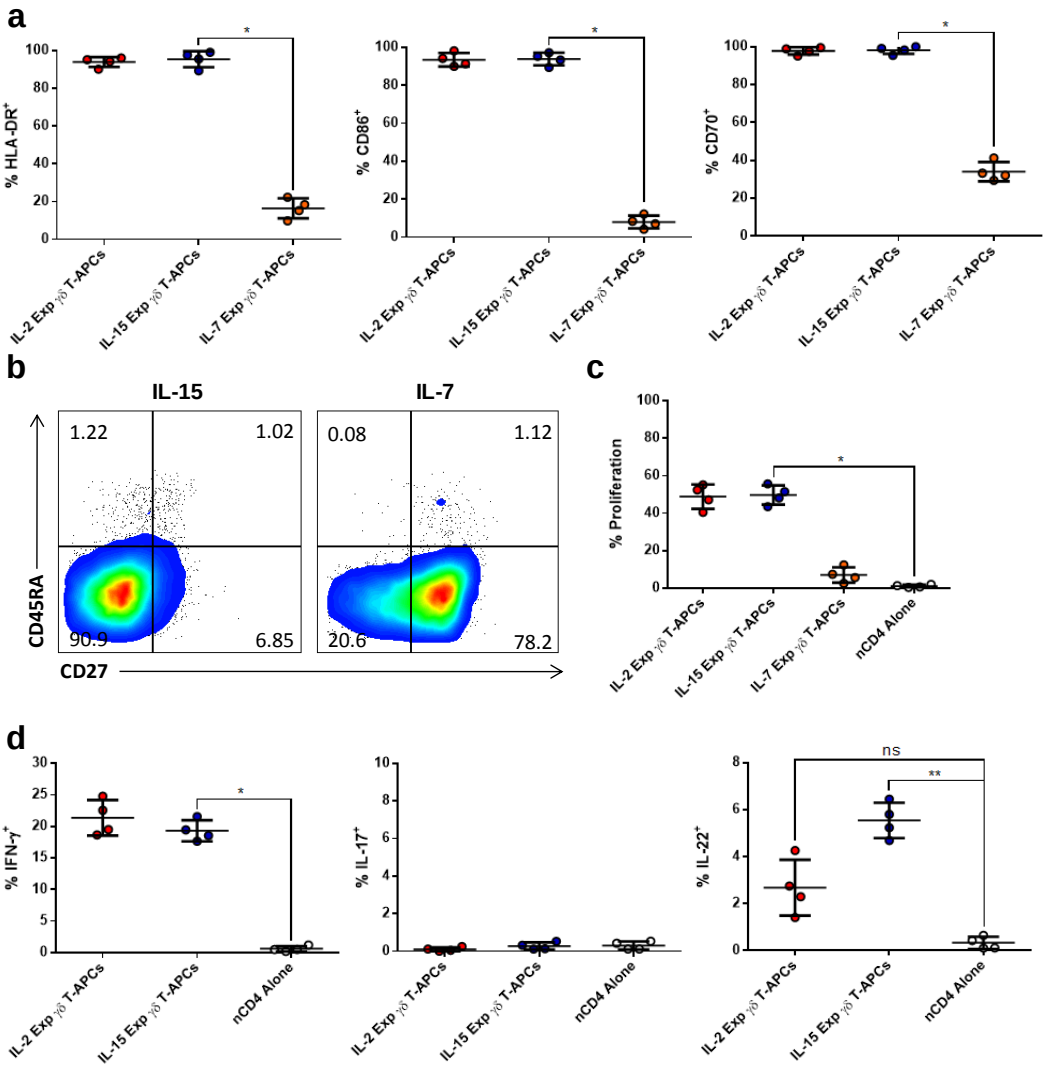

Figure 4.16 – Ability of expanded γδ T-APCs to induce naive CD4$^+$ T cell responses. (a) Expression of APC markers by Vγ9Vδ2$^+$ T cells expanded with zoledronate and common γ-chain cytokines for 14 days, and subsequently restimulated for 24 hours with 1 nM HMB-PP. Percentages of APC marker-expressing cells were determined after restimulation by flow cytometry. Percentage of HLA-DR$^+$ cells were determined by gating on live, single, Vγ9$^+$ T cells. CD86 and CD70 positive cells were determined by gating on live, single, HLA-DR$^+$Vγ9$^+$ T cells. **(b)** Expression of γδ T cell memory markers by IL-15 and IL-7 expanded Vγ9Vδ2$^+$ T cells. Expanded cells were stained for CD45RA and CD27 at day 14 of culture and expression of markers was assessed by flow cytometry. Plots are representative of 3 individual donors. Numbers on graphs display percentages of cells. **(c)** Comparison of naive CD4$^+$ T cell proliferation induced by expanded γδ T-APCs, generated under different conditions. Naive CD4$^+$ T cells were cultured with IL-2, IL-15, or IL-7 expanded γδ T-APCs, at a 1:10 APC:responder ratio for five days. Proliferation of naive CD4$^+$ T cells was determined by assessing CFSE dilution by flow cytometry, and percentages of proliferating, CFSE^{lo} cells were determined. **(d)** Comparison of naive CD4$^+$ T cell intracellular cytokine expression after polarisation by expanded γδ T-APCs. Naive CD4$^+$ T cells were cultured with IL-2 or IL-15 expanded γδ T-APCs at 1:10 APC:responder ratios for 9 days. Intracellular cytokine production was assessed by flow cytometry after cell restimulation at day 9 of culture. Percentages of cytokine positive cells were determined by gating of live, single, CD3$^+$CD4$^+$Vγ9$^-$ cells. Data points represent individual healthy donors from individual experiments. Horizontal bars display means of data sets. Error bars display standard deviation of samples. Statistical significance was determined using the Friedman test, followed by the Dunn's multiple comparison test, *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001, ns=not significant.

4.8 - Antigen-specific Polarisation of Autologous CD4$^+$ T Cells

With the demonstration that γδ T-APCs are able to differentially polarise naive and memory CD4$^+$ T cell responses in mixed lymphocyte reaction assays, experiments were next designed to replicate these findings in autologous culture systems. Mixed lymphocyte reactions are useful assays to determine the polarisation of naive CD4$^+$ T cells by APCs, due to the fact that approximately 10% of naive CD4$^+$ T cells will be alloreactive to allogeneic APCs. However, an allogeneic system may not be reflective of the types of responses which would occur *in vivo*, when antigen specific responses are induced. One limitation of autologous, antigen-specific systems utilising naive cells as responders is that the frequency of each antigen-specific cell is low, making accurate detection of responses difficult. To counter this limitation, two autologous systems were utilised to assess CD4$^+$ T cell 'antigen specific' responses to γδ T-APCs. Firstly, the bacterial superantigen TSST-1, which cross-links MHC class II molecules with TCRs expressing a Vβ2 chain[160], was used to stimulate a fraction of naive CD4$^+$ T cells upon encounter with γδ T-APCs. Alternatively, the complex antigen PPD was utilised to stimulate specific memory CD4$^+$ T cell responses in healthy, *Mycobacterium bovis* Bacillus Calmette–Guérin (BCG)-vaccinated donors[322]. Both of these culture systems have been widely used to assess CD4$^+$ T cell responses previously.

Figure 4.17 displays naive CD4$^+$ T cell responses to autologous APCs labelled with TSST-1 superantigen. Given that approximately 10% of naive CD4$^+$ T cells in peripheral blood would express a TCR containing a Vβ2 chain before stimulation with APCs, the percentages of Vβ2$^+$ naive CD4$^+$ T cells were assessed before and after co-culture with γδ T-APCs. Figure 4.17a displays representative flow cytometry plots of Vβ2 staining on naive CD4$^+$ T cell populations prior to and following co-culture with IL-15 γδ T-APCs, and Figure 4.17b displays a summary of Vβ2$^+$ cell percentages after all γδ T-APC co-cultures. Similarly to induction of proliferation, IL-2 and IL-15 γδ T-APC co-culture resulted in the highest levels of Vβ2$^+$ cell expansion across a range of TSST-1 concentrations, with IL-21 γδ T-APC co-culture resulting in a more limited expansion of specific cells. IL-7 γδ T-APCs were unable to expand Vβ2 TCR expressing naive CD4$^+$ T cells, and as such, percentages did not differ to unstimulated cells. Naive CD4$^+$ T cells were also cultured with TSST-1 in the absence of any APCs, where no expansion of specific cells was detected, highlighting the requirement for MHC class II-expressing APCs in this culture system.

Having identified Vβ2$^+$ specific expansion of cells in response to γδ T-APCs, the intracellular cytokine expression of such cells was determined. Figure 4.17c displays expression of IFN-γ, IL-22 and IL-10 induced by γδ T-APCs at two different concentrations of TSST-1. Similarly to results obtained in mixed lymphocyte reactions, IL-2 and IL-15 γδ T-APCs favoured potent IFN-γ responses, with IL-15 γδ T-APCs also supporting large populations of IL-22$^+$ cells. In contrast, IL-21 γδ T-APCs favoured IL-10 expression by naive CD4$^+$ T cell responders. A comparison of 10 ng/ml and 1 ng/ml TSST-1 revealed that IFN-γ induction was supported by higher concentrations of superantigen, whereas both IL-22 and IL-10 were increased at lower TSST-1 concentrations. Upon comparison of γδ T-APC mediated TSST-1 polarisation with alternative APC subsets, similar patterns of cytokine induction were observed as with mixed lymphocyte reaction culture systems. Of note, no IL-17 was detected in γδ T-APC-polarised cells, and low levels of Th2 cells were also identified (data not shown). In addition to these experiments with naive CD4$^+$ T cells, memory CD4$^+$ T cell responses to autologous, TSST-1 presenting γδ T-APCs were also assessed. Here, intracellular cytokine profiles did not differ significantly from the patterns obtained with memory CD4$^+$ T cells in MLR assays.

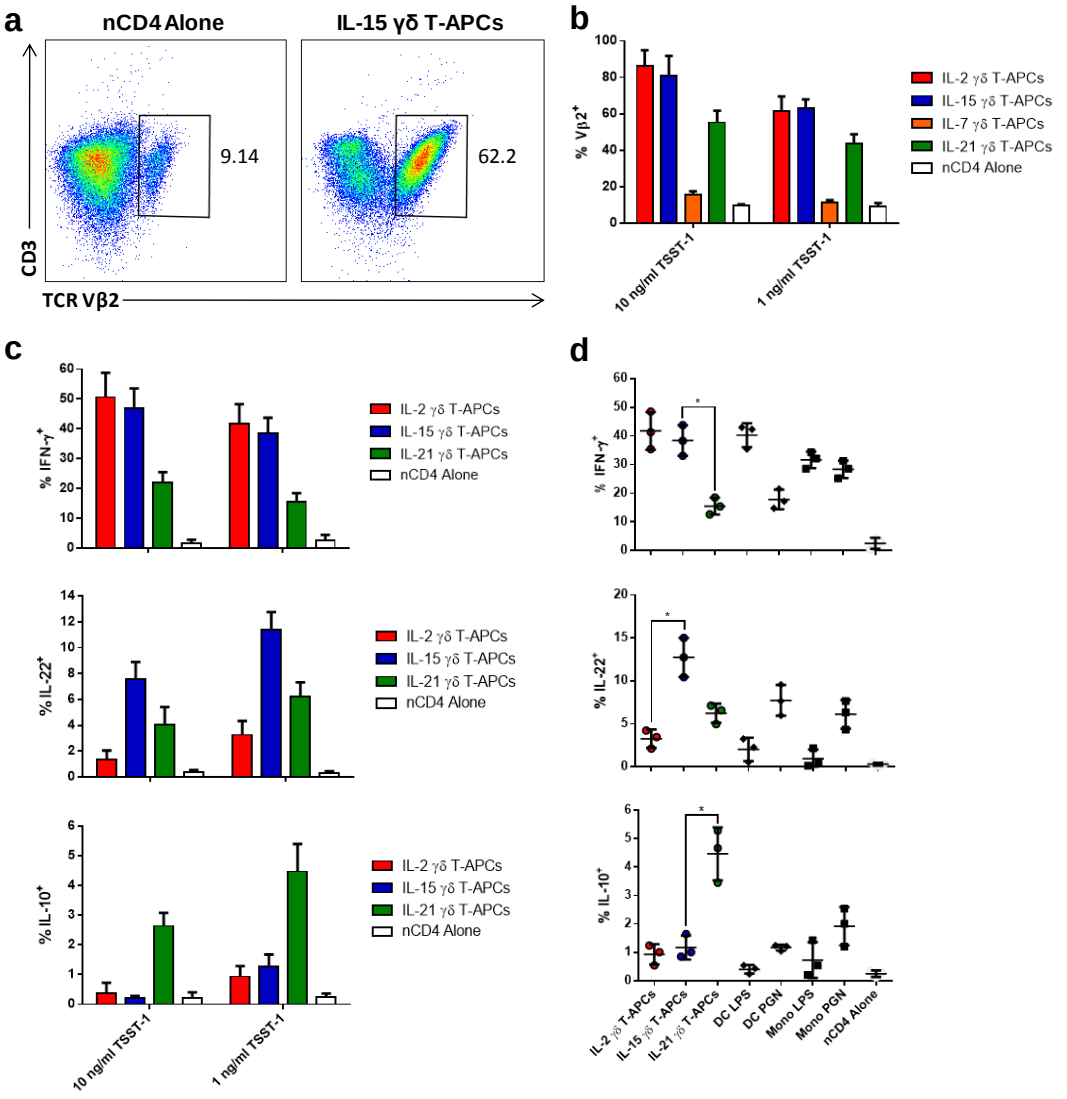

Figure 4.17 – Induction of naive CD4$^+$ T cell responses by TSST-1-presenting autologous APCs. (a) Representative flow cytometry plots displaying expression of TCR Vβ2 by naive CD4$^+$ T cells cultured either alone or with autologous IL-15 γδ T-APCs at a 1:10 APC:responder ratio. γδ T-APC cultures were supplemented with 1 ng/ml TSST-1 prior to co-culture, and were subsequently cultured for 9 days with naive CD4$^+$ T cells. Cells were then restimulated and stained for expression of TCR Vβ2 and intracellular cytokines. Percentages of positive cells were determined by flow cytometry. (b) Percentages of Vβ2$^+$CD4$^+$ T cells after co-culture with autologous γδ T-APCs for 9 days, labelled with either 10 ng/ml or 1 ng/ml TSST-1 prior to co-culture. Displaying data from 3 healthy donors from 3 individual experiments. (c) Expression of intracellular cytokines by naive CD4$^+$ T cells polarised by autologous γδ T-APCs, labelled with either 10 ng/ml or 1 ng/ml TSST-1. Displaying data from 3 healthy donors from 3 individual experiments. (d) Expression of intracellular cytokines by naive CD4$^+$ T cells polarised by autologous APCs, labelled with 1 ng/ml TSST-1. Data points represent individual healthy donors. Horizontal lines display means of data sets. Error bars display standard deviation of samples. Statistical significance was determined using the Kruskal-Wallis test, followed by the Dunn's multiple comparison test, *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001, ns=not significant.

Next, the antigen-specific memory response to PPD-presenting γδ T-APCs was investigated (Figure 4.18). While the naive $CD4^+$ T cell pool contains only very few PPD-specific cells, the memory compartment from BCG-vaccinated contains a sizeable population of PPD-specific $CD4^+$ T cells, thereby allowing the characterisation of antigen-specific memory responses in humans. PPD was added to γδ T-APCs for 24 hours to allow for antigen uptake, processing and presentation. Subsequently, these antigen-loaded γδ T-APCs were co-cultured with autologous memory $CD4^+$ T cells, and responding cells were assessed for proliferation and intracellular cytokine expression. As controls, $CD4^+$ T cells were cultured with PPD in the absence of APCs.

As shown in Figure 4.18a, IL-2, IL-15 and IL-21 γδ T-APCs were all able to induce proliferation of autologous memory $CD4^+$ T cells. Memory cells also displayed low levels of proliferation in response to IL-7 γδ T-APCs and in conditions where memory $CD4^+$ T cells were cultured with PPD in the absence of APCs. As observed, PPD was able to induce limited cell activation in purified cultures of $CD4^+$ T cells from BCG-vaccinated individuals, but responses were significantly enhanced in the presence of APCs. As such, the proliferation observed with IL-7 γδ T-APCs was likely due to the presence of PPD, rather than any APC activity by these cells. No memory $CD4^+$ T cell proliferation was observed when cultured with autologous γδ T-APCs in the absence of PPD (data not shown).

Upon assessing the intracellular cytokine production of responding memory cells, IFN-γ was predominantly expressed by cells co-cultured with IL-2 and IL-15 γδ T-APCs, as well as with LPS-treated DCs and monocyte controls (Figure 4.18b). Again, IL-22 production was favoured by IL-15 γδ T-APCs, and IL-10 by IL-21 γδ T-APCs. Interestingly, the increased levels of IL-22 and IL-10 detected in previous systems were less pronounced utilising this PPD culture system. This may be due to the inherent effects of PPD on the responding cells, which may favour non-IL-22 and non-IL-10 responses at the expense of other lineages. Alternatively, Th1 clones for example may possess relatively stable phenotypes which are more restricted in their plasticity. Regardless, the overall patterns observed resembled those obtained with other culture systems. Of note, Th2 populations were detected at low frequencies in all γδ T-APC co-cultures (data not shown).

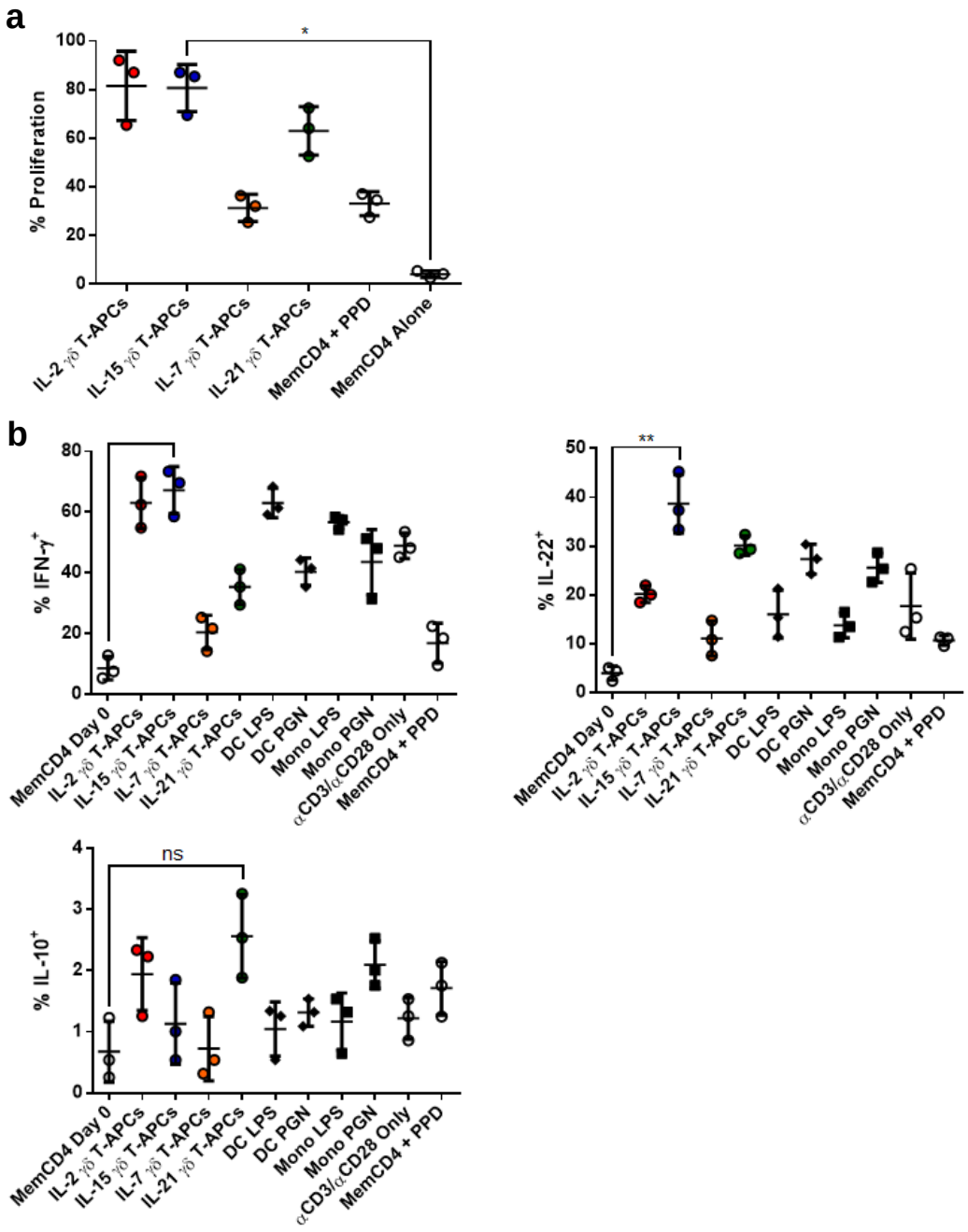

Figure 4.18 – Induction of PPD-specific memory CD4$^+$ T cell responses by PPD-presenting autologous APCs. (a) Proliferation of memory CD4$^+$ T cells cultured with γδ T-APCs for 5 days, in the presence of 1 µg/ml PPD. Proliferation of memory CD4$^+$ T cells was determined by assessing CFSE dilution by flow cytometry, and percentages of proliferating, CFSE^{lo} cells were determined. **(b)** Expression of intracellular cytokines by memory CD4$^+$ T cells polarised by autologous APCs, in the presence of 1 µg/ml PPD. Data points represent individual healthy donors. Horizontal lines display means of data sets. Error bars display standard deviation of samples. Statistical significance was determined using the Kruskal-Wallis test, followed by the Dunn's multiple comparison test, *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001, ns=not significant.

4.9 - Role of Cytokines in γδ T-APC Mediated CD4$^+$ T Cell Polarisation

As observed, γδ T-APCs are capable of driving naive and memory CD4$^+$ T cell responses *in vitro*, and are able to polarise distinct responses. The polarisation of naive CD4$^+$ T cells appears to depend on the cellular microenvironment that Vγ9Vδ2 T cells experience whilst undergoing APC generation. This observation led to three main findings; IL-2 and IL-15 γδ T-APCs polarise potent Th1 type responses, IL-15 γδ T-APCs also favour high levels of IL-22 production in responding cells, and IL-21 γδ T-APCs promote IL-10 responses in naive CD4$^+$ T cells. In addition, γδ T-APCs in general appear superior inducers of Th2 type responses over DCs. Given these observations, assays were next established to examine the mechanisms behind such polarisations.

The role of specific cytokines in the polarisation of naive CD4$^+$ T cells is well understood, with particular combinations promoting certain T helper lineages over others. As previously investigated , γδ T-APCs expressed markedly distinct repertoires of cytokines compared with dendritic cells or monocytes, with IFN-γ and TNF-α representing the predominant cytokines detected in γδ T-APC culture supernatants (Figure 3.17). However, Vγ9Vδ2 T cells are also capable of alternative cytokine expression, such as IL-4 under certain conditions[281,282], so the role of these cytokines could not be ruled out in the γδ T-APC-mediated polarisation of naive CD4$^+$ T cell responses. As such, γδ T-APC:naive CD4$^+$ T cell co-cultures were set up with the addition of blocking agents to various cytokines.

Due to the observations that IL-15 γδ T-APCs induced potent IFN-γ and IL-22 type responses in naive CD4$^+$ T cells, and IL-21 γδ T-APCs promoted the highest levels of IL-10 expression, these two cell populations were utilised for all subsequent assays, to investigate the mechanisms behind the induction of each individual cytokine. In identical experiments to those previously presented, 3-day generated γδ T-APCs were co-cultured at 1:10 ratios with naive CD4$^+$ T cells for a period of 9 days, in the presence or absence of blocking agents or isotype controls, after which the cultures were restimulated and intracellular cytokine expression was assessed (Figure 4.19). Figure 4.19a displays the intracellular cytokine production of naive CD4$^+$ T cells polarised by IL-15 γδ T-APCs. Using αIFN-γ neutralising antibodies, the percentage of IFN-γ-expressing CD4$^+$ T cells was significantly reduced as compared to controls. Small increases in the expression of IL-22 and IL-4 were also observed in responder populations, however these differences were not significant. In contrast, use of soluble TNF receptor to block TNF-α activity appeared to have

only a minimal effect on the expression of IFN-γ and IL-4 by naive $CD4^+$ T cells, but had a significant effect on the IL-22 induction, with IL-22 expression levels reduced in this condition. Blockade of IL-4 showed a trend to lower the induction of IL-4 expressing responder cells, although this decrease was not significant, due to the variability in expression levels. Blocking antibodies to IL-4 and IL-6 appeared to play no role in the polarisation of naive responses towards IFN-γ and IL-22.

When the polarisation of IL-10 responses was assessed in response to IL-21 γδ T-APCs, no significant effects could be observed with any of the cytokine blocking agents examined, indicating a lack of cytokine involvement in this polarisation pathway, at least in those assessed (Figure 4.19b). Of note, no significant effects on naive $CD4^+$ T cell proliferation or viability were observed upon blocking of cytokines (data not shown).

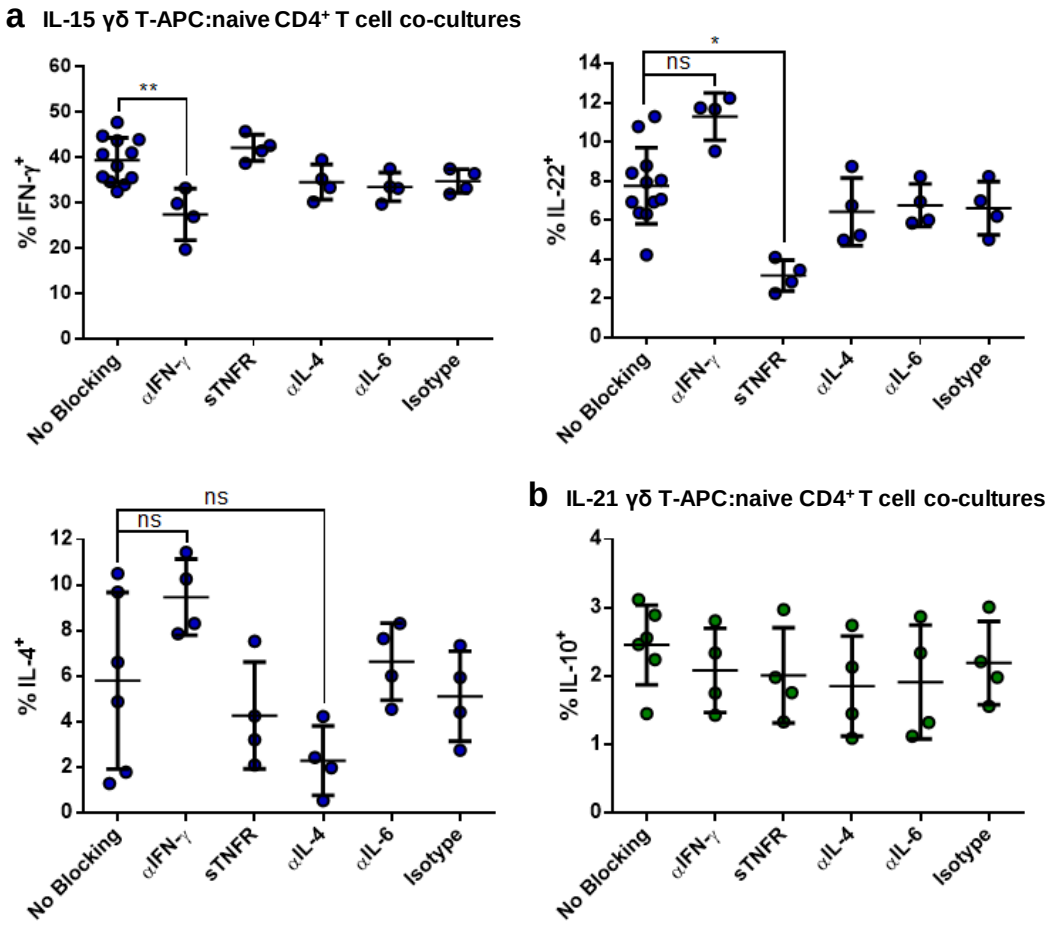

Figure 4.19 – Blockade of cytokines in γδ T-APC mediated polarisation of naive CD4$^+$ T cell intracellular cytokine production. (a) Naive CD4$^+$ T cells were cultured at 10:1 ratios with IL-15 γδ T-APCs for 9 days, in the presence or absence of blocking antibodies or isotype controls. At day 9 of culture, cultures were restimulated and intracellular cytokine production was assessed by flow cytometry. **(b)** Naive CD4$^+$ T cells were cultured with IL-21 γδ T-APCs for 9 days, in the presence or absence of blocking antibodies or isotype controls. At day 9 of culture, cultures were restimulated and intracellular cytokine production was assessed by flow cytometry. Percentages of cytokine positive cells were determined by gating on live, single, CD3$^+$CD4$^+$Vγ9$^-$CFSE^{lo} cells. Data points represent individual healthy donors. Horizontal lines display means of data sets. Error bars display standard deviation of samples. Statistical significance was determined using the Friedman test, followed by the Dunn's multiple comparison test, *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001, ns=not significant.

When cell culture supernatants were examined for secretion of cytokines by polarised naive CD4$^+$ T cells, similar observations could be made (Figure 4.20). Blockade of IFN-γ significantly reduced IFN-γ secretion by responder cells, and inhibition of TNF-α led to a reduction in IL-22 secretion (Figure 4.20a). Blockade of

IL-4 did in fact cause a significant reduction in IL-4 secretion by responder cells. Similarly to intracellular expression, no inhibition of IL-10 could be observed in IL-21 γδ T-APC co-cultures (Figure 4.20b), and IL-6 appeared to have no role in polarisation of cytokine secretion by naive $CD4^+$ T cells.

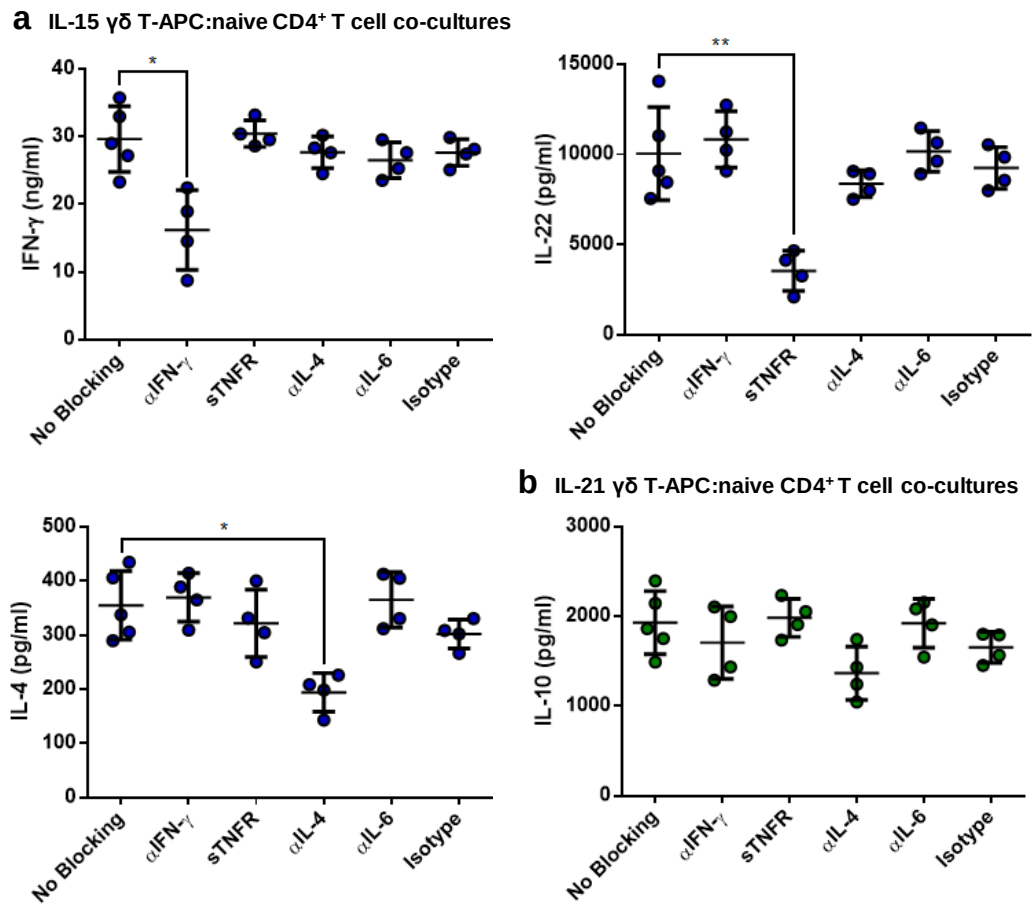

Figure 4.20 - Blockade of cytokines in γδ T-APC mediated polarisation of naive CD4$^+$ T cell cytokine secretion. (a) Naive CD4$^+$ T cells were cultured at 10:1 ratios with IL-15 γδ T-APCs for 9 days, in the presence or absence of blocking antibodies or isotype controls. At day 9 of culture, cultures were restimulated for 24 hours, after which supernatants were obtained and cytokine production was assessed by ELISA. **(b)** Naive CD4$^+$ T cells were cultured with IL-21 γδ T-APCs for 9 days, in the presence or absence of blocking antibodies or isotype controls. At day 9 of culture, cultures were restimulated for 24 hours, after which supernatants were obtained and cytokine production was assessed by ELISA. Data points represent individual healthy donors. Horizontal lines display means of data sets. Error bars display standard deviation of samples. Statistical significance was determined using the Friedman test, followed by the Dunn's multiple comparison test, *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001, ns=not significant.

Given the significant effects of IFN-γ and TNF-α on the induction of IFN-γ and IL-22 responses, the expression of transcription factors was examined to identify whether these factors were similarly affected by polarising cytokines. Figure 4.21 displays the transcription factor expression in response to blockade of IFN-γ or TNF-α in IL-15 γδ T-APC co-cultures. In a similar manner to cytokine expression, blockade of IFN-γ in co-cultures resulted in reduced expression of Th1 master regulator *TBX21*

by responder cells, in comparison with untreated and isotype control conditions. However, given the variation in expression of *TBX21*, this reduction was not significant. Furthermore, expression of *AHR* was dependent on the action of TNF-α, with addition of sTNFR resulting in a marked decrease in the expression of the Th22 master transcription factor. Upon examining the expression of *RORC* and *GATA3*, no significant effects were observed upon blockade of either cytokine examined. Overall, expression of *TBX21* and *AHR* appeared to respond similarly to blockade of IFN-γ and TNF-α, as did IFN-γ and IL-22 expression by polarised naive $CD4^+$ T cells.

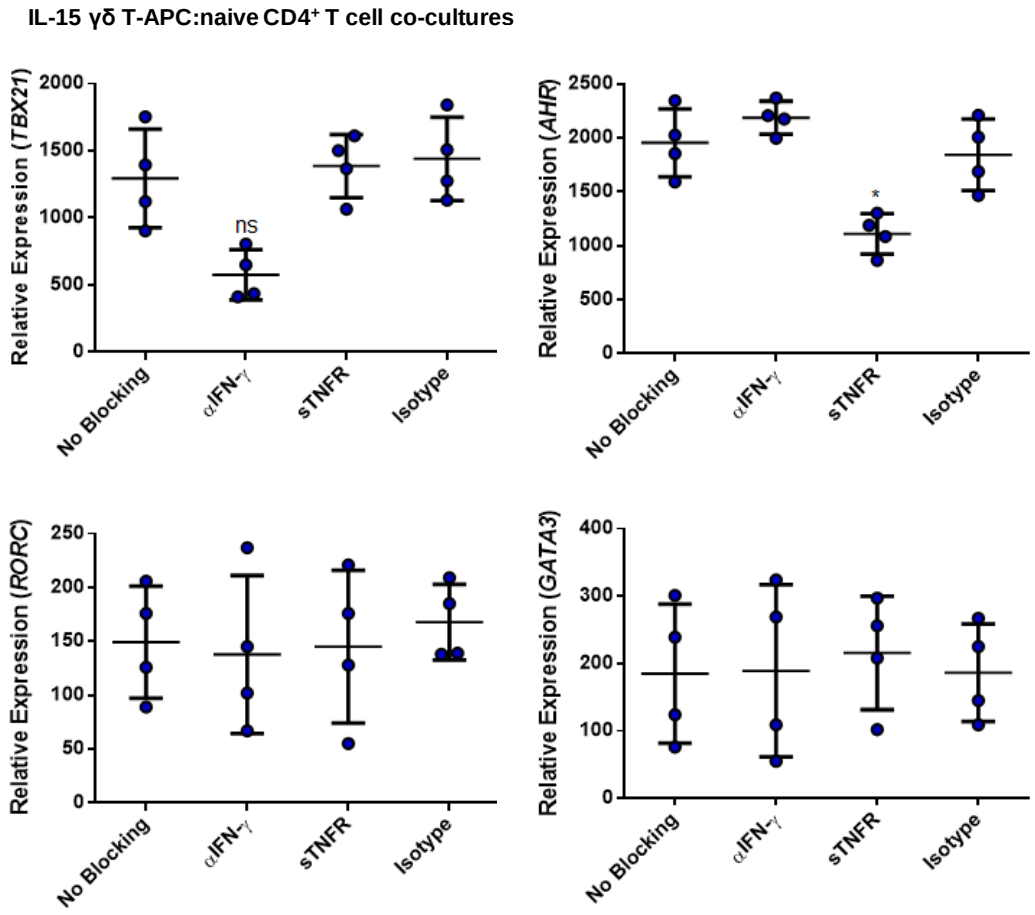

Figure 4.21 - Blockade of cytokines in γδ T-APC mediated polarisation of naive CD4$^+$ T cell transcription factor expression. Naive CD4$^+$ T cells were cultured at 10:1 ratios with IL-15 γδ T-APCs for 9 days, in the presence or absence of blocking antibodies or isotype controls. At day 9 of culture, cultures were assessed for transcription factor expression by real-time PCR. Relative expression was determined in reference to naive CD4$^+$ T cell control. Data points represent individual healthy donors. Horizontal lines display means of data sets. Error bars display standard deviation of samples. Statistical significance was determined using the Friedman test, followed by the Dunn's multiple comparison test, *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001, ns=not significant.

In summary, the role of cytokines appeared to have prominent roles in the γδ T-APC mediated polarisation of naive CD4$^+$ T cells. However, these cytokines alone did not account for the complete polarisation of responses, indicating other polarising factors may be involved.

4.10 - Role of Co-stimulatory Molecules in γδ T-APC Mediated $CD4^+$ T Cell Polarisation

The range of cytokines produced by γδ T-APCs appeared more limited than that observed with DCs and monocytes (Figure 3.17), despite the γδ T-APC potential to polarise different T helper lineages. In addition, the roles of IFN-γ, TNF-α and IL-4 did not account for the total polarisation of naive $CD4^+$ T cells observed, as evidenced by the fact that neutralisation of IFN-γ only had a partial effect on the polarisation toward Th1 cells and by the inability to block the generation of IL-10 producing $CD4^+$ T cells. These observations together suggested that other molecules might be important in γδ T-APC mediated $CD4^+$ T cell polarisation. As γδ T-APCs have been shown to express a wide range of co-stimulatory molecules in this study, the roles of each co-stimulatory molecule in γδ T-APC:naive $CD4^+$ T cell co-cultures were next investigated.

In similar experiments to the blocking of γδ T-derived cytokines, γδ T-APC co-cultures were set up with naive $CD4^+$ T cells, and cell proliferation, viability, cytokine production, and transcription factor expression were assessed. Blocking antibodies to each co-stimulatory molecule were added to γδ T-APC cultures for 1 hour prior to co-culture, then cells were washed and used in assays. Figure 4.22 displays the proliferation and viability of naive $CD4^+$ T cells after 5-day co-culture with IL-15 γδ T-APCs that had been pre-incubated with blocking antibodies to co-stimulatory molecules or isotype controls. In terms of naive $CD4^+$ T cell proliferation (Figure 4.22a), a significant reduction was observed in the presence of αCD80 and αCD86 blocking antibodies. In addition, blockade of CD48 led to an inhibition of naive $CD4^+$ T cell proliferation. Blockade of a range of other co-stimulatory molecules (CD70, ICOSL, 4-1BBL, OX40L, CD40) did not appear to cause a significant effect on responder cell proliferation. When viability was assessed, almost all treatments did not significantly affect cell viability as compared to controls (Figure 4.22b). However, blocking of CD48, expressed by IL-15 γδ T-APCs, led to a significant reduction in cell viability of responder cells. Similar effects on cell proliferation and viability were observed in assays utilising IL-2 or IL-21 γδ T-APCs (data not shown).

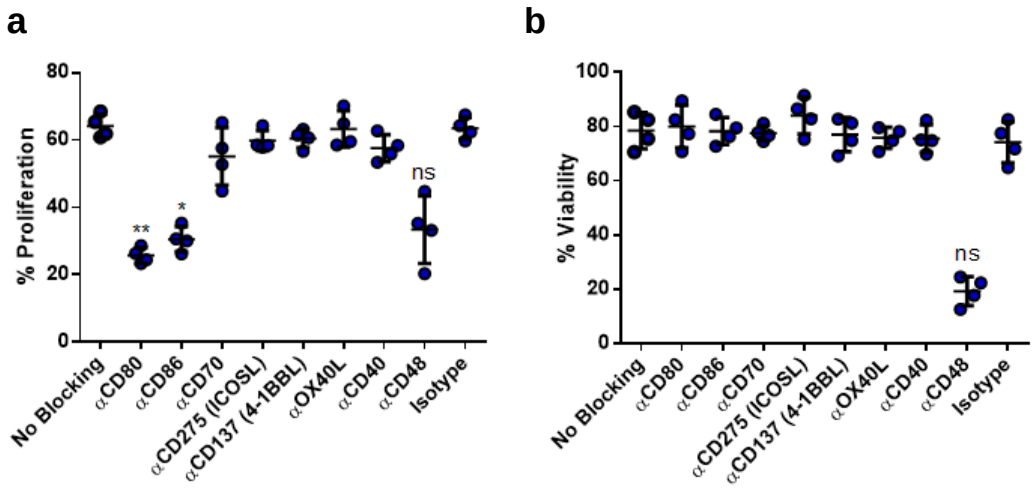

Figure 4.22 – Effects of γδ T-APC-expressed costimulatory molecule blockade on induction of naive CD4$^+$ T cell responses. Blockade of costimulatory molecules expressed by IL-15 γδT-APCs in naive CD4$^+$ T cell co-cultures. **(a)** Naive CD4$^+$ T cells were cultured at 10:1 ratios with IL-15 γδT-APCs for 5 days, in the presence or absence of blocking antibodies or isotype controls. At day 5 of culture, cells were assessed for proliferation by assessing CFSE dilution by flow cytometry. Percentages of proliferating, CFSE^{low} cells were determined. **(b)** Naive CD4$^+$ T cells were cultured with IL-15 γδT-APCs for 9 days, in the presence or absence of blocking antibodies or isotype controls. At day 9 of culture, cultures were restimulated and percentage viability (live cells) were determined based on live/dead staining by flow cytometry. Data points represent individual healthy donors. Error bars display standard deviation of samples. Statistical significance was determined using the Kruskal-Wallis test (non-parametric, one-way ANOVA), *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001, ns=not significant. Significance displayed in comparison to No Blocking control.

Subsequent assays focused on the role of co-stimulatory molecules in the induction of IFN-γ, IL-4, IL-22, and IL-10 in naive CD4$^+$ T cells. Figure 4.23 displays representative flow cytometry plots of intracellular cytokine staining and CFSE staining of naive CD4$^+$ T cells, polarised by IL-15 γδ T-APCs in the presence or absence of several blocking agents. Data displayed are for CD70 and ICOSL blocking, which appeared to show the most significant effects on CD4$^+$ T cell cytokine expression. In the presence of anti-CD70 blocking antibodies, naive CD4$^+$ responder cells expressed markedly lower levels of IFN-γ upon restimulation, indicating a crucial contribution of CD70 signalling to the ability of IL-15 γδ T-APCs to promote Th1 responses (Figure 4.23a). This reduction in IFN-γ expression was identified in the proportion of cells which had undergone proliferation, reflected by dilution of CFSE staining, with CFSE-stained cells expressing no IFN-γ in either

condition. In response to ICOSL blockade, a significant reduction of IL-22 responses in responder cells was observed (Figure 4.23b). Similarly to IFN-γ, the IL-22 expression was observed in CFSE negative cells, highlighting the requirement for proliferation and response to γδ T-APCs to express these cytokines. Given that previous stainings of IL-22 and IFN-γ highlighted two populations of IL-22^{+} cells, either expressing this cytokine alone or in combination with IFN-γ, co-stainings of these cytokines are displayed in Figure 4.23c. In the presence of ICOSL blocking antibodies, the reduction in IL-22 expression was observed in both the IFN-γ^{+} and IFN-γ^{-} populations, and as such affected both Th1 and Th22 differentiation. IL-17 was not expressed by responding CD4^{+} T cells upon blockade of any co-stimulatory pathway investigated.

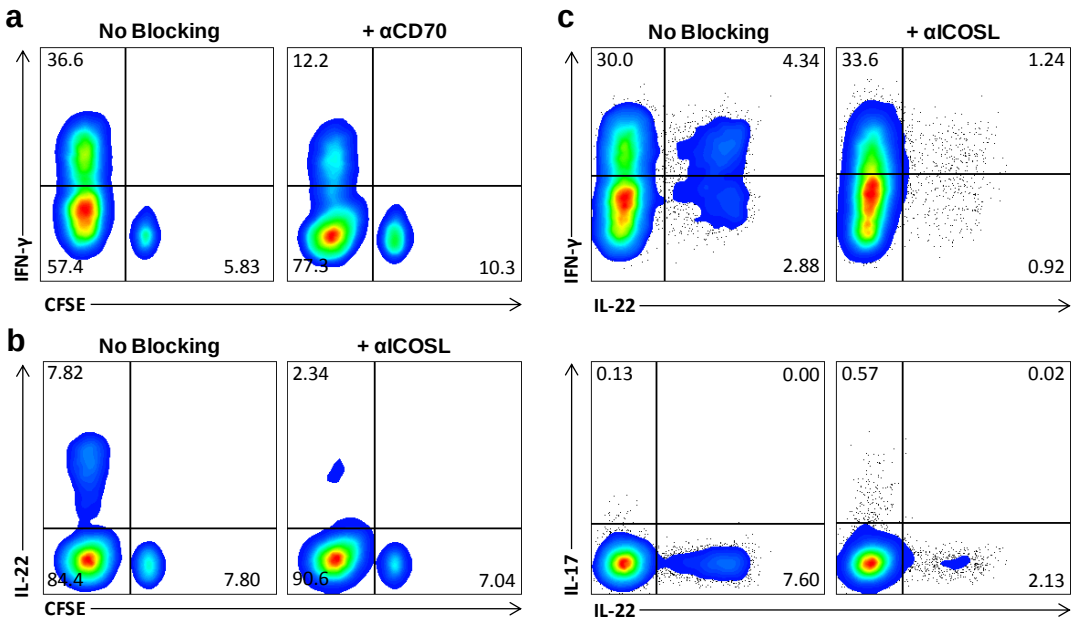

Figure 4.23 – Effects of γδ T-APC-expressed CD70 and ICOSL blockade on intracellular cytokine production by naive CD4$^+$ T cells. (a) Representative flow cytometry plots displaying intracellular expression of IFN-γ and CFSE staining in naive CD4$^+$ T cells, polarised by IL-15 γδ T-APCs either labelled or unlabelled with CD70 blocking antibodies. CFSE-stained naive CD4$^+$ T cells were cultured with IL-15 γδ T-APCs for 9 days, and subsequently restimulated at day 9 for analysis of intracellular cytokine expression by flow cytometry. **(b)** Representative flow cytometry plots displaying intracellular expression of IL-22 and CFSE staining in naive CD4$^+$ T cells, polarised by IL-15 γδ T-APCs either labelled or unlabelled with ICOSL blocking antibodies. CFSE-stained naive CD4$^+$ T cells were cultured with IL-15 γδ T-APCs for 9 days, and subsequently restimulated at day 9 for analysis of intracellular cytokine expression by flow cytometry. **(c)** Representative flow cytometry plots displaying intracellular expression of IFN-γ, IL-17, and IL-22 in naive CD4$^+$ T cells, polarised by IL-15 γδ T-APCs either labelled or unlabelled with ICOSL blocking antibodies. CFSE-stained naive CD4$^+$ T cells were cultured with IL-15 γδ T-APCs for 9 days, and subsequently restimulated at day 9 for analysis of intracellular cytokine expression by flow cytometry. Numbers on graphs display percentages of positive cells. Gated on live, single, CD3$^+$CD4$^+$Vγ9$^-$ cells. Representative of 4 individual γδ T cell donors from 4 individual experiments.

Figure 4.24 displays a summary of the intracellular expression of IFN-γ, IL-22, IL-4, and IL-10 by naive CD4$^+$ T cells polarised by IL-15 or IL-21 γδ T-APCs, in the presence or absence of blocking antibodies. In response to IL-15 γδ T-APCs (Figure 4.24a), blockade of co-stimulatory molecules displayed a range of effects on expression of each cytokine. CD80 and CD86 appeared to play similar roles in naive CD4$^+$ T cell polarisation, with blocking of these molecules leading to an overall

reduction in IFN-γ, IL-22, and IL-4. However, given the variation in these conditions, the reductions observed were not statistically significant. Blockade of CD70, as previously discussed, caused a significant reduction in the ability of IL-15 γδ T-APCs to promote IFN-γ type responses, and also appeared to increase the ability to polarise IL-4 and IL-22 type responses. In opposition to the effects of CD70, blockade of ICOSL resulted in a significant reduction of IL-22 expressing responder cells, although no real effects were observed on the induction of IFN-γ or IL-4. Blockade of other costimulatory molecules such as CD40 had only minimal effects on the expression of cytokines assessed. Blocking of CD48, in line with its reduction in $CD4^+$ T cell proliferation and viability, reduced the expression of all cytokines examined. The response of naive $CD4^+$ T cells to IL-21 γδ T-APCs, in terms of IL-10 intracellular expression, appeared to be unaffected by the majority of blocking agents examined (Figure 4.24b). Both CD80 and CD86 were essential for IL-10 induction by IL-21 γδ T-APCs, with blocking of either of these molecules almost abrogating the IL-10 response. CD48 function was also necessary in IL-10 expression, whereas no other molecules examined showed a significant effect on IL-10 induction. Similar responses to costimulatory molecule blockade were observed with memory $CD4^+$ T cell and γδ T-APC co-culture.

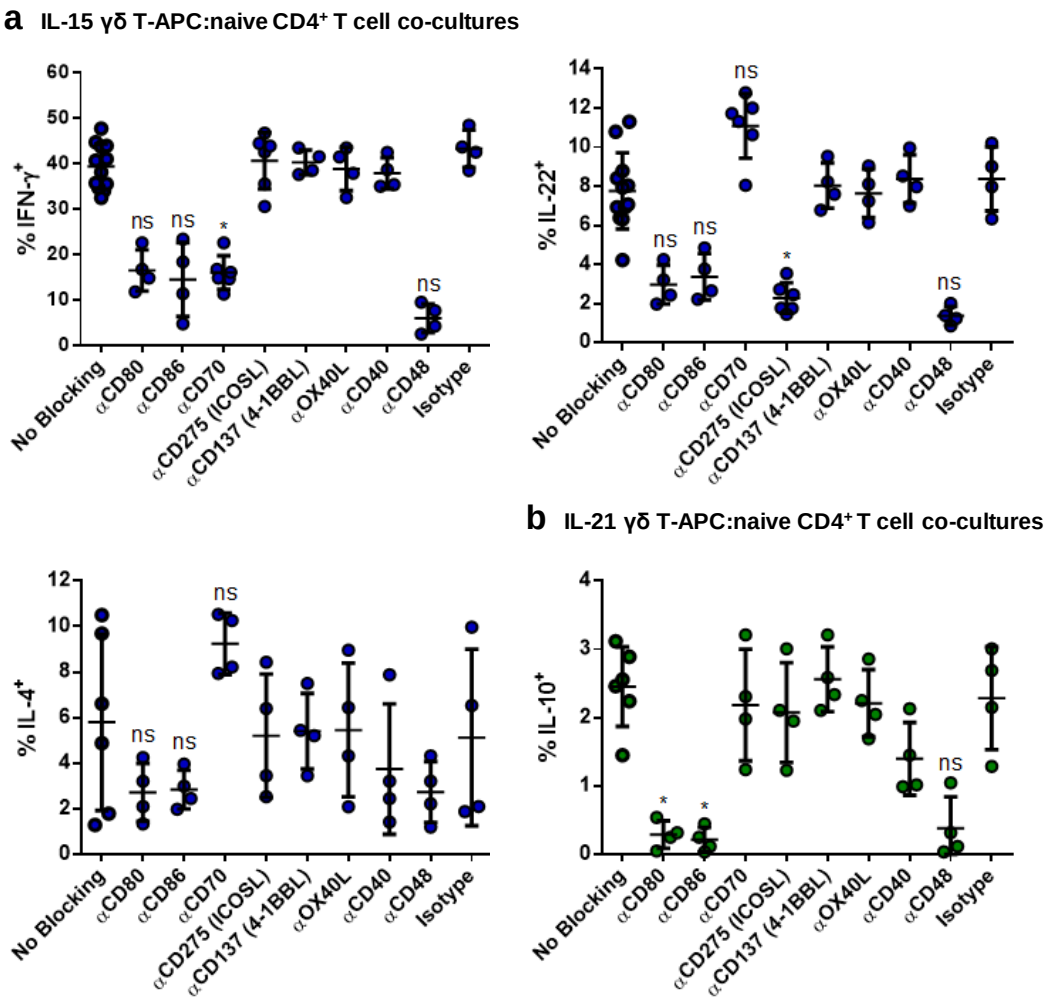

Figure 4.24 - Blockade of costimulatory molecules in γδ T-APC mediated polarisation of naive CD4$^+$ T cell intracellular cytokine production. Blockade of costimulatory molecules expressed by γδ T-APCs in naive CD4$^+$ T cell co-cultures. **(a)** Naive CD4$^+$ T cells were cultured at 10:1 ratios with IL-15 γδ T-APCs for 9 days, in the presence or absence of blocking antibodies or isotype controls. At day 9 of culture, cultures were restimulated and intracellular cytokine production was assessed by flow cytometry. **(b)** Naive CD4$^+$ T cells were cultured with IL-21 γδ T-APCs for 9 days, in the presence or absence of blocking antibodies or isotype controls. At day 9 of culture, cultures were restimulated and intracellular cytokine production was assessed by flow cytometry. Percentages of cytokine positive cells were determined by gating on live, single, CD3$^+$CD4$^+$Vγ9$^-$CFSE^{lo} cells. Data points represent individual healthy donors. Horizontal bars display means of data sets. Error bars display standard deviation of samples. Statistical significance was determined using the Friedman test, followed by the Dunn's multiple comparison test, *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001, ns=not significant. Significance displayed in comparison with No Blocking control.

To complement intracellular cytokine expression data, levels of cytokines in co-culture supernatants were measured in the presence or absence of co-stimulatory blocking antibodies (Figure 4.25). The effects of blocking antibodies observed were consistent with those observed previously. CD80 and CD86 were important in the induction of all cytokines, with blockade of these molecules decreasing overall cytokine production by polarised naive $CD4^+$ T cells. CD70 blocking again reduced the capacity of IL-15 γδ T-APCs to promote IFN-γ production by responding cells, and interference with the ICOSL signalling pathway reduced the secretion of IL-22 in naive $CD4^+$ T cells (Figure 4.25a). In response to IL-21 γδ T-APCs, IL-10 production by responding cells appeared to be reduced in the presence of CD40 blocking antibodies, although this decrease was not significant (Figure 4.25b). Lastly, CD48 was important for overall $CD4^+$ T cell responses, with blockade of this γδ T-APC-expressed costimulatory molecule resulting in a decreased production of all cytokines assessed.

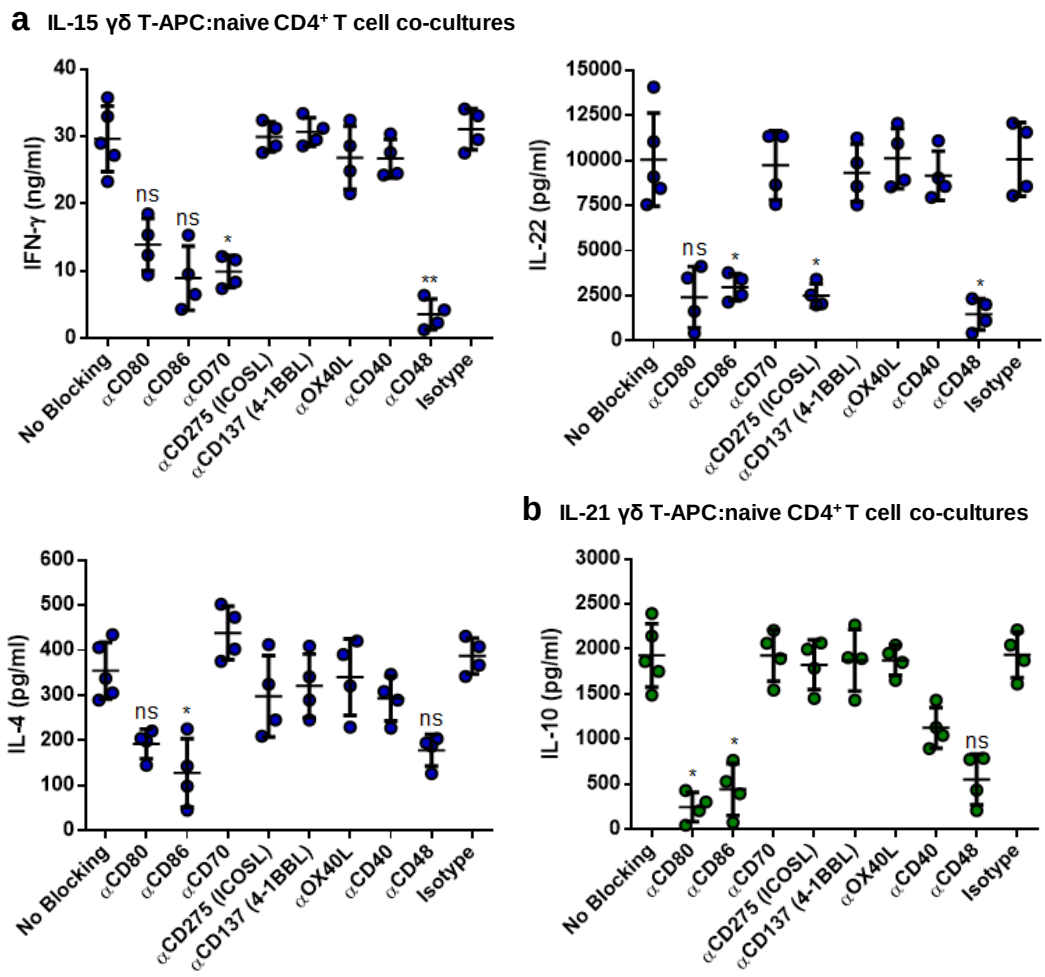

Figure 4.25 - Blockade of costimulatory molecules in γδ T-APC mediated polarisation of naive CD4$^+$ T cell cytokine secretion. Blockade of costimulatory molecules expressed by γδ T-APCs in naive CD4$^+$ T cell co-cultures. **(a)** Naive CD4$^+$ T cells were cultured at 10:1 ratios with IL-15 γδ T-APCs for 9 days, in the presence or absence of blocking antibodies or isotype controls. At day 9 of culture, cultures were restimulated for 24 hours, supernatants collected and cytokine concentrations were assessed by ELISA. **(b)** Naive CD4$^+$ T cells were cultured with IL-21 γδ T-APCs for 9 days, in the presence or absence of blocking antibodies or isotype controls. At day 9 of culture, cultures were restimulated for 24 hours, supernatants collected and cytokine concentrations were assessed by ELISA. Data points represent individual healthy donors. Horizontal bars display means of data sets. Error bars display standard deviation of samples. Statistical significance was determined using the Friedman test, followed by the Dunn's multiple comparison test, *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001, ns=not significant. Significance displayed in comparison with No Blocking control.

Having identified that CD70 and ICOSL play significant roles in the polarisation of naive CD4$^+$ T cells by γδ T-APCs, towards IFN-γ and IL-22 responses, respectively,

the role of these molecules in transcription factor gene expression was next examined. Figure 4.26 displays the expression of several transcription factors by naive $CD4^+$ T cells when polarised by IL-15 γδ T-APCs. Expression of *TBX21* followed a similar pattern of expression as did IFN-γ, in that blockade of CD70 led to a significant reduction in expression of this transcription factor. CD70 blocking also resulted in an increase in *RORC* expression, although this was not statistically significant. Conversely, ICOSL blocking led to a reduced expression of both *RORC* and *AHR* by polarised responder cells, reflecting a reduction in IL-22 expression. No significant effects were observed on *GATA3* levels when blocking agents were used, consistent with the unaffected expression of IL-4 in responder cells under these conditions.

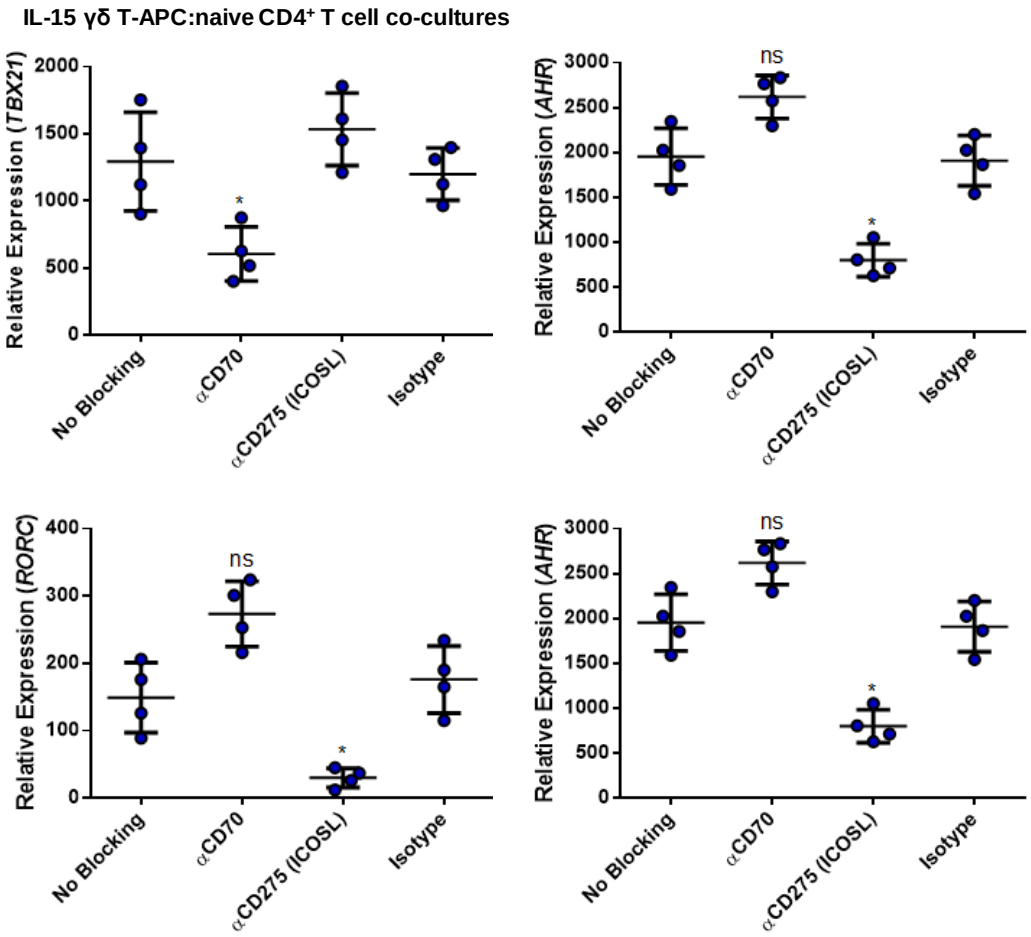

Figure 4.26 - Blockade of costimulatory molecules in γδ T-APC mediated polarisation of naive CD4$^+$ T cell transcription factor expression. Blockade of costimulatory molecules expressed by γδ T-APCs in naive CD4$^+$ T cell co-cultures. Naive CD4$^+$ T cells were cultured at 10:1 ratios with IL-15 γδ T-APCs for 9 days, in the presence or absence of blocking antibodies or isotype controls. At day 9 of culture, polarised CD4$^+$ T cells were assessed for transcription factor expression by real-time PCR. Relative expression was determined in reference to naive CD4$^+$ T cell control. Data points represent individual healthy donors. Horizontal bars display means of data sets. Error bars display standard deviation of samples. Statistical significance was determined using the Friedman test, followed by the Dunn's multiple comparison test, *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001, ns=not significant. Significance displayed in comparison to No Blocking control.

4.11 - Roles of TNF-α, ICOS-L, and CD70 in Polarisation of Naive $CD4^+$ T cells

By blocking the action of either TNF-α or ICOSL individually in co-cultures, partial but significant reductions in the expression of IL-22 and *AHR* were observed. Further to this, IL-6 was redundant in this system, in contrast to previous reports identifying a combination of IL-6 and TNF-α as required for efficient induction of optimal IL-22 responses in $CD4^+$ T cells[121]. Highlighting a potentially new role for ICOSL in IL-22 induction, blockade of both TNF-α and ICOSL in combination was assessed, to examine the overall effects on induction of IL-22-type responses. Figure 4.27 displays the intracellular expression of IL-22 (Figure 4.27a), secretion of IL-22 (Figure 4.27b), and expression of *AHR* (Figure 4.27c), by naive $CD4^+$ T cells polarised by IL-15 γδ T-APCs. In all readouts, a combination of TNF-α and ICOSL blockade led to an increased reduction in IL-22/*AHR* expression, highlighting the roles of these two molecules in IL-22-type responses. Of note, low levels of IL-22 and *AHR* were still detected in polarised responder cells after blockade of TNF-α and ICOSL , suggesting the presence of further unidentified polarising factors provided by γδ T-APCs.

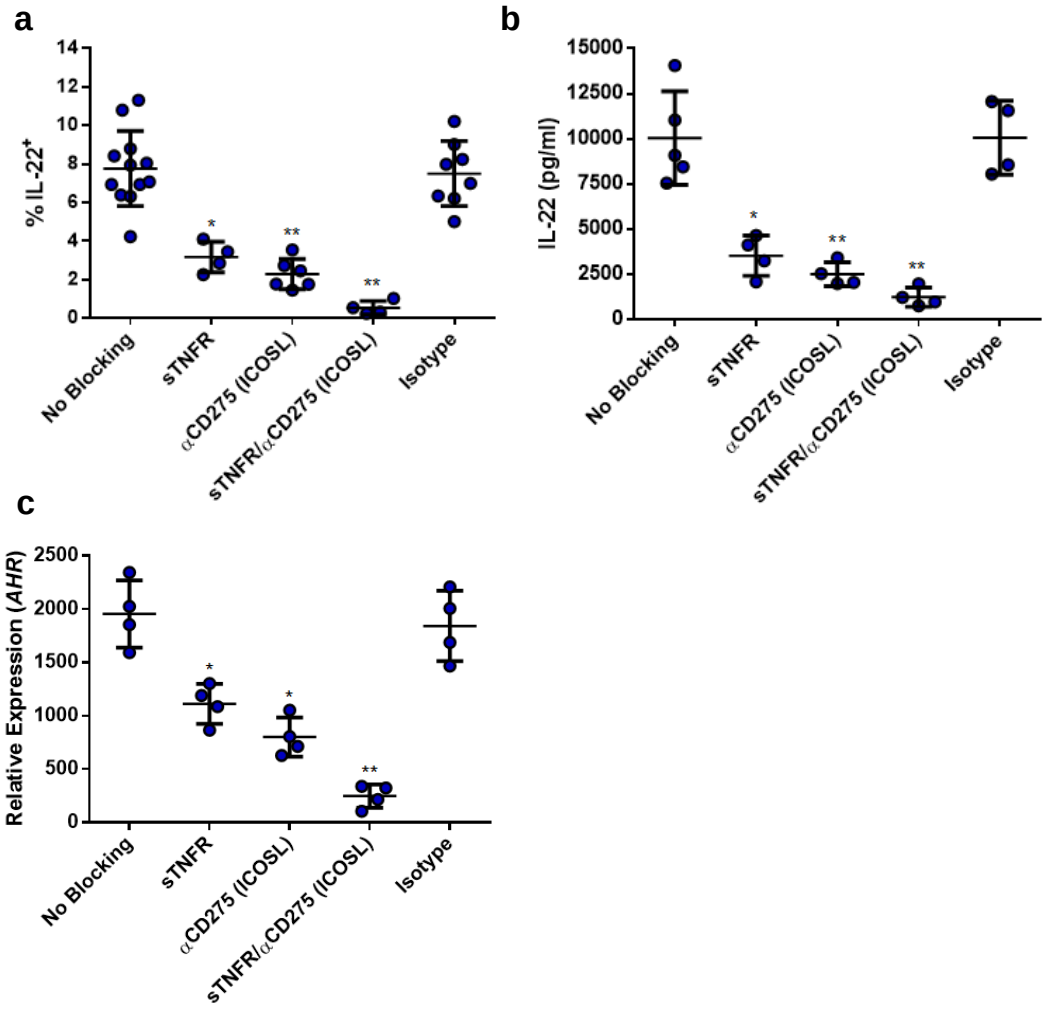

Figure 4.27 – Effects of γδ T-APC expressed TNF-α and ICOSL blockade on the polarisation of naive CD4$^+$ T cells. (a) Summary plot displaying intracellular cytokine expression of naive CD4$^+$ T cells, polarised by IL-15 γδ T-APCs in the presence of blocking antibodies to polarising factors. Naive CD4$^+$ T cells were cultured at 10:1 ratios with IL-15 γδ T-APCs for 9 days, in the presence or absence of blocking antibodies or isotype controls. At day 9 of culture, cultures were restimulated and intracellular cytokine production was assessed by flow cytometry. (b) Summary plot displaying cytokine secretion by naive CD4$^+$ T cells, polarised by IL-15 γδ T-APCs in the presence of blocking antibodies to polarising factors. Naive CD4$^+$ T cells were cultured at 10:1 ratios with IL-15 γδ T-APCs for 9 days, in the presence or absence of blocking antibodies or isotype controls. At day 9 of culture, cultures were restimulated for 24 hours, supernatants collected and cytokine concentrations were assessed by ELISA. (c) Summary plot displaying transcription factor expression by naive CD4$^+$ T cells, polarised by IL-15 γδ T-APCs in the presence of blocking antibodies to polarising factors. At day 9, transcription factor expression was determined by real-time PCR. Relative expression was determined in reference to naive CD4$^+$ T cell control. Data points represent individual healthy donors. Horizontal lines display means of data sets. Error bars display standard deviation of samples. Statistical significance was determined using the Friedman test, followed by the Dunn's multiple comparison test, *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001, ns=not significant.

With the demonstration that CD70 costimulation is able to promote Th1 type responses, and that a combination of TNF-α and ICOSL promotes γδ T-APC-driven IL-22 responses by naive $CD4^+$ T cells, assays were established to assess whether these effects were reproducible in APC-free cultures. Naive $CD4^+$ T cells were stimulated with anti-CD3 and anti-CD28, in addition to each polarising factor, and subsequent cytokine expression was determined.

Figure 4.28 displays the intracellular cytokine expression of naive $CD4^+$ T cells polarised by each factor. With the addition of soluble CD70, which stimulates cells via CD27, IFN-γ intracellular expression was increased over αCD3/αCD28 cultures only (Figure 4.28a). In addition, when naive $CD4^+$ T cells were cultured in the presence of polarising cytokines to each T helper lineage in addition to sCD70, responses were consistently skewed towards Th1 responses, with the levels of IFN-γ increased in all cultures. Further to this, sCD70 addition to cultures led to decreases in Th2 and Th22 populations, and particularly in Th17 populations (data not shown).

IL-22 responses were also investigated in response to TNF-α and ICOSL stimulation of naive $CD4^+$ T cells (Figure 4.28b). After stimulation with αCD3/αCD28 antibodies alone, naive $CD4^+$ T cells exhibited low levels of IL-22 polarisation. These levels of expression were marginally enhanced by the addition of either TNF-α or agonistic antibodies to ICOS. However, upon addition of both polarising factors in combination, IL-22 expression levels by naive $CD4^+$ T cells were significantly increased. With the observation that these two factors in combination could promote strong IL-22 responses in naive $CD4^+$ T cells, IL-22 expression levels were compared to the Th22 polarising combination of TNF-α and IL-6, which have been identified previously to promote the polarisation of Th22 cells[121]. Total percentages of IL-22-expressing cells were similar between each set of polarising conditions. However, when gated on $IL\text{-}22^+$ cells, the co-expression of IFN-γ differed between conditions (Figure 4.28c). In the presence of TNF-α and IL-6, the majority of cells expressing IL-22 did not co-express IFN-γ. In contrast, a large subset of TNF-α/αICOS stimulated cells, which were identified as $IL\text{-}22^+$, co-expressed IFN-γ but were also negative for all other cytokines examined. Under either condition, $IL\text{-}22^+$ cells were negative for IL-4, IL-17, IL-9 and IL-10.

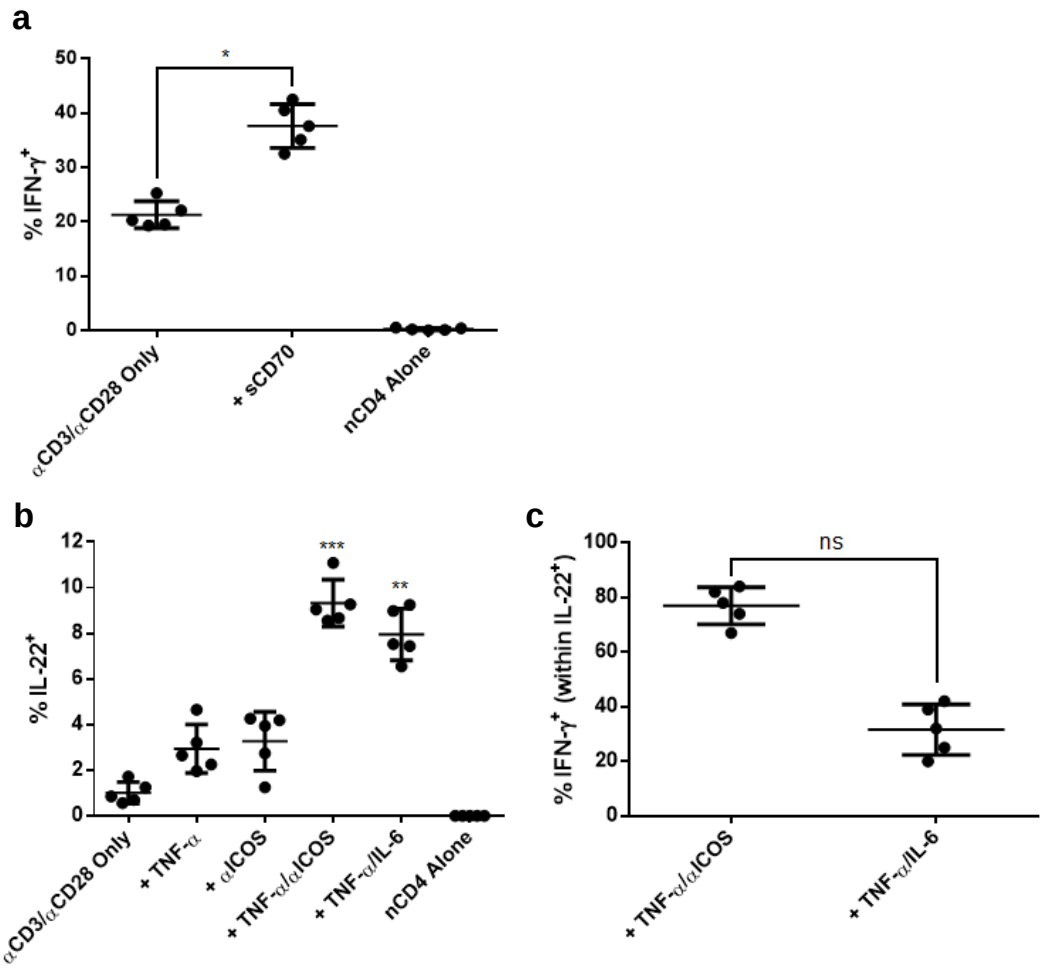

Figure 4.28 - Role of polarising factors in the polarisation of naive CD4$^+$ T cell intracellular cytokine expression. (a) Naive CD4$^+$ T cells were stimulated with αCD3/αCD28 antibodies for 9 days, in the presence or absence of soluble CD70. At day 9 of culture, cultures were restimulated and intracellular cytokine expression was assessed by flow cytometry. **(b)** Naive CD4$^+$ T cells were stimulated with αCD3/αCD28 antibodies for 9 days, in the presence or absence of different combinations of polarising factors. At day 9 of culture, cultures were restimulated and intracellular cytokine expression was assessed by flow cytometry. Significance displayed in comparison to αCD3/αCD28 only condition. **(c)** Expression of IFN-γ by IL-22-expressing CD4$^+$ T cells. Naive CD4$^+$ T cells were stimulated with αCD3/αCD28 antibodies for 9 days, in the presence or absence of different combinations of polarising factors. At day 9 of culture, cultures were restimulated and intracellular cytokine expression was assessed by flow cytometry. Percentages of IFN-γ$^+$ cells were determined by gating on IL-22$^+$ cells. Data points represent individual healthy donors. Horizontal bars display means of data sets. Error bars display standard deviation of samples. Statistical significance was determined using the Friedman test, followed by the Dunn's multiple comparison test, *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001, ns=not significant.

Following examination of intracellular cytokine expression, cytokine production and secretion was measured in identical culture conditions (Figure 4.29). Similar effects could be observed with each polarising factor; sCD70 favoured increased production of IFN-γ, whereas combinations of TNF-α/αICOS or TNF-α/IL-6 induced potent IL-22 induction. Of note, ICOS stimulation did appear to increase levels of IL-4 secretion in associated cultures, though this increase was not significant compared to control conditions (data not shown).

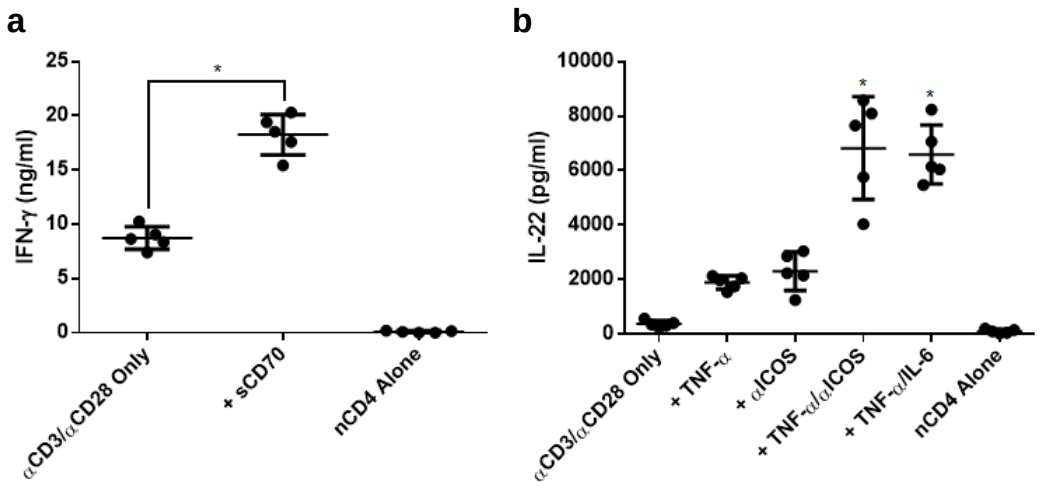

Figure 4.29 - Role of polarising factors in the polarisation of naive CD4$^+$ T cell cytokine secretion. (a) Naive CD4$^+$ T cells were stimulated with αCD3/αCD28 antibodies for 9 days, in the presence or absence of soluble CD70. At day 9 of culture, cultures were restimulated for 24 hours, supernatants collected and cytokine concentrations were assessed by ELISA.**(b)** Naive CD4$^+$ T cells were stimulated with αCD3/αCD28 antibodies for 9 days, in the presence or absence of different combinations of polarising factors. At day 9 of culture, cultures restimulated for 24 hours, supernatants collected and cytokine concentrations were assessed by ELISA. Data points represent individual healthy donors. Horizontal lines display means of data sets. Error bars display standard deviation of samples. Statistical significance was determined using the Friedman test, followed by the Dunn's multiple comparison test, *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001, ns=not significant. Significance displayed in comparison to αCD3/αCD28 only condition.

In addition to measuring the induction of IL-22 in naive CD4$^+$ T cells, transcription factor expression was assessed in response to the same polarising factors. *TBX21* displayed low level expression in response to CD3/CD28 stimulation alone, with only sCD70 stimulation via CD27 able to induce significantly higher expression of this transcription factor. Several other conditions induced higher levels of *TBX21* expression, although these increases were not significant. In addition, small increases of *TBX21* were observed in ICOS/TNF-α stimulated cells as compared with TNF-α and IL-6 treated cells. Naive CD4$^+$ T cells exhibited increased expression of *GATA3* in response to ICOS stimulation, either alone or in combination with TNF-α, or with TNF-α and IL-6 treatment. However, increased expression of *GATA3* was not statistically significant. *RORC* expression followed a similar trend to *GATA3*, showing upregulation in the majority of conditions, which again were not significant. Of note, sCD70 treatment led to reductions in both *GATA3* and *RORC* expression.

Lastly, *AHR* displayed similar patterns of expression to those observed with IL-22. Low levels of *AHR* were detected in populations stimulated with CD3/CD28 alone, and levels were not significantly affected by sCD70 or TNF-α treatment. ICOS stimulation alone led to small increases in *AHR* expression. However, highest expression levels of *AHR* were observed with either αICOS/TNF-α or TNF-α/IL-6 treatments.

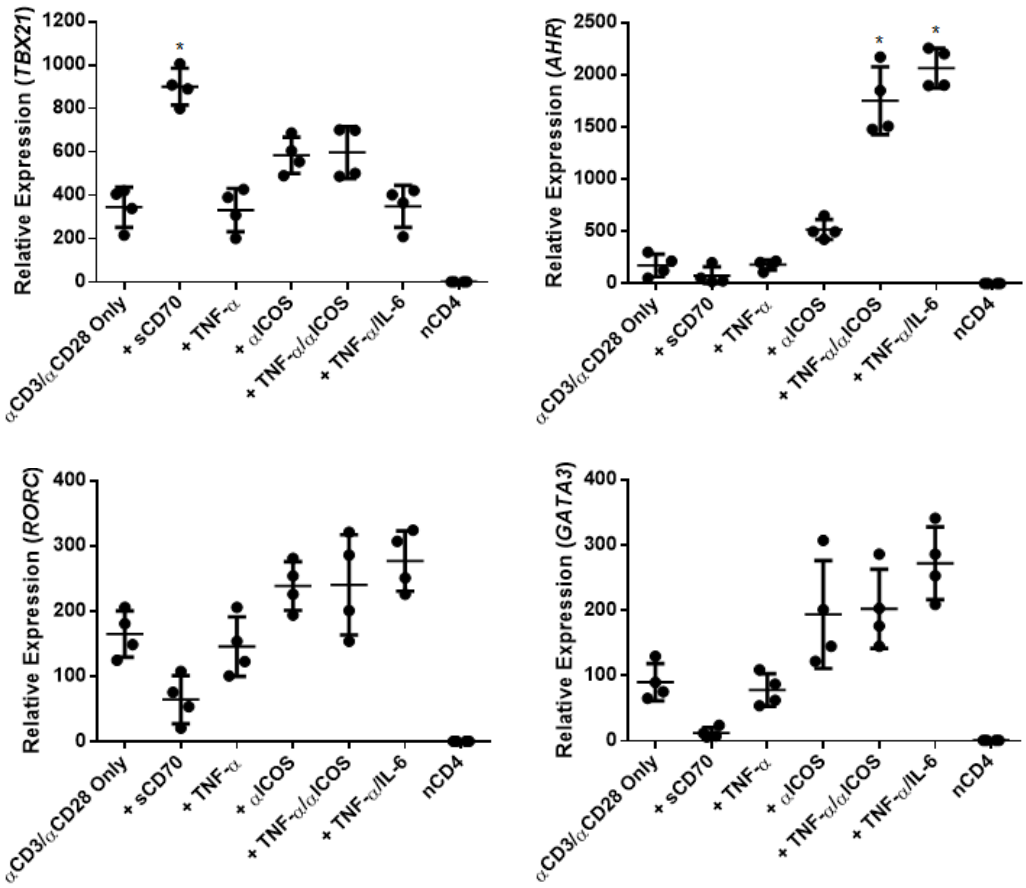

Figure 4.30 - Role of polarising factors in the induction of naive CD4$^+$ T cell transcription factors. Naive CD4$^+$ T cells were stimulated with αCD3/αCD28 antibodies for 9 days, in the presence or absence of different combinations of polarising factors. At day 9 of culture, RNA was isolated from cultures and assessed for transcription factor expression by real-time PCR. Relative expression determined in reference to naive CD4$^+$ T cell control. Data points represent individual healthy donors. Horizontal bars display means of data sets. Error bars display standard deviation of samples. Statistical significance was determined using the Friedman test, followed by the Dunn's multiple comparison test, *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001, ns=not significant. Significance displayed in comparison to αCD3/αCD28 only condition.

4.12 - Discussion

In summary, the data presented in this Chapter provide evidence for the ability of γδ T-APCs to polarise $CD4^+$ T cell responses. These findings demonstrate that not only are γδ T-APCs able to differentially induce T helper lineages, but also induce distinct responses as compared to DCs and monocytes.

Initial experiments were aimed at assessing the ability of differentially generated γδ T-APC subsets to initiate $CD4^+$ T cell responses. The γδ T-APCs presented here were potent inducers of naive and memory $CD4^+$ T cell responses and able to match DCs and outperform monocytes at APC:responder cell ratios of up to 1:100, in agreement with previous reports[322]. However, only γδ T-APCs cultured in the presence of IL-2 and IL-15 were able to induce such strong responses, whereas presence of IL-21 during γδ T-APC generation led to a population of APCs which induced relatively limited responses as characterised by only modest proliferation even at APC:responder cell ratios of up to 1:1. In contrast, IL-7 did not support the ability of γδ T-APCs to induce a response in naive or memory $CD4^+$ T cell populations. The fact that IL-7, a homeostatic cytokine, did not support an APC phenotype in Vγ9Vδ2 T cells, and as such did not allow for induction of $CD4^+$ T cell responses, highlights the requirement for a strong γδ T-APC phenotype to induce subsequent responses. Indeed, this was confirmed utilising blocking antibodies to adhesion molecules, which revealed that LFA-1 interaction was essential for γδ T-APC induction of naive $CD4^+$ T cell responses. Overall, γδ T-APCs were capable of inducing robust responses in $CD4^+$ T cell populations, and also displayed an adaptive nature to their microenvironment, in terms of APC function; in the presence of IL-2, IL-15, or IL-21, Vγ9Vδ2 T cells were able to become APCs and induce adaptive immunity, whereas in the presence of IL-7, Vγ9Vδ2 T cells displayed no APC function and would potentially play roles in other aspects of Vγ9Vδ2 T cell immunity. In addition to the induction of $CD4^+$ T cell responses, γδ T-APCs were able to promote proliferative responses in naive $CD8^+$ T cell populations, highlighting the overall role this novel APC subset can perform. In addition to these findings obtained with freshly isolated γδ T cells, expanded γδ T-APCs were equally capable of inducing naive $CD4^+$ T cell proliferation, indicating that γδ T-APCs maintain their functional potential over extended periods of time, and that the APC function is not a transient phenomenon.

In this Thesis, a crucial role for CD48 was identified in the initiation and maintenance of $CD4^+$ T cell responses by γδ T-APCs. When γδ T-APC-expressed CD48 was

prevented from interacting with CD2 on $CD4^+$ T cells, only limited naive $CD4^+$ T cell proliferation and survival were observed. The CD48 interaction with CD2 was described previously to provide many costimulatory effects to responding $CD4^+$ T cells, aiding cell adhesion, proliferation, survival, and cytokine production[354]. Blockade of CD48 has been shown to inhibit IL-2 production, IL-2 receptor expression, and proliferation of $CD4^+$ T cells[355], and as such observations made in γδ T-APC co-cultures are in accordance with those earlier studies.

With the demonstration that γδ T-APCs do indeed promote naive and memory $CD4^+$ T cell responses, the investigation focused on which T helper lineages were induced. Using either IL-2 or IL-15 γδ T-APCs, significant populations of both Th1 and Th2 cells were induced in naive and memory $CD4^+$ T cell responders, characterised by IFN-γ/*TBX21* and IL-4/*GATA3* expression, respectively. Previous studies had shown that IL-2 γδ T-APCs are able to promote both of these subsets (albeit with a strong preference for Th1 cells)[322], and in this study similar effects were additionally observed utilising IL-15 γδ T-APCs. LPS-matured DCs, which exhibited strong production of IL-12, were among the most potent inducers of Th1 responses, in agreement with the established role of IL-12 in directing Th1 responses. However, in comparison with LPS-matured DCs, IL-2 and IL-15 γδ T-APCs were able to match this level of IFN-γ induction in naive $CD4^+$ T cells, and induced high levels of *TBX21* expression, despite a complete lack of IL-12 production by γδ T cells. This Th1-promoting capacity was at least partially reliant on the combined effects of IFN-γ production and CD70 expression by γδ T-APCs. IFN-γ is able to directly induce *TBX21* in naive $CD4^+$ T cells, and as such polarises Th1 responses[59]. Given that both γδ T-APCs and $CD4^+$ T cells themselves produce IFN-γ, it is unclear whether production of this cytokine by each subset is important in cell polarisation, and is likely a combination of both. In addition, a large fraction of γδ T-APCs were identified as $CD70^+$, whereas monocyte-derived DCs appear to express CD70 at lower levels[173], suggesting γδ T-APC-mediated Th1 induction was much more reliant on the CD70-CD27 costimulatory pathway than DC-mediated Th1 responses.

In contrast to IL-2 and IL-15 γδ T-APCs, IL-21 γδ T-APCs were less capable of Th1 induction. Two reasons are evident for this effect; firstly a lower percentage of IL-21 γδ T-APCs expressed CD70 than that observed with IL-2 and IL-15 γδ T-APCs, and secondly IL-21 γδ T-APCs failed to produce IFN-γ. With a lower expression of both of these molecules, IL-21 γδ T-APCs were incapable of inducing strong Th1 responses. As such, it appears a combination of IFN-γ and CD70 expression

account for the induction of Th1 responses by γδ T-APCs, in the absence of IL-12. In addition to the action of these two molecules, it is likely that the strength of stimulation provided by γδ T-APCs also accounted for a certain level of Th1 induction as strong stimulations have been described to favour Th1 polarisation[160]. As judged by their potential to induce proliferation of naive $CD4^+$ T cells, IL-21 γδ T-APCs provided reduced strength stimulations over IL-2 and IL-15 γδ T-APCs.

Significant induction of Th2 responses was observed using all γδ T-APC subsets, as confirmed by expression of IL-4, IL-5, IL-13 and *GATA3*. However, such responses only constituted a relatively small proportion of all responding cells, and under no condition was the resulting $CD4^+$ T cell response dominated by Th2 cells. The levels of IL-4 induction appeared to exceed those induced by DCs, and were only matched by monocytes. In this respect, neutralisation studies demonstrated that IL-4 was at least partially responsible for the induction of Th2 responses by γδ T-APCs, highlighted by the reduced IL-4 secretion by naive $CD4^+$ T cells in γδ T-APC: $CD4^+$ T cell co-cultures in the presence of anti-IL-4 mAbs. However, it is unclear whether this IL-4 was actually produced by γδ T-APCs to prime Th2 responses, or by naive $CD4^+$ T cells during their initial stimulation. Vγ9Vδ2 T cells are indeed capable of IL-4 production, but the question remains as to whether γδ T-APCs utilised this ability during co-cultures. In addition, lack of IL-12 production by γδ T-APCs may have allowed for increased levels of Th2 differentiation over DC co-cultures to occur. Of note, a similar induction of IL-4 and IFN-γ responses was detected in naive $CD8^+$ T cell responders, highlighting the capacity of γδ T-APCs not only to promote Th1 and Th2 responses but to trigger Tc1 and Tc2 responses as well.

A significant contrast between responses induced by γδ T-APCs, and those induced by DCs and monocytes, was in the induction and promotion of Th17 responses. γδ T-APCs failed to induce any IL-17 in naive $CD4^+$ T cell co-cultures, and even did not expand IL-17 expressing cells in memory $CD4^+$ T cell populations. In contrast, DCs and monocytes were both able to induce significant populations of IL-17 producing cells, the highest levels observed with PGN-treated APCs, consistent with previous reports[95]. A lack of IL-17 induction by γδ T-APCs can be attributed to a number of factors, not least of which is the absence of polarising cytokine production to direct Th17 differentiation. The cytokine requirements for Th17 differentiation are well defined, involving a combination of IL-1β, IL-6, and TGF-β. These cytokines are produced by DCs and monocytes, particularly in the presence of PGN[95]. However, none of these cytokines were detected in γδ T-APC supernatants, indicating a lack of ability to promote Th17 differentiation. In addition, CD70 interaction with CD27,

expressed by $CD4^+$ T cells, has been identified to provide a Th1 promoting signal at the expense of Th17 polarisation[176]. Indeed, addition of soluble CD70 to naive $CD4^+$ T cells, cultured in the presence of Th17 polarising cytokines, reduced IL-17 expression in favour of IFN-γ expression (data not shown). Given the high expression of CD70 by γδ T-APCs, this presents a possible mechanism by which γδ T-APCs not only do not induce IL-17 production in responder cells, but also limit or even inhibit the expansion of Th17 cells, as observed with memory $CD4^+$ T cell responses. Of note, upregulation of the Th17 master transcription factor, *RORC*, was detected in all γδ T-APC co-cultures, with increased levels in IL-15 γδ T-APC polarising cultures. Given the lack of IL-17 production by $CD4^+$ T cells under those conditions, it is unlikely this *RORC* expression was identified in Th17 cells. One explanation is that any contaminating γδ T-APCs left at the time of RNA extraction may have provided the *RORC* signal. Indeed, Vγ9Vδ2 T cells have been described to produce IL-17 and express *RORC* albeit only under very long culture periods and with relatively poor efficiency[289,290]. In the present Thesis, no IL-17 production was detected under any culture condition, in accordance with previous findings from our laboratory[305,306]. It appears that any surviving γδ T-APCs did not possess a Th17-phenotype, and given the minimal contamination of sorted $CD4^+$ T cell populations (>99.1% purity), this explanation appears unlikely. Another, and potentially more likely explanation, is that IL-22 expressing cells, such as Th22 cells, also display limited expression of *RORC*, despite a lack of IL-17 production, as discussed below.

One of the most surprising effects of γδ T-APC mediated $CD4^+$ T cell polarisation was in the efficient induction of IL-22. Both IL-2 and IL-21 γδ T-APCs induced IL-22 populations in naive responder cells, with levels of IL-22 induction similar to those observed with alternative APC controls. In contrast, IL-15 γδ T-APCs were able to promote IL-22 responses to levels which exceeded those induced by all other APC subsets. Similarly, highest levels of *AHR* expression were observed in IL-15 γδ T-APC co-cultures. A large proportion of the $IL\text{-}22^+$ cells induced were in fact IFN-γ co-expressing Th1 cells, with the remaining cells representing Th22 cells. In addition to the increased levels of IL-22 induction, these subsets induced by IL-15 γδ T-APCs were completely IL-17 negative. IL-22 was originally identified as a Th1 type cytokine, but is also co-expressed by Th17 cells[101]. The identification that γδ T-APCs may be important in the induction of IL-22 mediated immunity presents a novel function for these cells. As previously discussed, *RORC* expression was detected in all γδ T-APC co-culture systems, with highest levels observed in IL-15 γδ T-APC co-cultures. However, *RORC* expression does not always identify $IL\text{-}17^+$

cells[98], and Th22 cells have been identified to express *RORC* in addition to *AHR*[121]. Having identified the highest expression of *RORC* in responder cells polarised by IL-15 γδ T-APCs, where highest levels of IL-22 were identified, it appears these IL-22$^+$ cells may provide the *RORC* expression identified.

With the observation that IL-15 γδ T-APCs promote IL-22 type responses, the mechanism behind such induction was examined. According to previous studies, a combination of IL-6 and TNF-α promote the differentiation of Th22 cells[121], which do not co-express IFN-γ or IL-17, although the authors of that study did state that other polarising factors may also account for the induction or enhancement of Th22 responses. Consistent with previous reports, TNF-α was partially responsible for the induction of IL-22 by IL-15 γδ T-APCs, with blockade of this cytokine accounting for a significant decrease in IL-22 and *AHR* expression by naive CD4$^+$ T cells. Although limited studies have suggested that Vγ9Vδ2 T cells are capable of IL-6 production[356] under certain conditions, IL-6 was undetectable in γδ T-APC supernatants suggesting these cells do not contribute IL-6 to the polarising environment. Also, the lack of IL-6 function was confirmed utilising specific blocking antibodies against IL-6, which had no effect on the polarisation of IL-22-type responses. Instead, a role for ICOSL was identified, given that blockade of this costimulatory molecule, expressed by γδ T-APCs, resulted in a reduced ability to promote IL-22 responses. The role of ICOSL was confirmed by subsequent assays stimulating naive CD4$^+$ T cells in the absence of APCs; anti-ICOS agonistic mAbs or recombinant TNF-α alone induced low levels of IL-22 and *AHR* expression, whereas a combination of these two factors appeared to have a synergistic effect. In comparison with IL-6/TNF-α, similar levels of IL-22 and *AHR* were observed, however a higher proportion of IL-22$^+$ cells induced by αICOS/TNF-α co-expressed IFN-γ. In support of these findings, increased *TBX21* expression was observed with αICOS/TNF-α treatment, mirroring expression of IL-22 and IFN-γ in these culture conditions. Of note, this is the first description of a crucial role of ICOSL in the polarisation of IL-22-type responses, the precise mechanism of which remaining to be resolved. As such it is unknown whether a combination of ICOS stimulation with cytokines other than TNF-α (for instance IL-6) is similarly able to promote IL-22 expression by CD4$^+$ T cells, or whether it exerts any other differential effects on CD4$^+$ T cell polarisation. Also, it remains unclear whether ICOS stimulation in the presence of other polarising factors, such as Th17 polarising cytokines, may promote different effects in CD4$^+$ T cell polarisation, or always skews responses towards IL-22 by default. With regards to why IL-15 γδ T-APCs may be more prominent inducers of IL-22 than their IL-2 γδ

T-APC and IL-21 γδ T-APC counterparts, significantly higher expression of TNF-α production by IL-15 γδ T-APCs was identified, possibly accounting for increased induction of IL-22. In addition, increased expression of ICOSL was identified on IL-15 γδ T-APCs compared with IL-21 γδ T-APCs, and to a lesser extent IL-2 γδ T-APCs. However, other factors cannot be ruled out which could account for differential IL-22 induction by γδ T-APCs. Indeed, several other factors have been identified as IL-22 promoting, such as FICZ[346] and the active form of vitamin D3 ($1,25(OH)_2D_3$)[121], indicating a complex regulation of IL-22 expression involving a multitude of factors. Interestingly, low levels of IL-22 and *AHR* expression could be identified in IL-15 γδ T-APC:naive $CD4^+$ T cell co-cultures, after blockade of both ICOSL and TNF-α. This suggests that further, as yet unidentified, factors may be involved in IL-22 induction; however, given the low level expression of both IL-22 and *AHR* after CD3/CD28 stimulation of naive $CD4^+$ T cells alone, these cells may possess a natural tendency to express IL-22, which is further enhanced by polarising factors such as ICOSL, TNF-α, and/or IL-6. Interestingly, IL-22 induction was not detected in naive $CD8^+$ T cell co-cultures, indicating an alternate mechanism of Tc22 polarisation. Indeed, IL-21 has been reported as an important factor in driving Tc22 responses[217], and production of this cytokine by Vγ9Vδ2 T cells was not examined. As such, it appears IL-22 production by $CD4^+$ and $CD8^+$ T cells requires different pathways, and Tc22 induction may not depend on ICOSL and TNF-α action.

Given the roles of cytokines and costimulatory molecules expressed by γδ T-APCs in the facilitation of $CD4^+$ T cell polarisation as identified in this Chapter, a model of this process can be proposed (Figure 4.31). This model highlights that the molecules LFA-1, CD80, CD86, and CD48, expressed by the γδ T-APC, are all essential in stimulating and maintaining a $CD4^+$ T cell response. In addition, the polarising factors IFN-γ and CD70 promote $CD4^+$ T cell polarisation towards a Th1 type response, whereas TNF-α and ICOS-L promote the production of IL-22. It is the balance of these factors which promote the IFN-γ/IL-22 phenotype observed when naive and memory $CD4^+$ T cells are stimulated by γδ T-APCs.

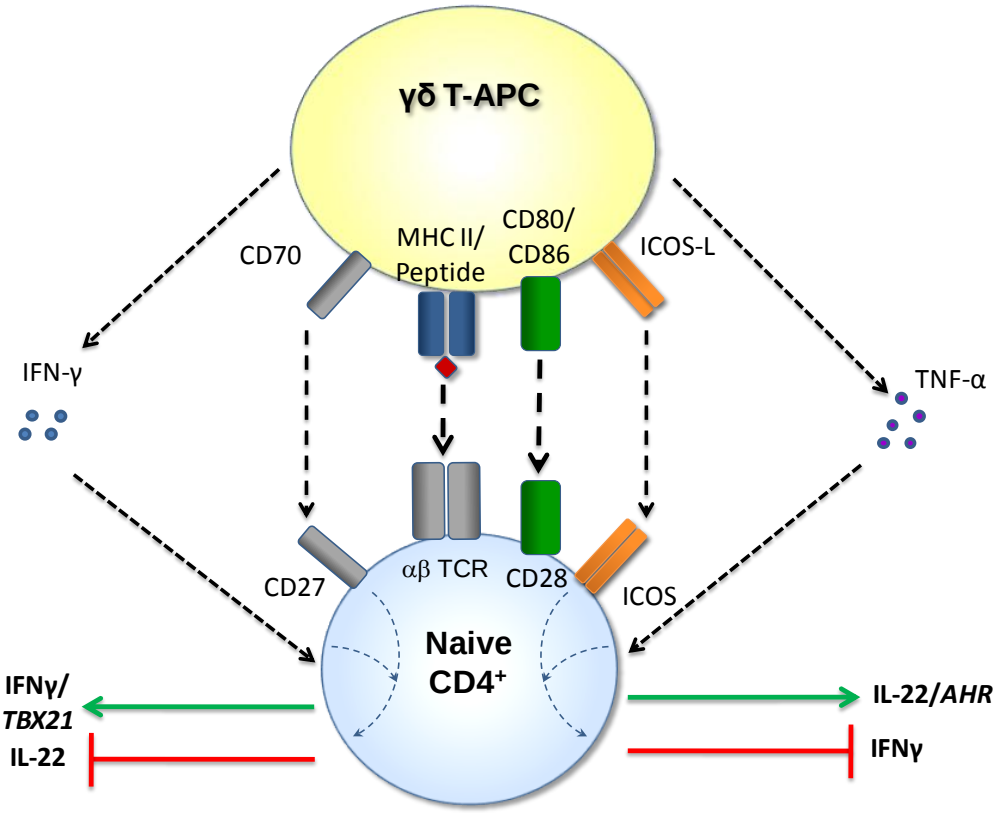

Figure 4.31 – Proposed model of γδT-APC expressed polarising factors and their roles in naive CD4$^+$ T cell polarisation.

In addition to the novel observation that γδ T-APCs can regulate IL-22 production, increased induction of IL-10 was observed in naive and memory CD4$^+$ T cells, mediated by IL-21 γδ T-APCs. The majority of IL-10$^+$ cells were negative for all other cytokines examined, while a small population were co-expressing IL-4. In addition, there were no significant increases in the expression of any of the transcription factors examined in IL-21 γδ T-APC co-cultures as compared to IL-2 or IL-15 cultures. Unfortunately, the specific mechanism and molecules behind the induction of IL-10 remain unclear. IL-10 induction was dependent on cell contact mediated by LFA-1, and the costimulation of both CD80 and CD86. However, all other costimulatory molecules examined appeared to have no effect on IL-10 induction. Additionally, blockade of a range of different γδ T cell-derived cytokines did not affect the ability of naive CD4$^+$ T cells to express IL-10. Prior to co-culture, IL-21 γδ T-APCs were identified to express only minimal levels of TNF-α and IFN-γ, explaining why the blockade of those cytokines had no effect on IL-10 induction. One potential mechanism by which IL-10 induction may have occurred is due to a lack of polarising cytokines, in combination with a relatively weak stimulation provided by IL-21 γδ T-APCs compared to other subsets, allowing for differentiation of CD4$^+$ T cells towards an IL-10$^+$ regulatory phenotype. However, no significant

increases in stable *FOXP3* expression were identified, and given the presence of IFN-γ^+, IL-4^+, and IL-22^+ cells induced alongside the IL-10^+ cells, a regulatory phenotype appears unlikely. Another possible explanation of IL-10 induction by IL-21 γδ T-APCs is a Tfh cell function. Vγ9Vδ2 T cells and $CD4^+$ T cells have been shown to interact with B cells to promote antibody class switching and humoral responses[280,292], and interactions were reliant on both IL-21 and IL-10. Given these findings, it is possible the IL-21 γδ T-APC function may allow for $CD4^+$ T cells to aid B cell responses, in accordance with the fact that IL-21 is a signatory cytokine expressed by Tfh cells themselves[280]. However, no increases in *BCL6* were identified in IL-21 γδ T-APC co-cultures, indicating that these cells did not represent Tfh cells. Further investigation is needed to identify which T helper lineage these IL-10^+ cells belong to, and where IL-21 γδ T-APCs may play a role in immunity.

The expression of chemokine receptor repertoires is an inherent component of T helper lineages, with each subtype displaying unique combinations of receptors. With the identification of a number of different T helper subsets induced by γδ T-APCs, characterised by cytokine and transcription factor expression, responder cells were examined for the expression of a number of chemokine receptors. CXCR3 staining, identifying cells likely to belong to the Th1 lineage, was expressed on a large proportion of responder cells. In contrast, only minimal CCR6 and CCR10 expression was detected in polarised $CD4^+$ T cells. CCR6 is expressed by Th17 cells[85], in combination with CCR4, and given the lack of IL-17 induced in responder populations, it is not surprising that no CCR6 was detected under the conditions examined. However, CCR10 has been reported to be expressed by Th22 cells[121], alongside CCR4 and CCR6. Small populations of Th22 cells were identified in co-cultures, making the lack of CCR10 expression a contrast to previous studies. The regulation of chemokine receptors on $CD4^+$ T cells is a complex process, involving antigen presenting cells and also external, often tissue specific, factors. CXCR3 appears to become upregulated on $CD4^+$ T cells as a result of cell activation[357], as opposed to being dependent on the cytokine microenvironment, perhaps explaining the prominent induction of this chemokine receptor on responder cells identified here. However, to define Th1 cells exclusively, a combination of CXCR3 and CCR5 is necessary[92], whereas CXCR3 staining gives an indication of the T helper subset identified. In contrast to CXCR3, upregulation of CCR10, an epidermotropic receptor[358], is controlled by several factors. IL-12 and the vitamin D3 metabolite, 1α,25-dihydroxyvitamin D_3, have both been described to be involved in the induction of CCR10 on T cells[358]. Given the lack of IL-12 production by Vγ9Vδ2 T cells, this

may account for the absence of CCR10 induction. In addition, in the absence of vitamin D3 metabolites, it is unclear whether γδ T-APCs may permit CCR10 induction under the necessary culture conditions, or whether responder cells may be directed to other peripheral sites in the absence of skin-specific metabolites.

The reliance of γδ T-APCs on co-stimulatory molecules instead of polarising cytokines appears consistent with their functional phenotype. For example, DCs and monocytes are capable of producing a range of polarising cytokines, and adapted their cytokine profiles depending on the ligands encountered. In contrast, Vγ9Vδ2 T cells in this system only produced IFN-γ and TNF-α and failed to produce classical polarising factors such as IL-1β, IL-6, IL-12, IL-23 and IL-10. Given the reduced range of cytokines produced by γδ T-APCs under the conditions examined, the expression of a wide range of co-stimulatory molecules indicates a much more prominent effect in γδ T-APC mediated $CD4^+$ T cell polarisation than in DC or monocyte mediated responses. It should be noted that while certain responses were promoted over others in these assays, multiple lineages were induced simultaneously in all APC co-cultures. This reflects the complexity of $CD4^+$ T cell polarisation in humans; in murine models, responses are much clearer, with only limited numbers of discrete lineages present during different immune scenarios. However, in human immunity, T helper lineage responses are often identified together in disease conditions, highlighting a collaboration between $CD4^+$ T cell subsets to mediate immune responses.

Overall, the data presented in this Chapter show that γδ T-APCs are indeed able to adapt to their cellular microenvironment, become antigen presenting cells, and direct the polarisation of naive $CD4^+$ T cells towards appropriate lineages, depending on the Vγ9Vδ2 stimulation. In addition, the responses generated by γδ T-APCs in this system were strikingly different to those observed with DCs and monocytes, indicating γδ T-APCs may occupy a 'functional niche', where they induce specific T helper responses under certain conditions, or at certain anatomical locations, that do not induce optimal generation of DCs. Alternatively, γδ T-APC polarisation of adaptive immunity may act in concert with DC mediated polarisation to facilitate the complex mix of $CD4^+$ T cell subsets often observed in human immunity. Further questions remain about the extent to which γδ T-APCs can adapt to their microenvironment and initiate different immune responses, whether there are cytokines outside the common γ-chain family which may support APC differentiation, and what responses these differentially generated γδ T-APCs may induce.

Chapter 5 - Role of γδ T-APCs in Intestinal Immunity

5.1 - Introduction

γδ T-APC studies to date have focused on utilising Vγ9Vδ2 T cells from blood to generate an APC phenotype in these cells[322,326]. This is largely due to the fact that Vγ9Vδ2 T cells are readily available to isolate from blood and display prominent APC characteristics following stimulation. However, this has highlighted a lack of knowledge regarding the presence and role of γδ T-APCs at peripheral sites other than blood. Having identified that IL-15 promotes the generation of APCs which polarise potent IL-22-type responses in responding $CD4^+$ T cells, subsequent experiments focused on relating this IL-15/γδ T-APC/IL-22 axis to one or more peripheral sites, with the aim of defining a potential site-specific role of γδ T-APCs in an immune response.

IL-22 as an effector cytokine plays a multitude of roles in many different anatomical sites, the functions of which can be general or organ-specific. Prominent locations of IL-22 action include the skin and the intestine. Acting on non-haematopoietic cells, IL-22 in the skin acts predominantly on keratinocytes to induce production of antimicrobial peptides, enhances proliferation, induces epidermal hyperplasia, and inhibits terminal differentiation of keratinocytes, to facilitate the maintenance of overall barrier integrity[359]. Similarly, IL-22 in the intestine facilitates antimicrobial peptide production, promotes inflammation and epithelial cell proliferation and repair, and maintains barrier integrity[360]. In addition, IL-22 has been shown to mediate immunity in the liver[361] and the lung[362]. Given the diverse peripheral locations of IL-22 function, there are many scenarios whereby γδ T-APCs may play a role in IL-22 induction. Similarly, IL-15 is produced by a number of different cell types and at different sites. IL-15 is produced by DCs, keratinocytes, and intestinal epithelial cells, among others[363]. As such, both the skin and intestine are sources of IL-15, and represent peripheral sites where IL-22 action is fundamental to tissue homeostasis and maintenance of barrier integrity.

Given the range of anatomical sites where an IL-15 dependent γδ T cell-mediated induction of IL-22 might occur under physiological conditions in health or disease, specific locations may be dependent on where Vγ9Vδ2 T cells have been observed to extravasate from the peripheral blood into the tissues, to facilitate immune responses. In this respect, the most compelling evidence of tissue-specific Vγ9Vδ2 T cell immunity derives again from the skin and the intestine. Given that studies of

skin and gut immunity in healthy individuals are limited due to restricted access to relevant tissues, much of the knowledge of the immune system in these locales comes from studies of autoimmune diseases, such as psoriasis, inflammatory bowel disease (IBD), and rheumatoid arthritis. Indeed, Vγ9Vδ2 T cells have been identified as important in human skin immunity; a subset of circulating Vγ9Vδ2 T cells expresses the skin homing marker CLA, in combination with chemokine receptors such as CCR6, and as such can be recruited to the skin[291]. In addition, psoriasis patients, who exhibit significant skin inflammation among other symptoms, show increased numbers of Vγ9Vδ2 T cells in psoriatic lesions. Functionally, these skin-homing Vγ9Vδ2 T cells produce several inflammatory mediators such as IFN-γ, TNF-α, and IL-17, and as such may contribute to disease pathogenesis or progression. Similarly, Vγ9Vδ2 T cells have been observed to play roles in intestinal immunity[242]. Subsets of Vγ9Vδ2 T cells exhibit expression of gut homing receptors such as integrin α4β7, and can be identified in intestinal biopsies. In addition, Vγ9Vδ2 T cells derived from the intestine produce pro-inflammatory cytokines such as IFN-γ and TNF-α, and have even been identified to express HLA-DR and CD70, and interact with intestinal αβ T cells[242]. Further to this, roles for Vγ9Vδ2 T cells have been proposed in IBD[345]. Lastly, Vγ9Vδ2 T cells have displayed APC potential in rheumatoid arthritis, expressing APC markers and contributing to the induction of $CD4^+$ T cell responses[324]. Given these observations, it appears likely that not only will Vγ9Vδ2 T cells be able to facilitate APC function in both the skin and intestine, but may also contribute to autoimmune pathologies in these peripheral sites. Indeed, both IL-15 and IL-22 have been shown to contribute to autoimmune disease pathology[137,138,364], and as such all factors in the IL-15/γδ T-APC/IL-22 axis appear to be involved in the pathogenesis of autoimmune diseases.

To investigate at which peripheral site Vγ9Vδ2 T cells may contribute to IL-22 immunity, the homing potential of γδ T-APC and their function in the intestine was to be assessed. In addition, the potential of γδ T-APCs to contribute to autoimmune disorders would be investigated, in particular IBD.

5.2 - Aims of Chapter

The following experimental aims will be discussed in this chapter:

- Investigate homing receptor expression on cellular subsets.
- Assess the role of Vγ9Vδ2 T cells in intestinal biopsies.
- Examine the ability of intestinal Vγ9Vδ2 T cells to become APCs and control $CD4^+$ T cell responses.
- Investigate the role of Vγ9Vδ2 T cells in IBD.

5.3 - Expression of Homing Receptors

As an initial step in identifying at which anatomical location γδ T-APCs may induce IL-22 type responses, the expression of several homing receptors was assessed on the surface of γδ T-APCs. Immune cells, which are able to extravasate from blood into peripheral tissues, express distinct receptors necessary for this process, and skin and intestinal extravasation in particular require distinct receptors. The cutaneous lymphocyte-associated antigen, CLA, is a requirement for cell entry into the skin. By binding its ligand, endothelial cell adhesion molecule (ELAM-1), CLA-expressing cells are able to enter the skin[365]. In addition, CLA is readily identified on T cells located in the skin, highlighting the importance of this molecule in skin homing[358]. Conversely, entry into intestinal sites requires expression of α4β7 integrin, which recognises its ligand mucosal addressin cell adhesion molecule 1 (MAdCAM-1), expressed by endothelial cells in the small intestine and colonic lamina propria[366]. Populations of α4β7 expressing cells are detectable in peripheral blood and comprise immune cells which are potentially involved in intestinal immunity. In addition to the expression of tissue specific adhesion molecules, chemokines and their receptors are also essential in directing immune cells to the skin or intestine. As previously discussed, CCR10 and its ligand CCL27, as well as CCR4 and its ligand CCL17, may be involved in skin homing of human T cells[358,366], among other locations. Lastly, CCR8 has been shown to mediate skin-resident memory T cell trafficking to the skin[358]. Similarly, specific chemokine receptors and ligands have been identified for intestinal homing. CCR9, and its ligand CCL25, contributes to homing of immune cells to the small intestine[366]. Overall, combinations of molecules are required for homing of immune cells to peripheral sites, and the expression of such molecules allows for the identification of subsets with distinct migratory properties.

To identify the homing potential of γδ T-APCs, Vγ9Vδ2 T cells were stimulated with HMB-PP and common γ-chain cytokines for 3 days to induce an APC phenotype, and subsequently stained for β7 integrin and CCR9 for intestinal homing, and CLA for skin homing (Figure 5.1). Figure 5.1a displays representative stainings of each homing marker by total γδ T-APCs, generated with IL-15. Significant staining of β7 integrin was observed, with the majority of γδ T-APCs expressing this receptor. Of note, β7 integrin is able to associate with α4 or αE, and as such the β7 staining in these experiments did not exclusively identify α4β7 expression. CCR9 staining was less pronounced on IL-15 γδ T-APCs, whereas CLA expression was very limited on this cell population. Comparisons of homing marker expression across γδ T-APCs generated under different conditions are presented in Figure 5.1b. Highest levels of β7 integrin were identified on γδ T-APCs generated with IL-2 and IL-15, exhibiting significantly higher percentages of positive cells compared to unstimulated Vγ9Vδ2 T cells. IL-7 γδ T-APCs displayed increased expression of β7 integrin as compared to unstimulated cells, although this increase was not statistically significant. CCR9 expression displayed a similar pattern, yet with the only significant upregulation detected on IL-15 γδ T-APCs. Of note, even unstimulated cells showed marked expression of CCR9 (approx. 30%). Lastly, CLA expression followed an opposite pattern, whereby CLA was actually downregulated on γδ T-APCs generated with IL-2, IL-15, or IL-7, displaying significantly lower percentages of CLA positive cells as compared to unstimulated cultures. Overall, it appeared that most γδ T-APCs generated under the influence of IL-2 and IL-15, and to some degree also IL-7, possessed an intestinal homing phenotype and did not show any skin homing properties.

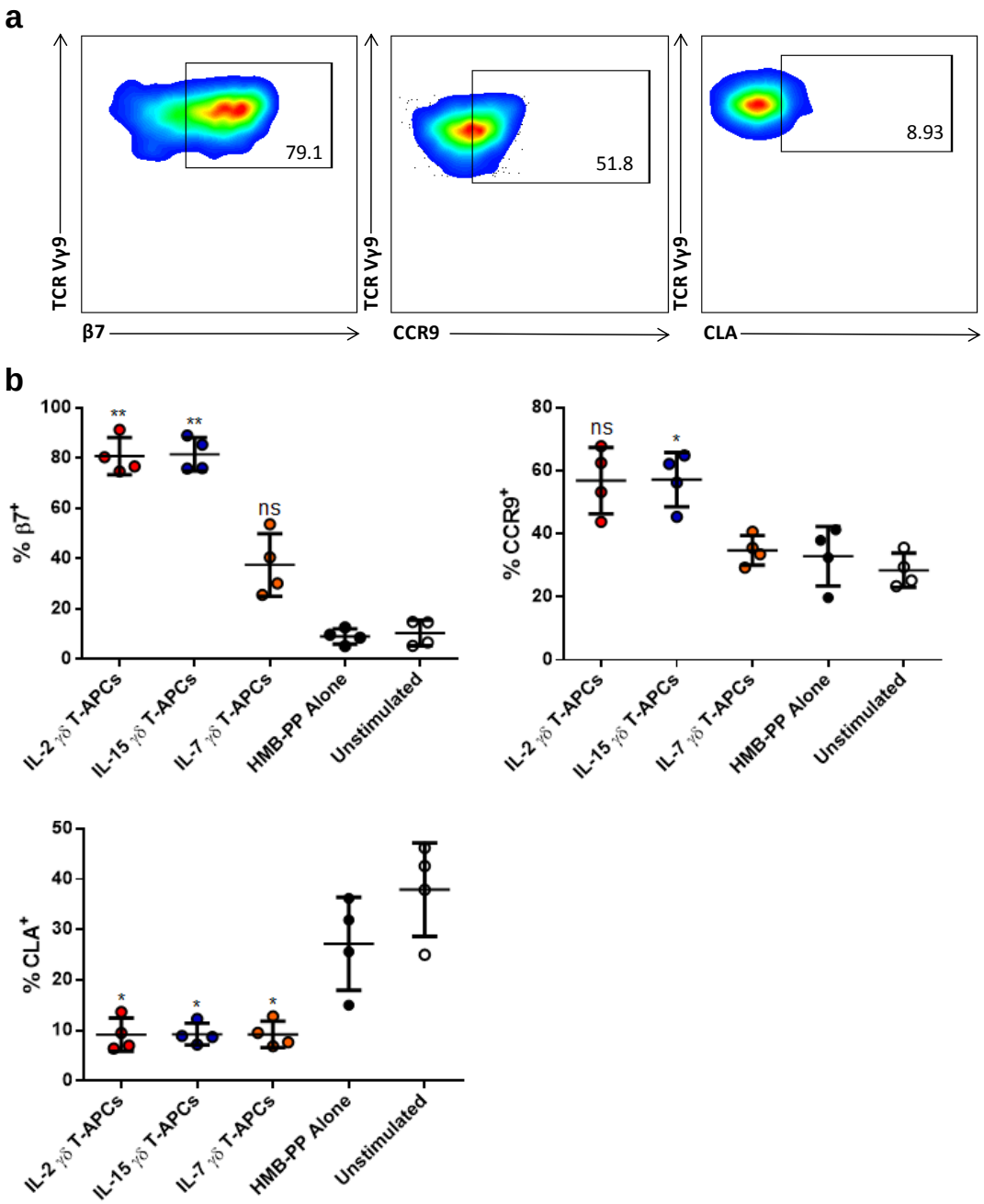

Figure 5.1 – Expression of homing markers by γδ T-APCs. (a) Representative flow cytometry plots displaying expression of β7 integrin, CCR9 and CLA on 3-day generated IL-15 γδ T-APCs. Vγ9Vδ2 T cells were stimulated with 10 nM HMB-PP in the presence or absence of IL-15 for 3 days, and assessed for expression of homing markers at day 3 of culture. Percentages of positive cells were determined by flow cytometry. **(b)** Summary of homing marker expression by γδ T-APCs generated in the presence or absence of different common γ-chain cytokines. Data points represent individual healthy donors from individual experiments. Horizontal lines display means of data sets. Error bars display standard deviation of samples. Statistical significance was determined using the Friedman test, followed by the Dunn's multiple comparison test, *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001, ns=not significant. Significance displayed in comparison to unstimulated control.

Given that CCR9 had been identified as upregulated on IL-15 γδ T-APCs, but that staining for this receptor did not identify distinct populations of positive and negative cells, migration assays were established to assess whether expression of CCR9 was functional (Figure 5.2). To assess migration to CCL25, the ligand for CCR9, IL-15 γδ T-APCs were generated over 3 days and placed in the top chamber of a transwell plate, and titrated concentrations of CCL25 were added in the bottom chamber. As γδ T-APCs utilised in migration assays were positive for CXCR3 (96.5%) and negative for CCR2 and CCR4 (data not shown), migration to CXCL10 (IP-10) and CCL2 (MCP-1) was assessed, representing ligands for CXCR3 and CCR2/CCR4, respectively. As such, CXCL10 represented a positive control for cell migration, and CCL2 a negative control. After 3 hours of culture, the numbers of cells which had migrated to the lower chamber were counted and the percentage of migrated cells was calculated.

Figure 5.2a displays migration of IL-15 γδ T-APCs toward each chemokine at a range of concentrations, for one individual donor examined. Migration to CXCL10 followed a dose-dependent response, with a peak migration observed at 100 ng/ml CXCL10. A similar dose-dependent response was observed to CCL25, with a peak migration at 100 ng/ml of chemokine. No increase in migration to CCL2 was detected in comparison with blank (no chemokine) controls. In terms of percentages of migrated cells, CXCL10 at 100 ng/ml led to approximately 20% of cell migration, compared to CCL25 which induced approximately 13% migration. The increased migration toward CXCL10 over CCL25 is was likely due to the higher expression levels of CXCR3 compared to CCR9. Figure 5.2b displays the expression of several APC markers on IL-15 γδ T-APCs, prior to and following migration to CCL25. Cells which had migrated to CCL25, in comparison to total input cells, showed higher levels of expression of HLA-DR and CD86, and to a lesser extent of CD70, indicating a selective enrichment of Vγ9Vδ2 T cells with an APC phenotype following CCR9 mediated migration.

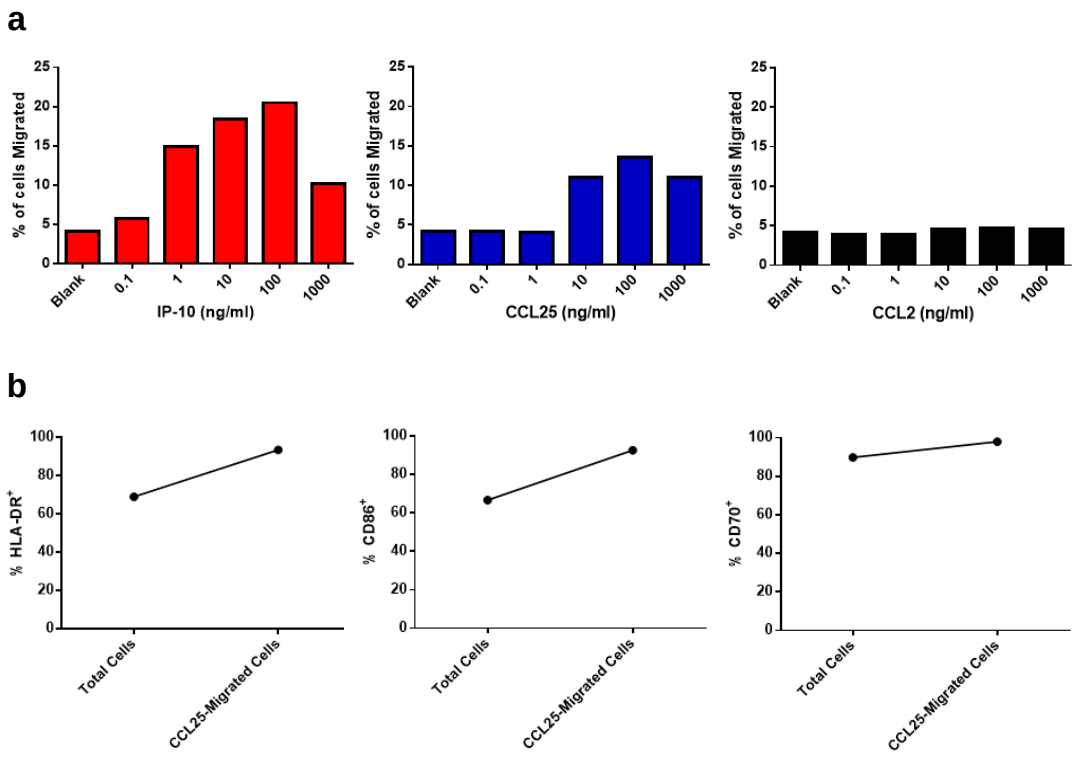

Figure 5.2 – Migration of IL-15 γδ T-APCs to chemokines. (a) Migration of IL-15 γδ T-APCs to IP-10, CCL25, and CCL2. IL-15 γδ T-APCs were cultured in the upper chamber of transwell plates, with different concentrations of each chemokine in lower chambers, and allowed to migrate for 3 hours. Numbers of migrated cells to each chemokine were calculated as a percentage of total input cells, determined by flow cytometry. **(b)** Expression of HLA-DR, CD86, and CD70 on IL-15 γδ T-APCs prior to use in migration assay and following 3 hour migration to CCL25. Percentages of APC marker positive cells were determined by flow cytometry. Data presented are derived from 1 healthy donor.

Having identified a preferential homing to the intestine by γδ T-APCs, assays were conducted to examine the homing potential of $CD4^+$ T cells upon polarisation by γδ T-APCs (Figure 5.3). Figure 5.3a displays the expression of each homing marker by naive $CD4^+$ T cells after 9 days of co-culture with different populations of γδ T-APCs. In response to IL-2 or IL-15 γδ T-APCs, naive $CD4^+$ T cells were induced to express significant levels of β7 integrin as compared to unstimulated naive $CD4^+$ T cells. IL-21 γδ T-APCs also induced β7 expression by responder cells, although this increase over naive cells was not statistically significant. CCR9 was not expressed by polarised naive $CD4^+$ T cells nor by unstimulated cells. Lastly, CLA expression by $CD4^+$ T cells was induced by IL-2 and IL-15 γδ T-APCs, although the expression levels observed were not significantly increased over unstimulated controls.

To identify which homing markers IL-22$^+$ CD4$^+$ T cells express, IL-15 γδ T-APC : naive CD4$^+$ T cell co-cultures were restimulated at day 9 and stained for intracellular IL-22, in combination with homing markers. Figure 5.3b displays representative stainings of IL-22 expression, and staining of β7, CLA, and CCR9 within the IL-22$^+$ population. The majority of IL-22 expressing cells also expressed β7, with a much smaller population being positive for CLA. No CCR9 expression was detected in IL-22 expressing cells. Of note, similar expression of β7 and CLA was detected on IFN-γ expressing CD4$^+$ T cells induced by IL-15 γδ T-APCs (data not shown). These data indicate that despite the capacity of giving rise to distinct CD4$^+$ T cell subsets as characterised by their cytokine profiles, under the experimental conditions chosen γδ T-APCs were unable to induce differential homing properties in those CD4$^+$ T cell subsets.

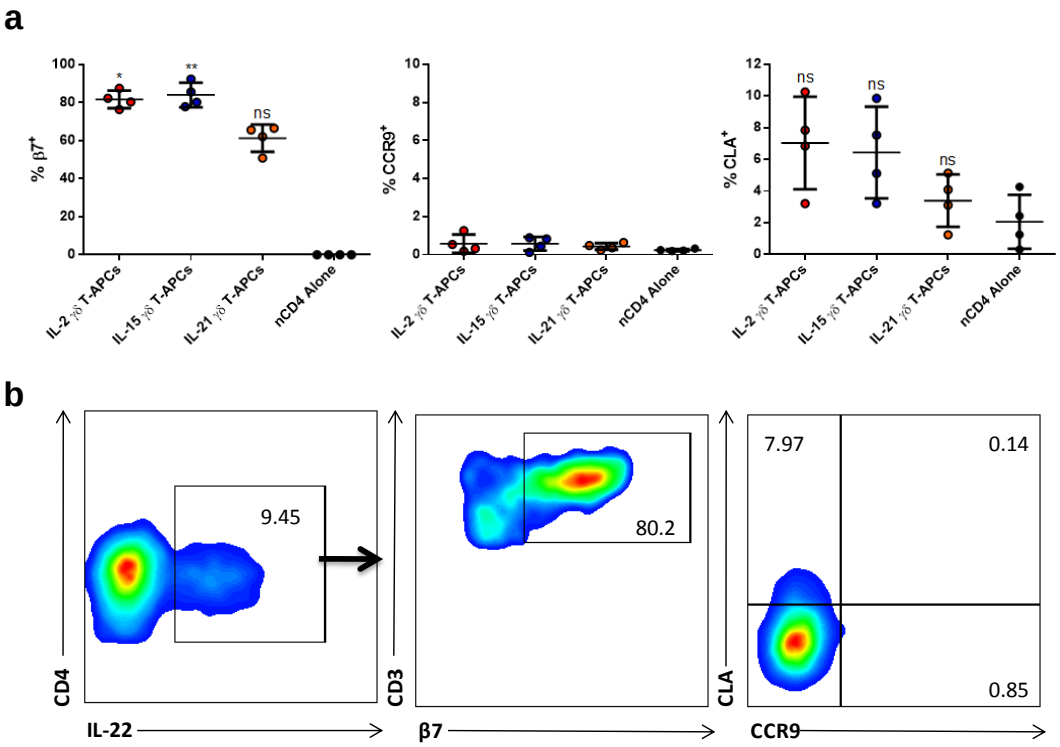

Figure 5.3 – Expression of homing markers by polarised naive CD4$^+$ T cells. (a) Expression of homing markers by naive CD4$^+$ T cells polarised by γδ T-APCs over 9 days of culture. At day 9, percentages of cells expression each homing marker by CFSE^{lo}CD4$^+$ T cells was determined by flow cytometry. **(b)** Expression of homing markers by IL-22-polarised CD4$^+$ T cells. Naive CD4$^+$ T cells were polarised by IL-15 γδ T-APCs over 9 days of culture, and at day 9 were restimulated and assessed for IL-22 and homing marker expression. Expression of β7 integrin, CLA, and CCR9 determined by gating on IL-22$^+$ cells. Plots representative of 4 healthy donors. Data points represent individual healthy donors. Horizontal lines display means of data sets. Error bars display standard deviation of samples. Statistical significance was determined using the Friedman test, followed by the Dunn's multiple comparison test, *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001, ns=not significant. Significance displayed in comparison to naive CD4 alone control.

5.4 - Vγ9Vδ2 T cells in Intestinal Biopsies

With the identification that IL-15 γδ T-APCs, and their responder cells, preferentially express intestinal homing receptors, attention focused on a potential role for γδ T-APCs in the intestine. Previous studies have identified that Vγ9Vδ2 T cells are indeed present in the intestine and can be obtained and studied in intestinal biopsies[242,345]. As such, assays were designed to assess the ability of Vγ9Vδ2 T cells to potentially act as APCs in the context of intestinal immunity. This work was

conducted in collaboration with Drs Neil McCarthy, James Lindsay and Andrew Stagg at the Blizard Institute, Queen Mary University of London.

To first examine this potential, human mucosal tissue was obtained from patients undergoing surgical resection for non-inflammatory disorders. Mucosal tissue, derived either from the terminal ileum or from colonic mucosa, was divided and cultured in 24 well plates in the presence of IL-2 and IL-15, with and without HMB-PP of culture. At day 3, supernatants from total mucosal cells were obtained and assessed for cytokine secretion by ELISA to assess the effect of specific Vγ9Vδ2 T cell activation in the context of total intestinal cells

Figure 5.4a displays the levels of cytokines expressed by total colon biopsy cells in the presence or absence of HMB-PP. In response to HMB-PP, increases in both IFN-γ and IL-22 were detected in cell supernatants. In contrast, no significant effects were observed on IL-17, IL-10, TNF-α or TGF-β. Considerable variation in the concentration of cytokines detected was observed between mucosal tissues obtained from different individuals. Given this inter-donor variation, the relative increase in IFN-γ and IL-22 was determined (Figure 5.4b). The increase in IFN-γ ranged from 20% to 75%, and increases in IL-22 ranged from 40% to 80%. Overall, addition of the Vγ9Vδ2 T cell-specific stimulus HMB-PP led to a significant induction of both IFN-γ and IL-22 in total, colon-derived cells.

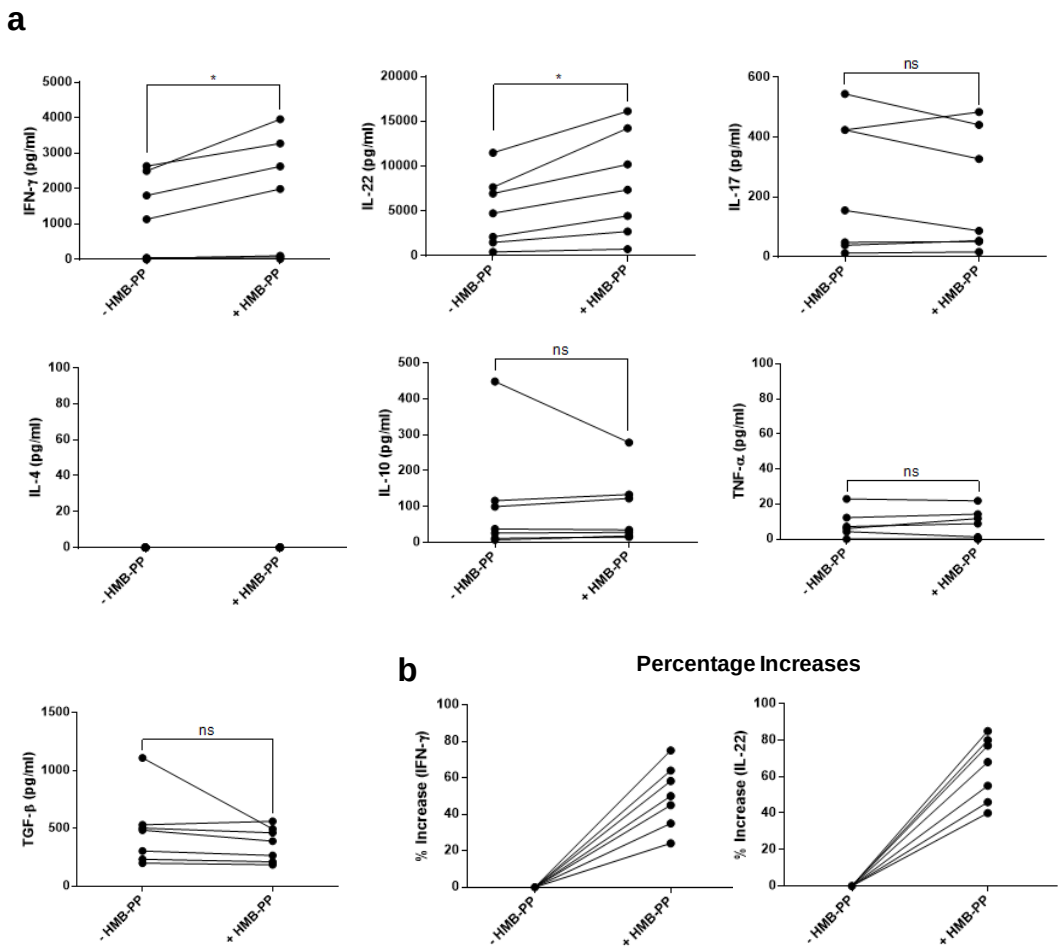

Figure 5.4 – Secretion of cytokines by total colon biopsy cells (a) Secretion of cytokines by total colon biopsy cells cultured in the presence or absence of HMB-PP. Colon biopsy tissue was cultured with IL-2 and IL-15, with or without 10 nM HMB-PP, for a period of 3 days. At day 3, supernatants were obtained and assessed for the presence of cytokines by ELISA. **(b)** Percentage increase in secretion of IFN-γ and IL-22 by total colon biopsy cells cultured in the presence or absence of HMB-PP. Data points represent individual pair-matched donors. Statistical significance was determined using the Mann-Whitney test, *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001, ns=not significant.

In identical experiments to those conducted with colon-derived cells, cultures of terminal ileum-derived cells were established, and cultured in the presence or absence of HMB-PP (Figure 5.5). Similar trends in IFN-γ and IL-22 secretion were detected, with increased levels in response to HMB-PP. However, with the limited number of terminal ileum samples obtained, increases were not statistically significant. Consistent increases were similarly not observed in any of the other cytokines assessed.

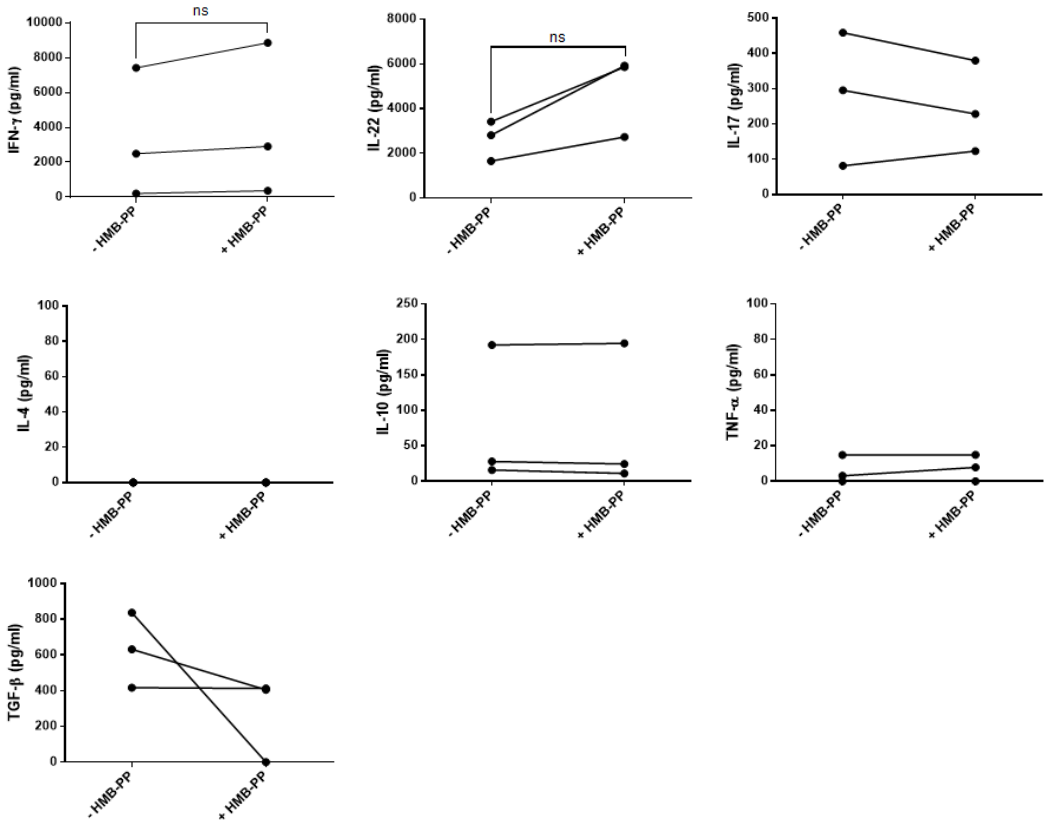

Figure 5.5 – Secretion of cytokines by total ileum biopsy cells (a) Secretion of cytokines by total ileum biopsy cells cultured in the presence or absence of HMB-PP. Ileum biopsy tissue was cultured with IL-2 and IL-15, with or without 10 nM HMB-PP, for a period of 3 days. At day 3, supernatants were obtained and assessed for the presence of cytokines by ELISA. **(b)** Percentage increase in secretion of IFN-γ and IL-22 by total ileum biopsy cells cultured in the presence or absence of HMB-PP. Data points represent individual pair-matched donors. Statistical significance was determined using the Mann-Whitney test, *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001, ns=not significant.

Overall, addition of HMB-PP to cultures of total cells, obtained from either colon or terminal ileum tissue, appeared to cause an increase in the production of IFN-γ and IL-22.

5.5 - Control of Naive $CD4^+$ T cell Responses by Intestinal γδ T-APCs

With the observation that simply stimulating Vγ9Vδ2 T cells in cultures of total biopsy cells led to increases in overall IFN-γ and IL-22 secretion, the two cytokines previously identified as induced by γδ T-APCs, the next stage was to assess the ability of intestinal Vγ9Vδ2 T cells to act as professional APCs in the same manner as blood-derived cells.

In the first instance, Vγ9Vδ2 T cells were sorted from cultures of total intestinal tissue samples, previously cultured for 7 days with IL-2 and IL-15 in the presence or absence of HMB-PP. Vγ9Vδ2 T cells were identified in cultures of total intestinal cells as previously described[242]. The percentages of $CD3^+Vδ2^+$ T cells pre-sort and cellular yields obtained post-sort are displayed in Table 5.1. With the limited number of samples obtained, it appeared increased numbers of Vγ9Vδ2 T cells were isolated from terminal ileum samples in comparison with colonic tissue, in paired donors. In addition, addition of HMB-PP to cultures over the 3 day period appeared to increase both the percentage and yield of Vγ9Vδ2 T cells in all samples. As such, significant numbers of cells could be isolated from tissue samples obtained.

Table 5.1 - Cell Sort Yield of Vδ2$^+$ T Cells from Intestinal Tissue Samples

Donor	Tissue	HMB-PP Treatment	CD3$^+$Vδ2$^+$ Cell Percentage	CD3$^+$Vδ2$^+$ Cell Yield
1	Colon	-	0.43%	2065
1	Colon	+	0.89%	4532
1	Ileum	-	0.55%	5189
1	Ileum	+	0.76%	8843
2	Colon	-	0.55%	5605
2	Colon	+	1.01%	11,534
3	Colon	-	0.24%	5039
3	Colon	+	0.37%	6765
4	Colon	-	0.44%	1489
4	Colon	+	0.54%	1885
4	Ileum	-	0.76%	4016
4	Ileum	+	0.82%	4574
5	Colon	-	0.05%	182
5	Colon	+	0.08%	318

Having confirmed the ability to isolate Vγ9Vδ2 T cells directly from intestinal tissue, attention shifted to the ability of these cells to become APCs. As previously discussed, total biopsy cells were cultured for 7 days in the presence of IL-2 and IL-15, with or without HMB-PP. At day 4, additional IL-2 and IL-15 was added to cultures. Upon reaching day 7 of culture, CD3$^+$ Vδ2$^+$ cells were sorted to high purity (>99.2% Vδ2$^+$) from total intestinal cells and assessed for expression of several APC markers.

Figure 5.6 shows the APC phenotype of intestinal-derived Vγ9Vδ2 T cells. Representative flow cytometry plots display the expression of APC markers by Vγ9Vδ2 T cells from cultures stimulated with or without HMB-PP (Figure 5.6a). In the absence of HMB-PP, Vγ9Vδ2 T cells exhibited moderate expression of all APC markers examined, and overall stainings mirrored those obtained with peripheral blood Vγ9Vδ2 T cells (Figure 3.15, Chapter 3). Cultures with HMB-PP displayed increased expression of HLA-DR, CD86, and ICOS-L, and CD70 expression appeared to be maintained. Figure 5.6b shows a summary of expression of APC markers by Vγ9Vδ2 T cells, derived from colon or terminal ileum tissue. In cultures of both colon and ileal cells, Vγ9Vδ2 T cells displayed an increase in expression of all APC markers, except CD70 for which percentages of positive cells remained

stable but high. There also appeared to be no difference between colon and ileum cells, although only a limited number of samples were analysed including 1 donor of terminal ileum tissue. Due to the low number of replicates, increases observed in APC marker expression were not statistically significant.

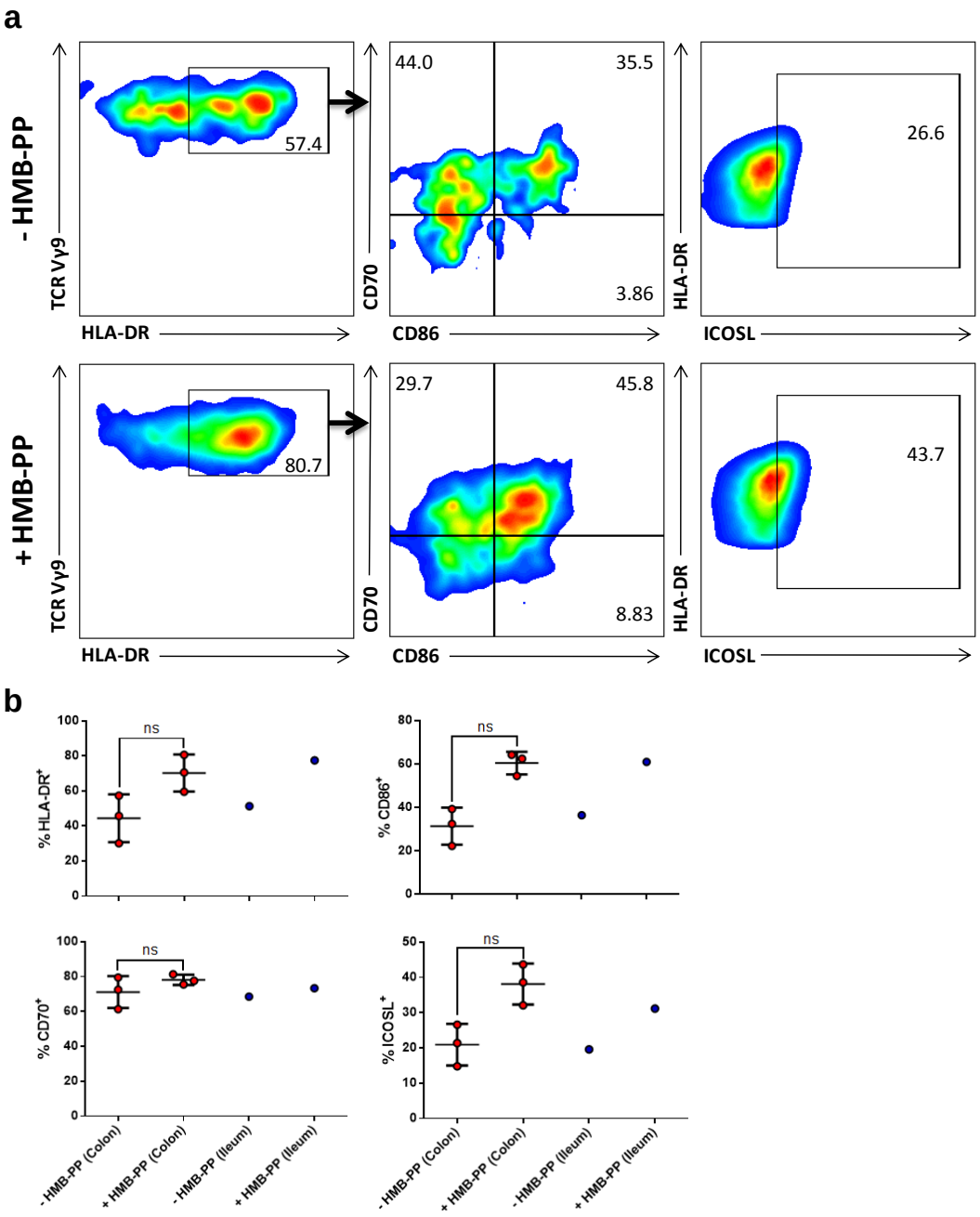

Figure 5.6 – Expression of APC markers by intestine-derived Vγ9Vδ2 T cells. (a) Representative flow cytometry plots displaying expression of APC markers by colon-derived Vγ9Vδ2 T cells. Total colon cells were cultured for 7 days with IL-2 and IL-15, in the presence or absence of 10 nM HMB-PP. At day 7, $Vδ2^+$ T cells were sorted by FACS and stained for expression of markers. Figures representative of 3 individual donors. Numbers on graphs display percentages of cytokine positive cells. CD86, CD70, and ICOSL stainings were obtained by gating on $HLA\text{-}DR^+$ cells. (b) Summary plots displaying expression of APC markers by $Vδ2^+$ T cells derived from colon and ileum biopsies. Dots display individual donors. Horizontal lines display means of data sets. Error bars display standard deviation of samples. Statistical significance was determined using the Mann-Whitney test, *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001, ns=not significant.

With the demonstration that intestinal Vγ9Vδ2 T cells are indeed capable of expressing an APC phenotype, the ability of these cells to initiate and polarise $CD4^+$ T cell responses was examined. In similar experiments to those conducted with peripheral blood-derived γδ T-APCs, intestinal Vγ9Vδ2 T cells were sorted from total intestinal cells at day 7, and subsequently cultured with CFSE stained naive $CD4^+$ T cells for a period of 9 days. Proliferation was assessed at day 9 instead of day 5 due to limited numbers of cells isolated from intestinal tissue.

Reflective of the enhanced APC phenotype, Vγ9Vδ2 T cells cultured in the presence of HMB-PP were stronger inducers of naive $CD4^+$ T cell proliferation, compared with HMB-PP negative cultures (Figure 5.7a). In addition, there did not appear to be any difference between colon and ileum derived Vγ9Vδ2 T cells, at least in the number of donors examined (Figure 5.7b). Similarly to experiments using blood-derived γδ T-APCs, polarised responder cells expressed both IFN-γ and IL-22, and no IL-17 expression was detected (Figure 5.7c). In addition, the majority of $IL\text{-}22^+$ cells were also $IFN\text{-}\gamma^+$. Vγ9Vδ2 T cells derived from either colon or ileum and cultured in the absence of HMB-PP, induced only low levels of cytokines in responder cells. The induction of both IFN-γ and IL-22 was increased when HMB-PP treated cells were used as APCs (Figure 5.7d). Again, no real differences could be identified between Vγ9Vδ2 T cells derived from either colon or terminal ileum tissue.

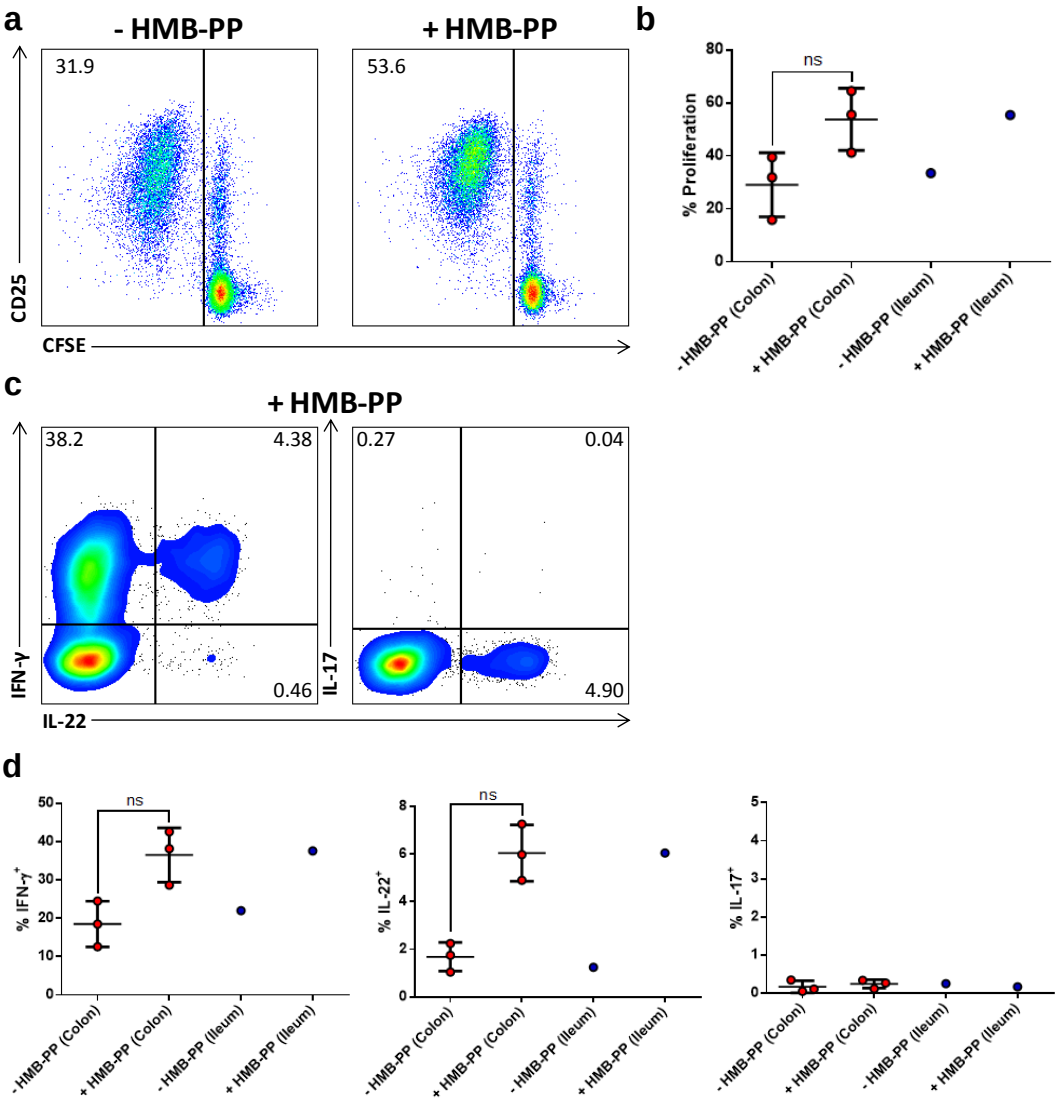

Figure 5.7 – Induction of naive CD4$^+$ T cell responses by intestinal γδ T-APCs. (a) Representative flow cytometry plots displaying proliferation of naive CD4$^+$ T cells, in response to co-culture with γδ T-APCs derived from a colon biopsy. γδ T-APCs were generated with IL-2 and IL-15 in the presence or absence of HMB-PP. Proliferation of naive CD4$^+$ T cells was determined at day 9 of culture by flow cytometry. **(b)** Summary plot displaying proliferation of naive CD4$^+$ T cells, in response to co-culture with γδ T-APCs derived from colon or ileum biopsies. **(c)** Representative flow cytometry plots displaying intracellular cytokine expression by naive CD4$^+$ T cells, polarised by γδ T-APCs derived from a colon biopsy. γδ T-APCs were generated with IL-2 and IL-15 in the presence of HMB-PP. Expression of cytokines was determined at day 9 of culture by flow cytometry. **(d)** Summary plots displaying intracellular cytokine expression by naive CD4$^+$ T cells, in response to co-culture with γδ T-APCs derived from colon or ileum biopsies. Data points represent individual donors. Horizontal bars display means of data sets. Error bars display standard deviation of samples. Statistical significance was determined using the Mann-Whitney test, *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001, ns=not significant.

5.6 - Roles of γδ T-APCs in Inflammatory Bowel Diseases

Given the results obtained in previous experiments, it appears that Vγ9Vδ2 T cells derived from the intestine are indeed able to act as APCs and trigger $CD4^+$ T cell responses. Further to this, potential roles of Vγ9Vδ2 T cells in the pathogenesis of IBD were postulated[345]. Since during this PhD study no intestinal tissue samples were available for an examination of local Vγ9Vδ2 T cell functions at inflamed sites, the APC function of circulating Vγ9Vδ2 T cells from the blood of IBD patients was examined instead.

A heterogeneous group of IBD patients presenting with either Crohn's disease or Ulcerative Colitis was recruited for the present studies. Given the recently identified effects of azathioprine treatment on γδ T cells[345], these patients were either untreated or had been off azathioprine treatment for >6 months. Patients were grouped into patients with either active or inactive disease, as determined symptomatically by the treating clinician, Dr James Lindsay.

In a similar manner to γδ T-APCs generated from the blood of healthy donors, Vγ9Vδ2 T cells were isolated from PBMC obtained from either inactive or active IBD patients and stimulated with HMB-PP and IL-15. At day 4 of culture, expression of APC markers was assessed by flow cytometry (Figure 5.8). Similarly to healthy γδ T-APCs, those derived from IBD patients displayed expression of several APC markers, including HLA-DR and CD86. Expression levels of these markers by γδ T-APCs derived from patients with inactive disease showed similar levels of expression to healthy γδ T-APCs (Figure 3.15, Chapter 3). In contrast, γδ T-APCs derived from patients with active disease showed significantly reduced expression of both HLA-DR and CD86 but not CD70 and ICOS-L (Figure 5.8).

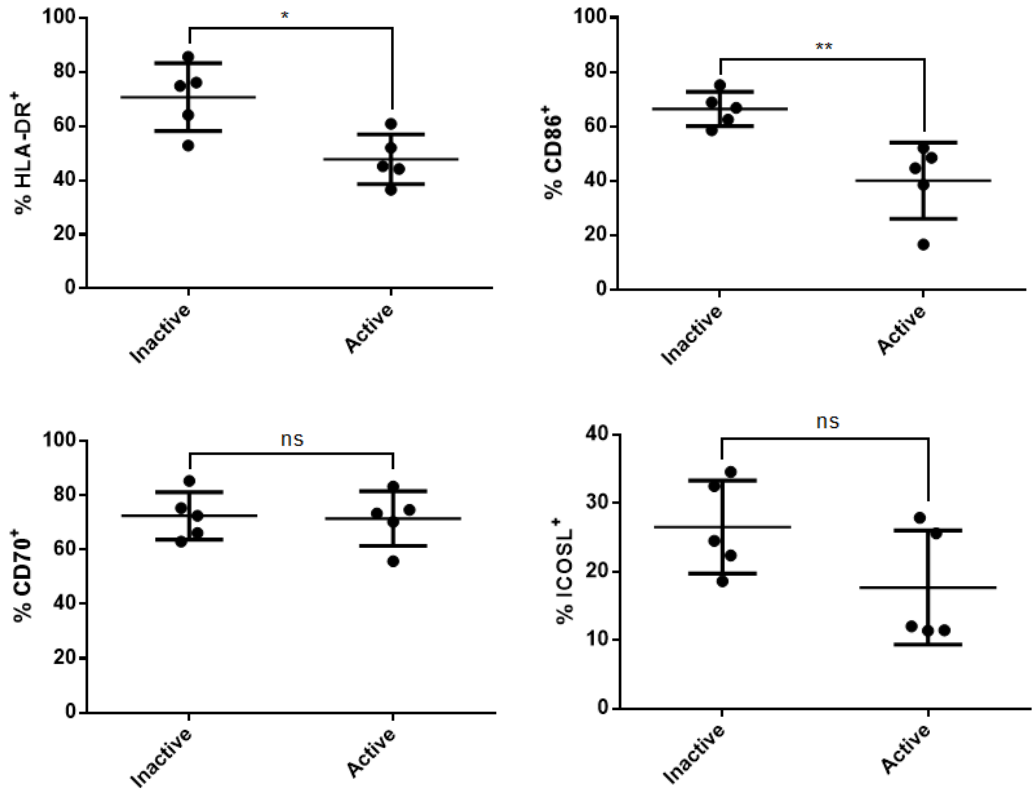

Figure 5.8 – Expression of APC markers by γδ T-APCs isolated from patients with IBD. Summary plots displaying expression of APC markers by Vγ9Vδ2 T cells, isolated from the peripheral blood of inflammatory bowel disease patients with either inactive or active disease. Vγ9Vδ2 T cells were isolated and cultured for 4 days in the presence of 10 nM HMB-PP and IL-15. At day 4 of culture, cells were assessed for expression of APC markers by flow cytometry. Results obtained by gating on live, single, $V\delta2^+$ cells. Dots on graphs display results obtained from individual donors. Horizontal lines display means of data sets. Error bars represent standard deviation of samples. Statistical significance was determined using the Mann-Whitney test, *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001, ns=not significant.

With the indication that γδ T-APCs derived from inactive and active IBD patients may possess a differential ability to act as APCs, these cells were subsequently co-cultured with allogeneic naive $CD4^+$ T cells obtained from healthy donors, and the polarisation of such responder cells was assessed (Figure 5.9). Expression of cytokines was determined by intracellular analysis. Similarly to healthy γδ T-APCs, induction of both IFN-γ and IL-22 was observed in responder cells when polarised by γδ T-APCs generated from inactive IBD patients. However, in accordance with the impaired APC phenotype observed with γδ T-APCs generated from active IBD

patients, the induction of both IFN-γ and IL-22 were significantly reduced. No IL-17 induction in naive $CD4^+$ T cells was observed under any condition tested.

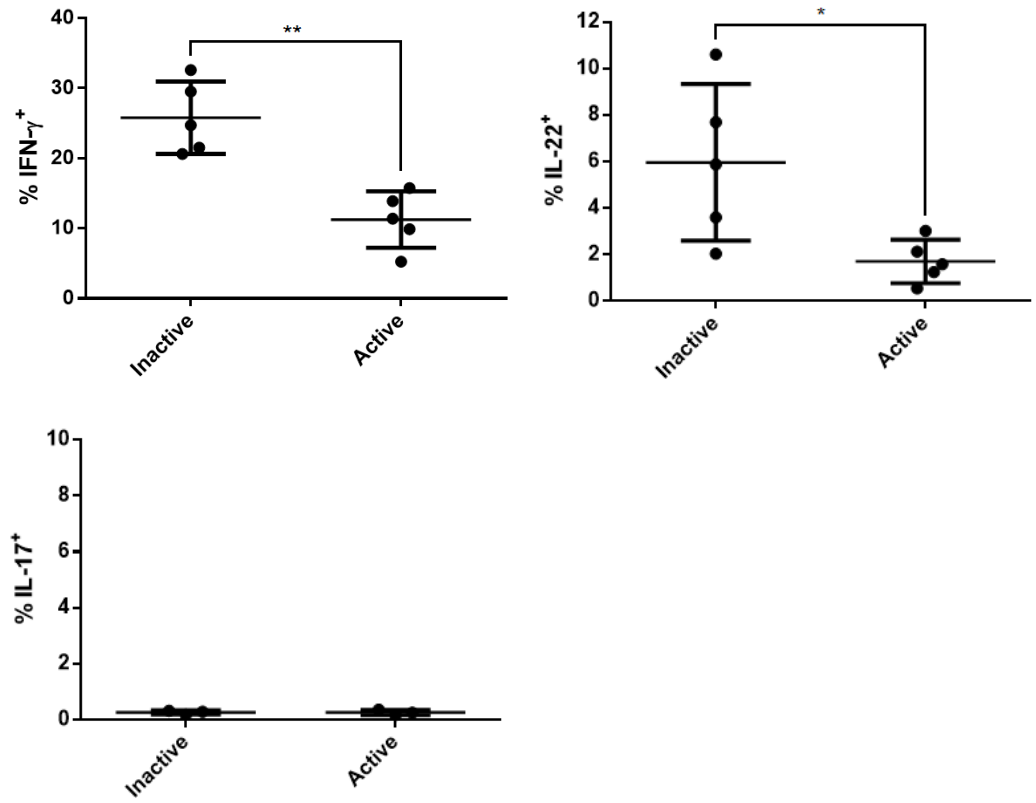

Figure 5.9 – Expression of cytokines by naive CD4$^+$ T cells polarised by IBD-derived γδ T-APCs. (a) Summary plots displaying expression of cytokines by naive CD4$^+$ T cells, polarised by IL-15 γδ T-APCs obtained from IBD patients with either inactive or active disease. Vγ9Vδ2 T cells were isolated from peripheral blood of IBD patients and stimulated with HMB-PP and IL-15 for 4 days, and were subsequently cultured with allogeneic naive CD4$^+$ T cells obtained from healthy donors for 9 days. At day 9, intracellular expression of cytokines by CD4$^+$ T cells was assessed by flow cytometry. Results obtained by gating on live, single, CD3$^+$CD4$^+$Vγ9$^-$CFSE^{lo} cells. Points on graphs display results obtained from individual donors. Horizontal lines display means of data sets. Error bars represent standard deviation of samples. Statistical significance was determined using the Mann-Whitney test, *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001, ns=not significant.

The secretion of cytokines by polarised naive CD4$^+$ T cells was also examined by ELISA (Figure 5.10). Similarly to intracellular expression, IFN-γ and IL-22 were both identified in cell culture supernatants of naive CD4$^+$ T cells, cultured IBD patient-derived γδ T-APCs. Again, decreased secretion of both cytokines was detected in co-cultures with γδ T-APCs generated from active patients as compared to cells from inactive IBD patients, although given the small number of donors examined such differences were not statistically significant. Similar trends were also observed for the secretion of IL-4 and TNF-α into the culture supernatants, indicating an

overall reduction in the ability of γδ T-APCs derived from active IBD patients to stimulate naive $CD4^+$ T cell responses. Only minimal levels of IL-17 and IL-10 were detected in these culture supernatants.

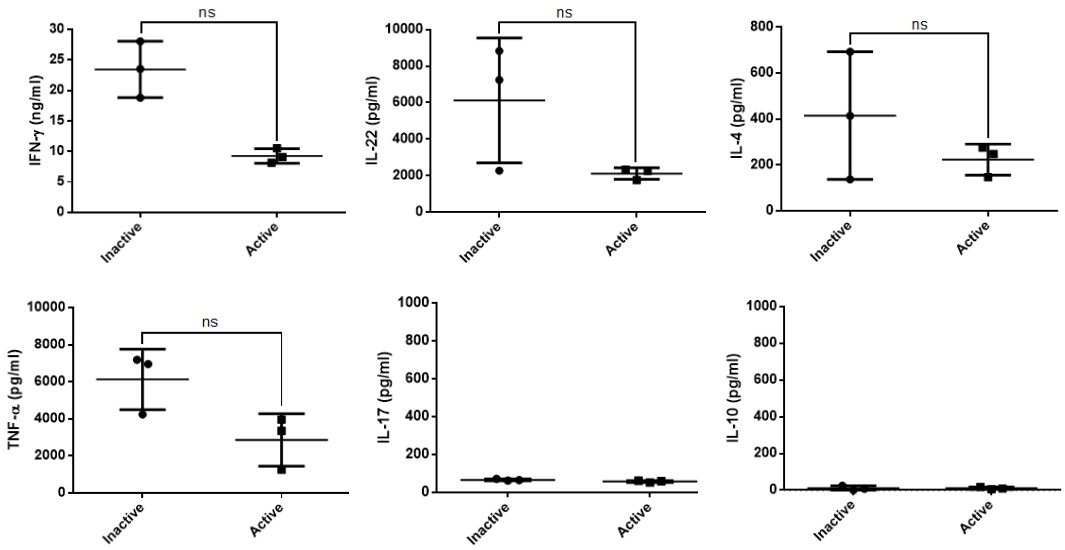

Figure 5.10 – Secretion of cytokines by naive CD4$^+$ T cells polarised by IBD-derived γδ T-APCs. (a) Summary plots displaying secretion of cytokines by naive CD4$^+$ T cells, polarised by IL-15 γδ T-APCs obtained from IBD patients with either inactive or active disease. Vγ9Vδ2 T cells were isolated from peripheral blood of IBD patients and stimulated with HMB-PP and IL-15 for 4 days, and were subsequently cultured with allogeneic naive CD4$^+$ T cells obtained from healthy donors for 9 days. At day 9, cells were restimulated for 24 hours and cytokine secretion was assessed by ELISA. Points on graphs display results obtained from individual donors. Horizontal lines display means of data sets. Error bars represent standard deviation of samples. Statistical significance was determined using the Mann-Whitney test, *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001, ns=not significant.

5.7 - Discussion

A number of studies have demonstrated that blood-derived Vγ9Vδ2 T cells are capable of becoming potent APCs and initiating $CD4^+$ and $CD8^+$ T cell responses, findings which have been replicated in this study. However, data presented here indicate for the first time that γδ T-APCs may play a role locally in intestinal immunity, with blood-derived cells expressing receptors for the migration to the intestine, and also the ability of intestine-derived Vγ9Vδ2 T cells to become APCs and drive IFN-γ and IL-22 responses.

Based on the expression of several homing markers expressed by both γδ T-APCs and responder $CD4^+$ T cells, the present findings demonstrate that, under the conditions examined, both APCs and responder cells preferentially home to the intestines as opposed to skin. However, a number of additional homing receptors were not examined in the present study, most importantly CCR8, which if expressed would direct recruitment to the skin[358]. γδ T-APCs and $CD4^+$ T cells displayed high levels of β7 integrin expression, and additionally a population of CCR9-expressing cells was identified among γδ T-APCs. In contrast, expression of the skin-specific receptor CLA was downregulated on γδ T-APCs, and only a small population of $CD4^+$ T cells expressed this receptor. Circulating Vγ9Vδ2 T cell populations have been identified to express both α4β7 and CLA upon isolation from human peripheral blood[242,366], and as such culture of γδ T-APCs led to shift in expression of these receptors towards α4β7 and away from CLA expression. Of note, β7 integrin is able to combine with either α4 or αE subunits to form distinct receptors, which recognise MAdCAM-1 and E-cadherin, respectively. To confirm that the $\beta7^+$ cells described here co-express the α4 subunit, and are able to home to the intestine, MAdCAM-1 binding could be examined in future studies, which would also confirm that expression of α4β7 by both γδ T-APCs and responding $CD4^+$ T cells is functional. Indeed, Vγ9Vδ2 T cells have previously been identified to express functional α4β7 in MAdCAM-1 binding assays[242]. In combination with β7 expression, CCR9 was identified in the present study on a population of γδ T-APCs, and proven to be functional in migration assays toward its ligand CCL25. In comparison with CXCR3-mediated migration to CXCL10, migration to CCL25 was less pronounced, likely reflecting the lower expression of CCR9 (~60%) compared with CXCR3 (~95%). However, further donors are required to confirm the functionality of CCR9 expressed by γδ T-APCs.

The regulation of homing receptor expression is a complex process and relies on a number of environmental factors. Retinoic acid, which is produced by dendritic cells in the intestine and associated lymphoid organs[367], has been shown to upregulate α4β7 expression by γδ T cells, as well as αβ T cells[242]. In addition, inhibition of retinoic acid leads to decreased expression of α4β7 and increased expression of CLA. Of note, the RPMI-1640 medium that was used for culturing γδ T-APCs in this study contains traces of retinoic acid[242], and as such may have directly allowed for the expression of α4β7 by Vγ9Vδ2 T cells and $CD4^+$ T cells. Regulation of CCR9 expression by T cells is less well defined. Retinoic acid appears important for CCR9 induction on responder cells, and DC subsets derived from mesenteric lymph nodes are able to induce CCR9 in αβ T cell populations[368], although the specific mechanisms behind this induction are unknown. As such, it is unclear why in this study CCR9 was identified on Vγ9Vδ2 T cells but not on responding $CD4^+$ T cells. Given the role of intestinal DCs in the induction of CCR9 in αβ T cell populations, it may be possible that only intestine-derived γδ T-APCs are capable of inducing CCR9 whereas blood or skin derived γδ T-APCs may induce expression of other chemokine receptors on $CD4^+$ T cells. Of note, CCR9 expression does identify cells which are able to migrate to the intestine, however CCR9-independent recruitment of cells to this site has been identified[369].

Given the gut-tropism of the cells used in these assays, the ability of γδ T-APCs to mediate intestinal immunity was investigated further. By examining the response of total intestinal-derived cells to HMB-PP, significant increases in the secretion of IFN-γ and IL-22 were observed, drawing parallels between the cytokines induced in naive $CD4^+$ T cells by γδ T-APCs in MLR cultures and these intestinal cell cultures. In contrast, IL-17 and IL-10, among other cytokines, were not increased in intestinal culture supernatants. Given the short culture period of 3 days compared to polarisation of naive $CD4^+$ T cells over 9 days, it is likely that memory responses were stimulated in these cultures rather than naive responses. Significant levels of IFN-γ and IL-22 were already detected in culture supernatants in the absence of HMB-PP. However, the HMB-PP negative conditions did not represent a true negative control, as cells were treated with IL-2 and IL-15 to support cell survival, and may have become exposed to HMB-PP or other cell ligands as a result of the issue processing. As such, a comparison of untreated cells (no cytokines) with HMB-PP treated cells in future experiments may present a clearer picture of the effects of HMB-PP on total intestinal cell cultures. As no mechanism was identified behind the induction of these two cytokines in total intestinal cell cultures, the

promotion of these responses by γδ T-APCs is but one explanation for the effects seen. Regardless, it is an indication that stimulation of Vγ9Vδ2 T cells drives IFN-γ and IL-22 responses. Indeed, the lack of TNF-α identified in intestine biopsy cultures contrasts with the potent secretion of this cytokine exhibited by stimulated Vγ9Vδ2 T cells, indicating TNF-α uptake and action on $CD4^+$ T cells.

With the indication that Vγ9Vδ2 T cell activation in the context of total intestinal cell populations leads to increases in IFN-γ and IL-22 secretion, colon and ileum-derived Vγ9Vδ2 T cells were assessed for their ability to become APCs. Indeed, these cells were able to exhibit strong expression of APC markers, and induced robust naive $CD4^+$ T cell responses, favouring IFN-γ and IL-22 induction. Interestingly, the expression of APC markers by Vγ9Vδ2 T cells, to similar levels to those observed with blood Vγ9Vδ2 T cells, required a longer culture period. This was likely due to increased populations of contaminating cells in intestinal cultures. A previous study had attempted to generate γδ T-APCs from intestine-derived Vγ9Vδ2 T cells[242], and observed induction of HLA-DR and CD70 but no CD86. Again, this may have been due to the shorter culture period utilised as compared to this study, and the lack of IL-15 as stimulant. The magnitude of IFN-γ and IL-22 responses in naive $CD4^+$ T cell populations identified were similar to those observed using blood derived γδ T-APCs. However, an appropriate control would be to use intestine-derived DCs to polarise naive $CD4^+$ T cell responses, as this would allow direct comparison of several intestinal APC populations and determine if intestine-derived γδ T-APCs have a preferential ability to promote IFN-γ and IL-22 type immunity. In addition, it would be useful to use γδ T-APCs to polarise memory $CD4^+$ T cells derived from the intestine, to identify any differences in responses as compared to memory $CD4^+$ T cells derived from the peripheral blood.

Having identified a role for γδ T-APCs in intestinal immunity, a potential contribution of this cell population to IBD pathology was postulated. Indeed, Vγ9Vδ2 T cells are recruited to the intestine during inflammatory conditions and may play roles in the pathogenesis of intestinal autoimmune diseases[242]. Given the lack of access to inflamed bowel samples, the ability of Vγ9Vδ2 T cells derived from the peripheral blood of patients with IBD to become APCs was examined. IBD of course show significant inflammation in the intestine, but several systemic effects can be observed in these patients, in the blood and skin, among other locales[370,371]. Similarly, changes in Vγ9Vδ2 T cell populations in the blood have been observed in patients with IBD, from expression of homing markers to memory subsets observed[242,345]. Given the systemic inflammatory state of patients with active

disease, it was expected that Vγ9Vδ2 T cells derived from peripheral blood may possess an increased ability to become APCs. Instead, data obtained revealed Vγ9Vδ2 T cells derived from patients with active disease showed a less pronounced ability to become APCs as compared with inactive disease, displaying significantly reduced expression of HLA-DR and CD86. This reduced ability was reflected in a lower induction of IFN-γ and IL-22 in naive $CD4^+$ T cell responders. One explanation for this is that central and effector memory cell populations of Vγ9Vδ2 T cells have been reported to decrease in frequency in the blood of Crohn's disease patients, whereas these populations are increased in inflamed intestinal sites[345]. With the effect of IL-2 and IL-15 somewhat restricted to the effector and memory stages of Vγ9Vδ2 T cells[277], it may be that the Vγ9Vδ2 T cells remaining in the peripheral blood of active disease patients are those less capable to becoming APCs, with the more capable cells being recruited to inflammatory sites.

While the division between active and inactive disease has identified differences between Vγ9Vδ2 T cell ability to become APCs, this division is arbitrary and could be improved upon recruitment of further patients. The diagnosis of IBD includes both Crohn's disease and Ulcerative Colitis; two distinct autoimmune diseases affecting different intestinal sites and differing in their immunological background[370]. Consequently, stratifying patients according to the type IBD may highlight disease-specific differences between Vγ9Vδ2 T cells and allow for a better definition of their involvement in the pathogenesis of each condition. In addition, the active/inactive division is based on symptomatic assessment by clinicians, and as such ranking patients based on expression of inflammatory markers or scoring of disease severity may highlight further effects of Vγ9Vδ2 T cells.

Overall, the data presented in this Chapter have highlighted a potential role for Vγ9Vδ2 T cells to become APCs in the intestine, and mediate local immunity during infection and disease scenarios.

Chapter 6 - General Discussion

6.1 - Summary

Vγ9Vδ2 T cells have been previously reported to represent a novel type of professional APC that is able to take up, process and present antigens to naive and memory $CD4^+$ and $CD8^+$ T cells. The aims of this study were to investigate the different conditions which induce an APC phenotype in Vγ9Vδ2 T cells, and the different T helper responses these APCs trigger in naive $CD4^+$ T cells. Potential sites or functional niches where γδ T-APCs may facilitate such $CD4^+$ T cell responses were to be investigated using appropriate samples from healthy individuals and patients.

Expanding on previous studies into γδ T-APCs, data presented here show that cytokines belonging to the common γ-chain family differentially regulate the APC phenotype of Vγ9Vδ2 T cells. In combination with TCR stimulation by the Vγ9Vδ2 T cell ligand HMB-PP, both IL-2 and IL-15 induced strong APC phenotypes in Vγ9Vδ2 T cells as identified by expression of key APC markers and co-stimulatory molecules in addition to high levels of proliferation, cytokine production, and antigen uptake. In contrast, IL-7 did not support the generation of γδ T-APCs, favouring cell proliferation and cytokine production but not expression of APC markers. Lastly, IL-21 supported a suboptimal APC phenotype, with Vγ9Vδ2 T cells cultured in the presence of this cytokine displaying reduced levels of proliferation, cytokine production and expression of APC markers as compared to cells cultured with IL-2 or IL-15. Given the different capacity of each cytokine to support the APC function of Vγ9Vδ2 T cells, APCs generated under these conditions were utilised for functional assays to investigate their $CD4^+$ T cell stimulating and polarising potential.

Using γδ T-APCs generated with IL-2, IL-15, IL-7, or IL-21, responses of naive and memory $CD4^+$ T cells were examined. Both IL-2 and IL-15 γδ T-APCs, in agreement with their expression of key APC markers, induced robust naive and memory $CD4^+$ T cell proliferative responses, which matched the $CD4^+$ T cell responses induced by mature monocyte-derived dendritic cells (mDCs). When the resulting effector phenotype of responder $CD4^+$ T cells was assessed, significant induction of Th1 cells was observed by both IL-2 and IL-15 γδ T-APCs, to comparable levels as those obtained when using mDCs. The most unexpected finding was that of IL-22 induction in naive and memory $CD4^+$ T cells; IL-15 γδ T-APCs induced the highest levels of IL-22 expression in both naive and memory responder cells, compared with

all other APC types examined. These IL-22 expressing cells were negative for IL-17 and hence did not represent Th17 cells. While many of these IL-22^{+}IL-17^{-} T cells co-expressed IFN-γ and were reminiscent of Th1 cells, there was a proportion of single positive IL-22^{+} cells induced, representing true Th22 cells. In accordance with the potent induction of IFN-γ and IL-22, IL-15 γδ T-APCs also favoured the expression of the Th1 and Th22 associated transcription factors TBX21 and AHR, respectively.

In striking contrast to the effects ofIL-2 and IL-15 γδ T-APCs on naïve CD4^{+} T cells, IL-21 γδ T-APCs induced a significant population of IL-10 producing CD4^{+} T cells, which were not identified under any other culture conditions assessed. Unfortunately, the resulting function of these IL-10^{+} cells could not be identified during the course of this PhD thesis, with no preferential expression of any of the lineage-specific transcription factors tested. Finally, all γδ T-APC populations that were able to induce CD4^{+} T cell responses triggered increased levels of IL-4, indicative of an increased generation ofTh2 cells, as compared to DCs and monocytes.

With the identification that IL-15 γδ T-APCs favoured high levels of IL-22 and AHR expression by responder CD4^{+} T cells, attention turned to the mechanism behind such induction. In terms of polarising cytokines, IFN-γ and TNF-α played important roles in CD4^{+} T cell polarisation, favouring Th1 (IFN-γ, TBX21) and Th2 (IL-22, AHR) type responses, respectively. However, the induction and polarisation of CD4^{+} T cell responses by γδ T-APCs were also reliant on co-stimulatory interactions, in addition to cell-cell contact between APCs and responder cells via LFA-1. CD80, CD86, and CD48 expressed by γδ T-APCs were all required for robust induction of CD4^{+} T cell proliferation and survival. In addition to these molecules, CD70 and ICOSL expression by γδ T-APCs promoted IFN-γ and IL-22 induction in CD4^{+} T cells, respectively. Of note, such a role for ICOSL has not been described before.

Subsequent investigations were oriented towards potential peripheral sites or disease conditions where such interactions may take place *in vivo*. Both γδ T-APCs and naive CD4^{+} T cells displayed preferential expression of intestinal homing receptors as opposed to skin homing molecules, and with the knowledge that Vγ9Vδ2 T cells play important roles in the intestinal immune response[242,345,366], the ability of intestinal Vγ9Vδ2 T cells to function as APCs was assessed. Indeed, simply stimulating total colon or terminal ileum-derived cells with HMB-PP, and therefore only directly activating Vγ9Vδ2 T cells, led to significant increases in both IFN-γ and IL-22 secretion. This indicated that Vγ9Vδ2 T cells may indeed favour

Th1 and Th22 responses in the intestinal environment. Subsequently, intestine-derived Vγ9Vδ2 T cells proved capable of becoming APCs and stimulating naive $CD4^+$ T cell responses to produce IFN-γ and IL-22. Without access to inflamed tissue from IBD patients, the systemic effects on blood Vγ9Vδ2 T cells were investigated in IBD patients with active or inactive disease, with reference to APC function of Vγ9Vδ2 T cells. Given the systemic inflammation observed in patients with IBD[242], it was expected that peripheral blood-derived Vγ9Vδ2 T cells from patients with active disease may exhibit increased APC and inflammatory ability over patients with active inactive disease. In contrast to expectations, Vγ9Vδ2 T cells derived from patients with active IBD were less capable of becoming APCs, inducing lower $CD4^+$ T cell responses in terms of proliferation and cytokine production, than in patients with inactive disease.

Overall, data obtained during this study highlighted the capacity of Vγ9Vδ2 T cells to polarise naive and memory $CD4^+$ T cells towards distinct responses, depending on the original Vγ9Vδ2 T cell culture microenvironment. Further to this, a potential role for Vγ9Vδ2 T cells to act as APCs in the intestine has been revealed, although this novel function may not be restricted to this site alone.

6.2 - Regulation of Vγ9Vδ2 T Cell APC Function

Whilst data presented in this study have elucidated several factors which are able to regulate the APC potential of Vγ9Vδ2 T cells, the exact mechanisms behind this function remain unknown. It is well documented that cellular activation is required for the induction of APC function in Vγ9Vδ2 T cells, with studies either activating cells directly using HMB-PP or IPP, or indirectly with zoledronate[322,325,326]. It is unclear whether it is TCR stimulation in general, or the specific action of HMB-PP, which allows for upregulation of APC characteristics. To investigate this, activation of Vγ9Vδ2 T cells with different stimuli, such as HMB-PP, αCD3 or αTCR antibody stimulation, in combination with cytokines may help to solve this question. In addition, while several cytokines investigated here, such as IL-2, IL-15, and IL-21, promoted the APC function of Vγ9Vδ2 T cells, other cytokines which were not investigated here may be important in the regulation of the APC function. What also remains unclear is the mechanism behind the differential regulation of APC phenotype by different common γ-chain cytokines. The clearest contrast between IL-2/IL-15 and IL-7 on induction of APC function was their differential ability to induce an APC phenotype, as well as their effects on the memory phenotype of expanded Vγ9Vδ2 T cells. Microarray analysis of Vγ9Vδ2 T cells stimulated with each common

γ-chain cytokine may thus reveal pathways that are important in the decision of Vγ9Vδ2 T cells to become APCs or not. Besides common γ-chain cytokines, cytokines such as IL-6 and IL-23 have been described as important regulators of other Vγ9Vδ2 T cell functions[281,289], and as such may have similar effects on γδ T-APC generation. Lastly, the role of co-stimulation in the induction of APC phenotype is largely unknown. While some studies have presented data to suggest opsonising antibodies induce and aid certain aspects of Vγ9Vδ2 T cell APC function via interaction with CD16[327,328], the role of molecules such as CD28 and CD27 expressed by Vγ9Vδ2 T cells have not been studied with respect to APC phenotype[372]. Given the importance of co-stimulation in the immune system, the question as to whether stimulation via these pathways would permit or alter APC function is an interesting area to investigate.

6.3 - Vγ9Vδ2 T cells as an APC Subset

One of the main findings of this study was the identification that IL-15 γδ T-APCs are more efficient at promoting IL-22 expression in naive and memory $CD4^+$ T cell populations than all other types of APC examined. These findings evoke similar studies that examined the ability of different types of DCs to polarise $CD4^+$ T cells. Indeed, while DCs are capable of remarkable plasticity with reference to directing $CD4^+$ T cell responses depending on the context[206], some DC subsets have proven capable of promoting certain T helper lineages over others. With particular respect to IL-22[121]; blood plasmacytoid DCs stimulated with CpG have been shown to act as potent Th22 inducers that outperform LPS-stimulated conventional DCs. In agreement with the important roles of IL-22 in skin homeostasis and immunity, human skin Langerhans cells are much better in promoting IL-22 responses, by both $CD4^+$ T cells and $CD8^+$ T cells than dermal DCs and monocyte-derived DCs[201]. Of note, Langerhans cell-induced IL-22^+CD4^+ T cells are negative for IFN-γ, IL-4, and IL-17, although other populations positive for each of these other cytokines were also identified in total cell cultures. However, no mechanism has been identified for this preferential induction of IL-22 by Langerhans cells. Given the potent induction of IL-22 by IL-15 γδ T-APCs, a direct comparison of γδ T-APCs with plasmacytoid DCs and Langerhans cells would identify whether these different types of APCs are equivalent in their IL-22 promoting function, and whether the same molecular pathways are involved in inducing IL-22 in $CD4^+$ T cells. Further to this, all DC subsets identified as Th22 inducing APCs also induced varying levels IL-17 and IFN-γ expression in responder cells[121,201]. The overall phenotype of IL-15 γδ T-APC-

induced naive $CD4^+$ T cell responder populations, identified in this study as containing IFN-γ^+, IL-4^+, and IL-22^+ cells, in the absence of IL-17^+ cells, appears to vary in comparison with all DC polarising data presented, where IL-17 is consistently induced to varying degrees. To date, no specific intestinal DC subsets have been identified as optimal Th22 or IL-22 inducers in humans, potentially identifying a functional niche where γδ T-APC mediated IL-22 induction may be facilitated.

With respect to the mechanism behind γδ T-APC induction of IL-22, combinations of TNF-α and ICOSL were identified as essential in this response, and consequently stimulation of naive $CD4^+$ T cells with TNF-α and ICOS stimulation leads to induction of IL-22 and *AHR* expression. This role of ICOSL in promoting IL-22 responses has not been reported before. Earlier studies utilising blood and skin-derived DCs in humans identified IL-6 and TNF-α as Th22 cell promoting factors, in addition to tissue specific factors such as the active form of vitamin D3, produced in the skin[121]. DCs of course have been shown to express ICOSL, inducing Th17, Treg, or Th2 cells depending on the culture conditions[373,374]. A study of skin-derived human DC subsets did investigate the role of ICOSL in the polarisation of IL-17, IL-21, and IL-22 responses in $CD4^+$ T cell populations[202], although no significant effects of ICOSL blockade were identified on IL-22 induction, whereas ICOSL stimulation appeared to inhibit IL-21 responses. Given the fact that Langerhans cells and other DC subsets produce IL-6[375], a factor important in Th22 induction, it may be that DCs rely less on ICOSL and other co-stimulatory molecules compared with γδ T-APCs, and are able to induce Th22 cells even when ICOSL action is inhibited, via IL-6. Indeed, as discussed previously DCs favour Th1 induction by secreting IL-12[206], whereas γδ T-APCs induce Th1 cells via CD70 expression in addition to IFN-γ, supporting the observation that γδ T-APC mediated $CD4^+$ T cell polarisation is more dependent on co-stimulatory molecules than polarising cytokines. Given the lack of IL-6 production by γδ T-APCs shown in this study, and a failure to block IL-22 production by anti-IL-6 treatment, γδ T-APC induction of IL-22 type responses appears to rely on ICOSL function. In addition to these observations, IL-22 responses promoted by Langerhans cells and plasmacytoid DCs are predominantly IFN-γ^-, representing Th22 cells[121,201], whereas in γδ T-APC co-cultures, IL-22 expressing cells were predominantly IFN-γ^+. Supported by the observation that ICOS/TNF-α stimulated naive $CD4^+$ T cells express predominantly IL-22^+IFN-γ^+ cells, whereas IL-6/TNF-α stimulated cells were split between IL-22^+IFN-γ^+ and IL-22^+IFN-γ^- cells, the main difference between γδ T-APC and DC induced IL-22 responses may be in the co-expression of IFN-γ. As such, these responder cell populations may be involved in

different aspects of immune responses or tissue homeostasis. It is important to note that all APCs express a range of polarising factors, which all act agonistically or antagonistically to induce the overall $CD4^+$ T cell responses induced. As such, while specific induction of IL-22 can be supported by the action of factors such as ICOSL and IL-6, other molecules may also be involved in directing IL-22 and other responses as well.

A function of γδ T-APCs that has not been investigated in detail before, and was only touched on in this study, is the ability of γδ T-APCs to induce specific homing receptor expression on responder cells. Several factors are important in the induction of chemokine receptors and tissue-specific integrins on $CD4^+$ and $CD8^+$ T cells following stimulation, not least of which are environmental factors, often metabolised by APCs such as DCs. For example, retinoic acid produced in the intestine by DCs is important in the induction of CCR9 and α4β7 expression by both DCs and αβ T cells[376,377]. As such, αβ T cell activation by retinoic acid-producing DCs in the mesenteric lymph nodes is able to induce an intestinal-homing phenotype in responder αβ T cells[378]. However, this ability to confer intestine-homing capacity on responder cells is restricted to subsets of DCs identified by CD103 expression. It remains unclear whether γδ T-APCs are able to metabolise factors important in induction of homing receptors, such as retinoic acid. Indeed, CD103-expressing Vγ9Vδ2 T cell populations can be identified in the intestinal mucosa[345]. In addition, skin specific factors derived from keratinocytes have been identified as essential in the induction of the skin-specific chemokine receptor CCR8[358]. Given that the source of these factors is not the APC itself, it may be that γδ T-APC induction of chemokine receptors on $CD4^+$ T cells is dependent on external factors produced by local tissue cells including epithelial cells and keratinocytes.

While the present study focused mainly on the induction of IL-22 by γδ T-APCs, γδ T-APCs may be able to promote alternative T helper cell responses, under different conditions. Indeed, significant induction of IL-10 was observed with IL-21 γδ T-APCs, although the underlying mechanism remains unclear. To further investigate this plasticity in inducing distinct qualities of $CD4^+$ T cell responses, gene expression profiling of IL-21 γδ T-APCs in comparison with IL-15 γδ T-APCs may identify novel factors involved in this IL-10 induction, and as such may also aid in defining the IL-10 producing $CD4^+$ T cell subset. Another area of interest could be the induction of Treg cells by γδ T-APCs. While no evidence of Treg induction was found in this study under the conditions examined, γδ T cells themselves have been shown to

exhibit regulatory potential[295]. As the generation of such suppressor γδ T cells requires the action of TGF-β and IL-15, a comparison of γδ T-APCs generated in the presence of IL-15 with and without addition of TGF-β may yield interesting insights into the underlying pathways of driving suppressive and APC functions, and may define conditions under which γδ T-APCs may promote the induction of suppressive Tregs.

6.4 - Characterising the IL-22 Response

With the demonstration that a combination of ICOSL and TNF-α induces populations of IL-22$^+$IFN-γ$^+$ cells in naïve CD4$^+$ T cells, and that γδ T-APCs favour a similar induction of such double-positive cells, questions remain as to why these IL-22 expressing cells also co-express IFN-γ, rather than expressing IL-22 alone. Given that IL-22 is often found co-expressed with IL-17, the wealth of studies examining Th17 cells may give insights into the potential roles of these different IL-22$^+$ sub-populations. It has been reported that different subsets of human Th17 cells, depending on their co-expressed cytokines, are more suited to combat particular pathogens over others. For example, *Candida albicans*-induced Th17 cells express IL-17 in combination with IFN-γ, whereas *Staphylococcus aureus*-specific Th17 cells co-express IL-17 and IL-10[159]. The induction of these different Th17 subsets is largely reliant on IL-1β control. Using this concept, it is conceivable that IL-22$^+$IFN-γ$^+$ cells participate in the eradication of certain pathogens, whereas IL-22$^+$IFN-γ$^-$ cells may be involved in other aspects of immunity. In addition, only a relatively limited range of cytokines has been examined in this study, and it cannot be ruled out that IL-22$^+$IFN-γ$^-$ cells may co-express other cytokines. IL-13 in particular has been reported to be co-expressed by IL-22$^+$ cells[379], and these cells are found in human atopic dermatitis. In this respect, increased levels of IL-13 were detected in ELISA supernatants of γδ T-APC-polarised naive CD4$^+$ T cells, in support of a potential induction of IL-22$^+$IFN-γ$^-$IL-13$^+$ cells.

Another potential concept, which may apply to IL-22 expressing cells, derives from the study of murine Th17 cells. Extensive study of this subset has revealed sub-populations of “pathogenic” and “non-pathogenic” Th17 cells, as characterised by the cytokines they secrete and their involvement in autoimmune diseases[380]. Of particular interest is the finding that the IL-17$^+$IFN-γ$^+$ cells identified in humans reportedly possess a profile similar to the “pathogenic” Th17 cells involved in autoimmunity in mice, whereas IL-17$^+$IL-10$^+$ cells are more similar to non-pathogenic cells[381]. By applying this concept to IL-22 producing cells, it could be postulated that

IL-22^{+}IFN-γ^{+} cells, induced by γδ T-APCs, may represent a more pathogenic T helper subset involved in inappropriate immune responses and autoimmunity, whereas *bona fide* Th22 cells induced by Langerhans cells and plasmacytoid DCs may be involved in protective immunity or homeostasis. Indeed, IL-22 has been identified as having both homeostatic[132] and pro-inflammatory roles[359], and as such is both beneficial and damaging depending on the immunological context, similarly as IL-17[89], highlighting the 'double-edged' aspects of these cytokines.

6.5 - Potential Roles of γδ T-APCs in Intestinal Immunity and Inflammatory Bowel Disease

A number of factors in the IL-15 γδ T-APC induction of IL-22 in CD4^{+} T cells are of interest for intestinal immunity. IL-15[364], IL-22[382], and Vγ9Vδ2 T cells[242] have all been implicated in intestinal immune responses and in inflammatory bowel diseases. As such, the *in vitro* mechanisms identified in this study appear to resemble a potential interaction, which may occur in the intestinal lamina propria or the mesenteric lymph nodes. Such a role is supported by the expression of the intestinal-homing molecules CCR9 and β7 integrin by IL-15 γδ T-APCs. With the identification that colon and ileum-derived Vγ9Vδ2 T cells are indeed capable of acting as APCs, and that Vγ9Vδ2 T cells isolated from the peripheral blood of IBD patients with active disease are restricted in their ability to become APCs as compared with inactive patients, the following model of the potential Vγ9Vδ2 T cell function in intestinal immunity and IBD is proposed (Figure 6.1).

The first step in Vγ9Vδ2 T cell-mediated intestinal immunity is the entry of cells into the intestinal lamina propria. Given that the peripheral blood contains the largest population of Vγ9Vδ2 T cells[322], migrating cells to the intestine likely derive from the peripheral blood. Circulating Vγ9Vδ2 T cells express inflammatory chemokine receptors such as CXCR3 and CCR5 for rapid recruitment to inflammatory sites[241]. Ligands for these chemokine receptors such as CXCL10 are secreted during inflammatory episodes of IBD[383], and as such represent one mechanism by which Vγ9Vδ2 T cells may be recruited to an already inflamed site. In addition, sub-populations of Vγ9Vδ2 T cells express α4β7 integrin and as such are able to enter intestinal sites without any prior activation. The expression of CCR9 by Vγ9Vδ2 T cells identified in this study was dependent on cell activation, and as such Vγ9Vδ2 T cells *in vivo* would likely require recognition of antigen prior to upregulation of this chemokine receptor. A significant feature of IBD is an increased intestinal barrier permeability during inflammatory episodes[345]. This potentially increases the

availability of stimulating ligands to circulating cells, thereby allowing for immune cell activation by microbial compounds including HMB-PP derived from the intestinal microflora. Upon recognition of HMB-PP, Vγ9Vδ2 T cells would potentially upregulate CCR9 and α4β7 to allow entry into the intestinal lamina propria. Preferential recruitment of T_{EM} cells in particular would describe the reduced APC phenotype of cells remaining in the peripheral blood of patients with active disease[345]. In addition to the recruitment of Vγ9Vδ2 T cells to inflamed sites, small populations are already present locally in the intestine, and as such may contribute to the initiation of inflammatory episodes.

Once Vγ9Vδ2 T cells have entered the inflamed sites, the necessary components to induce an APC phenotype would be present. HMB-PP can be provided by commensal bacteria upon increased barrier permeability, and IL-15 has been reported to be over-expressed in IBD[364]. Alternatively, Vγ9Vδ2 T cells can be activated by TCR independent mechanisms such as stress receptors[258], or type I IFNs[384,385]. With these factors present, Vγ9Vδ2 T cells would be able to upregulate APC markers and present antigens to $CD4^+$ T cells. Two routes of $CD4^+$ T cell stimulation would then exist; migration to mesenteric lymph nodes to stimulate naive $CD4^+$ T cell responses, or stimulation of local memory $CD4^+$ T cells in inflamed tissue. Vγ9Vδ2 T cells have been shown in this study and previously to be able to upregulate CCR7[241], which is necessary for lymph node homing. Upon upregulation of CCR7, γδ T-APCs would be able to enter the T cell zone of the mesenteric lymph nodes[241,322], and interact with naive $CD4^+$ T cells to induce IFN-γ and IL-22-expressing T helper cells. These responder cells would then be able to enter the peripheral blood and contribute to the systemic inflammation observed in IBD, or alternatively under the required conditions in the mesenteric lymph nodes, these activated $CD4^+$ T cells would be able to upregulate intestinal-homing receptors and migrate to inflamed sites to contribute to local inflammation. Alternatively, γδ T-APCs would be able to stimulate local memory $CD4^+$ T cell responses, skewing their effector phenotypes and promoting IFN-γ and IL-22 type responses. This model describes a potential mechanism by which γδ T-APCs are able to contribute to the initiation and pathogenesis of inflammatory bowel diseases. A number of unknowns remain with regards to this mode. It is unclear whether γδ T-APCs may be located in the same area of the intestinal lamina propria as $CD4^+$ T cells, and also whether the induction of IFN-γ and IL-22 responses would be beneficial or detrimental to the intestinal environment. More work is required to investigate these questions and further identify the roles of γδ T-APCs in intestinal immunity.

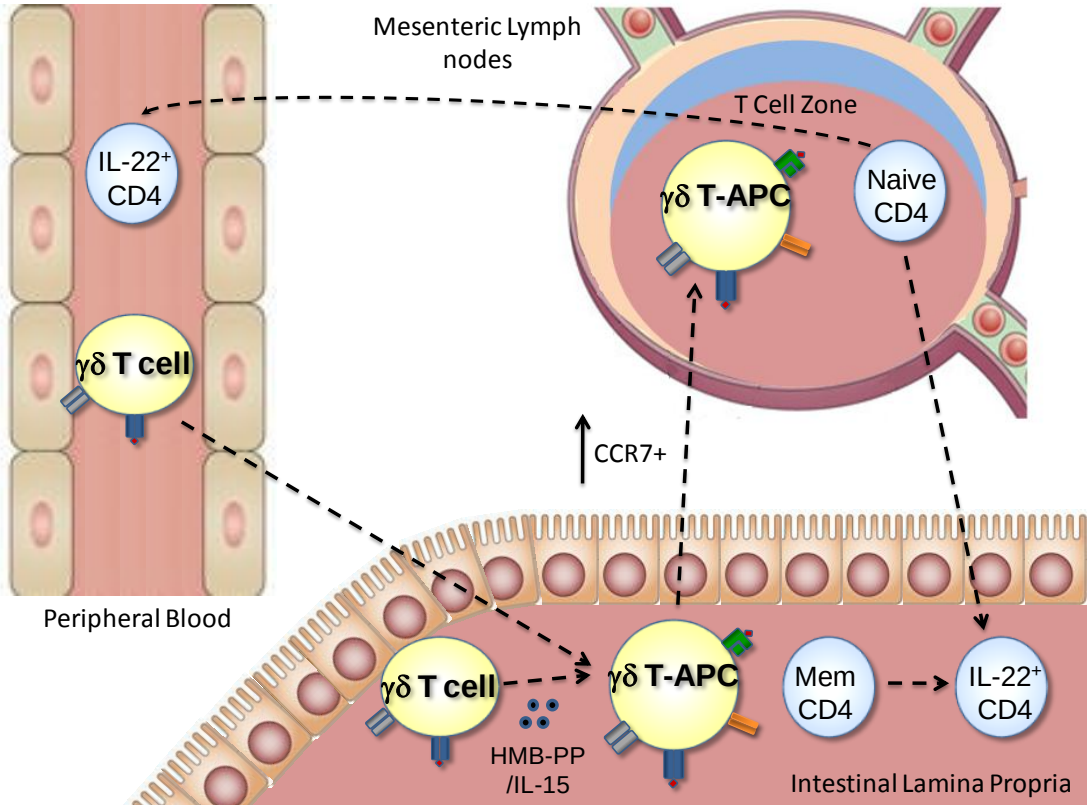

Figure 6.1 - Proposed model of γδ T-APC involvement in intestinal inflammation.

This model describes a potential role of γδ T-APCs in IBD, as well as in immune responses of the intestine. Of course, the diversity of cells in the intestinal lamina propria[386] highlights the fact that γδ T-APCs and induction of IFN-γ and IL-22 would only represent a relatively small component of the mechanisms involved. Indeed, Th17 cells in particular have been highlighted for their central role in autoimmune disorders[104], including IBD[387]. In many models of autoimmune conditions and in numerous patient groups, IL-17 has been identified as a central molecule driving the inflammatory environment[104]. Anti-IL-17 therapy is just beginning to be utilised in the therapy of autoimmune diseases, and has shown significant efficacy in diseases such as psoriasis, with treatment for moderate to severe psoriasis now approved[388]. However, use of Secukinumab, a monoclonal anti-IL-17 therapy, has to date proven ineffective in the treatment of patients with moderate to severe Crohn's disease. In fact, increased rates of adverse events were detected in some patient groups. The role of IL-17 in the pathogenesis of IBD is therefore currently unclear, and other cytokines such as IFN-γ may be equally as important in disease pathogenesis[387]. As such, this highlights the importance of molecules other than IL-17 in the pathogenesis of IBD and autoimmunity in general.

One of the questions not investigated in this study is the difference between intestinal sites, the colon and ileum, in terms of effector cells and potential Vγ9Vδ2 T cell involvement. The intestines are a complex organ with numerous sections that differ in their functions and immunological capacity. The intestines are divided into the small and large intestine, which are again divided into discrete sections[386]. Given that the most compelling evidence of Vγ9Vδ2 T cell involvement in intestinal immunity is derived from studies of inflammatory bowel diseases[242,345], which are restricted to the distal parts of the small intestine, termed the ileum, and the large intestine, predominantly formed of the colon, discussion of Vγ9Vδ2 T cell function will be restricted to these sites[382]. Large populations of $CD4^+$ T cells are found in both the ileum and colon, and as such represent areas of potential γδ T-APC function[386]. In addition, as shown in this study, Vγ9Vδ2 T cells can be isolated from both the terminal ileum and colon. Interestingly, ILC3 cells, which have been shown to be essential producers of IL-22 in the intestine, are found in reduced numbers in the colon in particular[386], and as such IL-22 production in this area of the intestine is likely derived from other sources. In addition, the bacterial colonisation is most apparent in the distal parts of the intestine, such as the colon and ileum, supporting the involvement of commensal bacteria in the pathogenesis of IBD. These factors together suggest Vγ9Vδ2 T cells may facilitate IFN-γ and IL-22 responses predominantly in these distal areas of the intestine, although their function may not be restricted to these areas alone. The ability of Vγ9Vδ2 T cells to become APCs did not appear to differ between cells derived from the colon or ileum, although with the limited number of donors examined it is not possible to confirm whether there are any functional differences between Vγ9Vδ2 T cells found in different areas of the intestine. Narrowing down the anatomical locations in which the APC function of Vγ9Vδ2 T cells is supported may aid in identifying their contribution to IBD and immune responses to infections.

Aside from potential differences in intestinal sites in terms of Vγ9Vδ2 T cell function, the differences between Crohn's disease and ulcerative colitis have not been addressed in this study. Crohn's disease can affect the distal small intestine and colon, whereas colitis is restricted to the colon[382]. Limited evidence suggests that Vγ9Vδ2 T cells may contribute to both Crohn's and colitis[345], although no data are available comparing the action of these cells in both diseases. Interestingly, increased numbers of IL-22 producing cells are observed in both Crohn's disease and ulcerative colitis compared with healthy controls, although Crohn's disease have increased $IL\text{-}22^+$ populations over ulcerative colitis in the intestinal lamina propria[389].

The majority of these IL-22 producing cells are of the T helper subtype, highlighting the importance of $CD4^+$ T cell infiltration into inflamed sites during inflammatory episodes. In contrast, a study has shown that $IL\text{-}22^+CD4^+$ T cells are actually depleted in the inflamed tissue of ulcerative colitis patients, as compared with healthy and Crohn's disease controls[390]. It is possible that, given the distinct pathologies of Crohn's disease and ulcerative colitis, Vγ9Vδ2 T cells and their APC function involving the induction of IL-22 and IFN-γ type responses may differ between the two conditions. With larger patient cohorts, any differences in involvement of Vγ9Vδ2 T cells in Crohn's disease and ulcerative colitis may become more apparent. In addition, study of intestine-derived Vγ9Vδ2 T cells from inflamed sites of patients with Crohn's disease and ulcerative colitis would identify differences in recruited cells and their involvement in disease pathogenesis.

With the evidence of IL-15 γδ T-APC induction of IFN-γ/IL-22 responses in IBD, the question remains as to whether these responses would be beneficial or damaging to the afflicted individual. Much evidence has identified positive roles for IL-22 in intestinal immunity and during IBD activity; in murine models, inhibition of IL-22 led to increased tissue damage, and IL-22 mediates protective effects and tissue repair via proliferation of epithelial cells, among other mechanisms[382]. However, IL-22 possesses several pro-inflammatory effects, not least of which is the induction of pro-inflammatory cytokine production such as IL-6 and CXCL8 by colonic myofibroblasts[389]. Given this evidence, IL-22 appears to possess both beneficial and detrimental effects during IBD. The co-expression of IFN-γ, which has an established role in autoimmunity[391], by IL-22-expressing cells induced by γδ T-APCs may differentiate the pathogenic effects of these effector cells from the beneficial effects. It could be suggested that local IL-22 production by cells such as ILC3s acts in a beneficial, homeostatic and tissue reparatory mechanism, whereas infiltration of inflammatory Vγ9Vδ2 T cells and $IFN\text{-}\gamma^+IL\text{-}22^+$ $CD4^+$ T cells leads to intestinal damage. Given the limited evidence presented in this study, more work is required to address this question.

A final question remains as to which antigens γδ T-APCs may present to $CD4^+$ T cells during inflammatory episodes of IBD. Whether these antigens may be autoantigens, such as the recently identified FAM84A[392], or foreign antigens from the gut microbiota remains to be investigated. Indeed, the antigenic target in addition to the APC has significant roles on the resulting T helper effector phenotype, and presents an interesting area of further research[393].

6.7 - Conclusion

This study has identified potential functional niches whereby γδ T-APCs may direct $CD4^+$ T cell responses towards effector phenotypes, distinct from those induced by other APC subsets. These γδ T-APCs likely cooperate with multiple different APC subsets such as DCs to facilitate suitable adaptive immunity to pathogens, and may also contribute to immune pathologies under appropriate circumstances. While much attention has focused on the role of γδ T-APCs and IL-22 in intestinal immunity, there are alternative scenarios where this interaction may be observed under physiological conditions. A potential role in skin immunity appears likely, as both Vγ9Vδ2 T cells[291] and IL-22[359] appear to be involved in the pathogenesis of psoriasis. Further to this, expanded γδ T-APCs are currently being considered for treatment of certain cancers[304,325], and given the data presented in this study, IL-22 responses could be induced by these cells. IL-22 itself has been identified in the regulation of tumour growth, and as such the knowledge that IL-22 responses may be increased in treated patients is a valuable insight for future γδ T cell treatments. While γδ T-APC research may be lagging 30 years behind that of dendritic cells, evidence has shown these novel APCs are an important component of immune responses.

Chapter 7 - References

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Appendix

Publications During PhD Studies

- **Tyler, C.J.,** Doherty, D.G., Moser, B., Eberl, M. Human Vγ9/Vδ2 T cells: Innate adaptors of the immune system. Cellular Immunology (2015), accepted January 2015.

- Davey, M.S., Morgan, M.P., Liuzzi, A.R., **Tyler, C.J.**, Khan, M.W.A., Szakmany, T., Hall, J.E., Moser, B., Eberl, M. Microbe-specific unconventional t cells induce human neutrophil differentiation into antigen cross-presenting cells. Journal of Immunology (2014) 193 (7), 3704-3716.

Presentations During PhD Studies

- Oral Presentation - Federation of American Societies for Experimental Biology (FASEB) Autoimmunity Conference, Itasca, Illinois (July 2015). Title 'γδ T-APCs promote IL-22 responses in CD4+ αβ T cells; implications for Inflammatory Bowel Disease'

- Oral Presentation - 6th International Gamma Delta T cell Conference, University of Illinois, Chicago, Illinois (May 2014). Title: 'Control of αβ T cell responses by γδT-APCs; novel induction of IL-22'.

- Further Oral Presentations - Institute of Infection and Immunity Annual Meeting 2014 (Cardiff University), Institute of Infection and Immunity Seminar Series 2015 (Cardiff University).

- Poster Presentations - British Society for Immunology Congress 2014 (Brighton), Postgraduate Research Day 2013 (Cardiff University), British Society for Immunology Summer School 2013 (Newcastle University).