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Identification of super-agonist peptides for cancer vaccines

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Summary

Background – T-cell-based immunotherapies are considered the biggest breakthrough in cancer treatment in recent decades. T-cells recognise peptide epitopes derived from tumour associated antigens (TAAs) displayed on human leukocyte antigen (HLA) molecules on the surface of tumour cells. Cancer peptide vaccines have attempted to use TAA-derived epitopes to activate cytotoxic T-cells, however these therapies have been largely unsuccessful. TAA are often self-proteins and therefore the resulting peptide epitopes are poorly immunogenic. Tumour infiltrating lymphocyte (TIL) therapy has resulted in remarkable cancer remission in some stage IV metastatic melanoma patients. Through an ongoing collaboration with the CCIT in Copenhagen, I had access to samples from long-term (7+ years) survivors following TIL therapy treatment for metastatic melanoma. Dissection of the TCR clonotypes of one patient, MM909.24, identified dominant and persistent TCR clonotypes in the TIL infusion product and PBMC post-cure. The aim of my study was to use one such TCR clonotype, ST8.24, to design super-agonist peptides to improve the efficacy of cancer peptide vaccines.

Results – Combinatorial peptide library (CPL) screening of ST8.24 identified ten candidate super-agonist peptides. The optimal super-agonist peptide was determined using HLA-EAAGIGILTV-pMHC tetramer staining of peptide primed polyclonal CD8 T-cells from healthy HLA A2+ individuals. The super-agonist peptide primed more EAAGIGILTV-tetramer positive cells than the wild type antigen in every individual tested. Super-agonist primed T-cells from healthy and melanoma patient samples were then found to be functionally superior. I sought to decipher the mechanism of the super-agonist peptide and discovered that it possessed remarkable structural mimicry to EAAGIGILTV. Furthermore, priming with the super-agonist peptide elicited T-cells specific for other TAA-derived epitopes. Finally, the peptide was shown to elicit superior T-cells in samples from renal cell carcinoma (RCC), chronic lymphocytic leukaemia (CLL) and acute myeloid leukaemia (AML) patients.

Conclusions – I showed that it is possible to design a potent super-agonist peptide using CPL screening of CD8 T-cells. This peptide can break T-cell tolerance, eliciting functionally superior T-cells which recognise epitopes from different TAAs. These data show that super-agonist peptides make attractive candidates for pan-cancer peptide vaccines and warrant further investigation.

Work incorporated in this thesis

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List of abbreviations

β2M – Beta 2 microglobulin
Ab – Antibody
ACT – Adoptive cell therapy
AIRE – Autoimmune regulator
ALL – Acute lymphoblastic leukaemia
AML – Acute myeloid leukaemia
APC – Antigen presenting cell
APL – Altered peptide ligand
BAGE – B melanoma antigen
BST2 – Bone marrow stromal antigen 2
C – Celsius
CAR-T – Chimeric antigen receptor T-cell
CD – Cluster of differentiation
CDR – Complementary determining region
CEA – Carcino-embryonic antigen
CFSE – Carboxyfluorescein succinimidyl ester
CLL – Chronic lymphocytic leukaemia
CO_2 – Carbon Dioxide
CPL – Combinatorial peptide library
CMV – Cytomegalovirus
Cr – Chromium
CRISPR – Clustered regularly-interspace short palindromic repeats
CTL – Cytotoxic T lymphocyte
CTLA-4 – Cytotoxic T-lymphocyte-associated protein 4
CyTOF – Cytometry in time-of-flight
DC – Dendritic cell
DMEM – Dulbecco modified eagle's minimal essential medium
DMSO – Dimethyl Sulphate
DNA – Deoxyribonucleic acid
EBV – Epstein Barr virus
EDTA – Ethylenediaminetetraacetic acid
ELISA – Enzyme-Linked Immunosorbent Assay
ELISpot – Enzyme-linked Immunospot
ER – Endoplasmic reticulum
ERAP – Endoplasmic reticulum aminopeptidase associated with antigen processing
FACS – Florescent activated cell sorting
FBS – Foetal Bovine Serum
FDA – Food and Drug Administration
FITC – Fluorescein isothiocyante
FPKM – Fragmetns per kilobase million
GAGE – G antigen 1
GEM – germline encoded mycolyl lipid reactive T-cell
GLUT1 – Glucose transporter 1
GM-CSF – granulocyte/monocyte cell stimulating factor
HBV – Hepatitis B virus
HCT – Hematopoietic stem cell transplant
HCV – Hepatitis C virus
HIV – Human Immunodeficiency virus

HLA – Human leukocyte antigen
H – Hour
HRP – Horseradish peroxidase
HSP – Heat shock protein
HSV – Herpes simplex virus
HPV – Human papillomavirus virus
IAP – Inhibitor of apoptosis protein
ICS – Intracellular cytokine staining
IFA – incomplete Freuds adjuvant
IFN-γ – Interferon gamma
LAA – Leukaemia associated antigen
IL-2 – Interleukin 2
IL-4 – Interleukin 4
IL-15 – Interleukin 15
IMP2 (IF2B2) – Insulin-like growth factor 2
LAG-3 – Lymphocyte activating gene 3
MACS – Magnetic cell separation
MAIT – mucosal associated invariant T-cell
MAGE – Melanoma-associated antigen
MART1 – Melan-A/Melanoma antigen recognised by T-cells
MBP – Myelin basic protein
MHC-I – Major histocompatibility complex class 1
MHC-II – Major histocompatibility complex class 2
MIP-1β – Macrophage inflammatory protein 1 beta
Min – Minute
mL – millilitre
MR1 – Major histocompatibility complex, class I related
MRD – Minimal residual disease
MS – Mass spectrometry
MUC1 – Mucin 1
NK - Natural killer
PAD – peptidylarginine deiminase
PB – Pacific blue
PBMC – Peripheral blood mononuclear cells
PBS – Phosphate buffered saline
PCR – Polymerase chain reaction
PD-1 – Programme-death receptor 1
PHA – Phytohemagglutinin
PFA – Paraformaldehyde
PI – Protease inhibitor
PKI – Protein kinase inhibitor
PR3 – proteinase 3
PRAME – preferentially expressed antigen in melanoma
PSA – Prostate specific antigen
RCC – Renal cell carcinoma
rCD2 – Rat CD2
RNA – Ribonucleic acid
RNAi – Ribonucleic acid interference
RNAseq – RNA sequencing
RPHM – Reads per kilobase million
RT – Room temperature

SD – Standard deviation
TAA – Tumour associated antigen
TAP 1/2 – Transporters associated with antigen processing 1 and 2
TAPI – TNF alpha protease inhibitor
TCR – T-cell receptor
TEC – Thymic epithelial cell
TIGIT – T-cell immunoglobulin and ITIM domain
TIL – Tumour infiltrating lymphocyte
TIM-3 – T-cell immunoglobulin-3
TME – Tumour microenvironment
TNF-α – Tumour necrosis factor alpha
TSA – Tumour specific antigen
T-VEC – Talimogene laherparevpec
VDJ – Variable, diversity, joining region
VEGF – Vascular endothelial growth factor
WBS – Welsh Blood Service
WT – Wild-type
WT1 – Wilms tumour protein 1

Table of Contents

Statements and declarations ..... *i*

Acknowledgements ..... *i*

Summary ..... *i*

Work incorporated in this thesis ..... *ii*

Other work published during my PhD ..... *ii*

Presentations of the results described in this thesis ..... *ii*

List of abbreviations ..... *iii*

Table of Contents ..... *i*

1. Introduction *1*

1.1. The Immune system **1**

1.1.1. Conventional αβ T-cells 1

1.2. MHC processing and presentation **2**

1.2.1. Major histocompatibility complex 2

1.2.2. Peptide processing and presentation 3

1.3. Generation of TCR diversity **5**

1.3.1. The αβ T-cell receptor 5

1.3.2. Gene rearrangement at the αβ TCR loci 7

1.3.3. Thymic selection of T-cells 9

1.4. CD8 T-cells and cancer **10**

1.5. CD8 T-cell epitopes and cancer **13**

1.5.1. Tumour antigens 13

1.5.1.1. Overexpressed antigens 14

1.5.1.2. Oncofetal antigens 14

1.5.1.3. Cancer-testis antigens 15

1.5.1.4. Differentiation antigens 15

1.5.1.5. Heat shock proteins 16

1.5.1.6. Tumour specific antigens 16

1.5.2. Modified peptides 17

1.5.2.1. Glycosylation 17

1.5.2.2. Citrullination 18

1.6. Cancer Immunotherapy **19**

1.6.1. Cytokine therapy 19

1.6.2. Adoptive cell therapy 20

1.6.3. Checkpoint Inhibitor therapy 21

1.6.4. Engineered T-cell therapy 23

1.7. Cancer vaccines **26**

1.7.1. Dendritic cell vaccines 27

1.7.2. Viral vaccines 28

1.7.3. Whole tumour or subunit vaccines 30
1.7.4. Nucleic acid vaccines 31
1.7.5. Peptide vaccines 32

1.8. Project Aims 34

2. Materials and Methods 35

2.1 Patient and healthy donor samples 35
2.1.1 Patient samples 35
2.1.2 Healthy donor samples 35

2.2 Cell Culture 36
2.2.1 Cell Culture Reagents 36
2.2.2 Antigen Presenting Cells (APC) 37
2.2.3 Primary tumour culture 39
2.2.4 Cell counting 39
2.2.5 Cryopreservation and thawing 39

2.3 Maintenance and expansion of T-cell cultures 40
2.3.1 Isolation of peripheral blood mononuclear cells (PBMC) 40
2.3.2 Expansion and culture of T-cells 40
2.3.3 Single Cell Cloning 40

2.4 Functional Assays 41
2.4.1 Peptides 41
2.4.2 Chromium release assay 41
2.4.3 Enzyme-Linked Immunosorbent Assay (ELISA) 42
2.4.3.1 Activation Assay for ELISA 42
2.4.3.2 ELISA protocol 42
2.4.4 Combinatorial Peptide Library (CPL) 43
2.4.4.1 Epitope Identification 43

2.5 Magnetic activated cell sorting 43
2.5.1 CD8 T-cell isolation 43
2.5.2 Isolation of reactive CD8 T-cells using TNF and IFNγ 44
2.5.3 Isolation of tetramer positive cells 44

2.6 CD8 T-cell Peptide Priming 45

2.7 Flow Cytometry 45
2.7.1 Labelling cells 45
2.7.2 Intracellular Cytokine Staining (ICS) assay 48
2.7.3 TNF Alpha Protease Inhibitor (TAPI) Assay 48
2.7.4 pMHC- Tetramer Staining 48
2.7.4.1 Manufacture of pMHC-tetramers 48
2.7.4.2 pMHC-tetramer staining 49
2.7.5 T2 Binding Assays 50
2.7.6 Flow Cytometry Based Killing Assay 51
2.7.6.1 Set up of flow cytometry based killing assay 51
2.7.6.2 Running flow cytometry based killing assay 51

2.8 Molecular Biology 52
2.8.1 Lentiviral transduction of cell lines 52
2.8.1.1 Vectors 52
2.8.1.2 Sequences and primers 53
2.8.1.3 Molecular cloning 53

2.8.1.4 Lentivirus production 55
2.8.1.5 Lentiviral transduction of tumour lines 56
2.8.2 Gene-silencing using CRISPR/Cas 9 system 56

2.9 Protein expression, refolding, purification and crystallisation 57
2.9.1 Vectors and protein sequences 58
2.9.1.1 pGMT7 Vector 58
2.9.1.2 Sequences 58
2.9.2 Transformation of E. coli cells 58
2.9.3 Expression of inclusion bodies in Rosetta *E. coli* cells 58
2.9.3.1 Spectrophotometry to estimate protein concentration 59
2.9.4 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) 59
2.9.5 Soluble peptide-MHC class I molecules 60
2.9.6 Fast protein liquid chromatography (FPLC) 60
2.9.7 Crystallisation, diffraction data collection and model refinement 60

2.10 TCR clonotyping 61
2.10.1 mRNA extraction from T-cells 61
2.10.2 cDNA synthesis 61
2.10.3 Step-out and Nested PCR 62
2.10.4 Agarose gel electrophoresis 63

2.11 Data Analysis 63

3. Peptide super-agonist enhances T-cell responses to melanoma 65

3.1. Background 65
3.1.1. Altered peptide ligands (APL) for treatment of melanoma 65
3.1.2. Aims 67

3.2. Results 68
3.2.1. Isolation and validation of ST8.24 T-cell clone 68
3.2.2. Previously identified agonist peptide **F**A**T**GIGI**I**TV fails elicit a response from the TIL used to successfully treat patient MM909.24 70
3.2.3. Designing candidate super-agonist peptides for T-cell clone ST8.24 using a combinatorial peptide library (CPL) screen 72
3.2.4. Testing the activity of the top ten predicted super-agonist peptides 74
3.2.5. Testing the potency of T-cell priming by ST8 super-agonist ligands 76
3.2.6. Validation of optimal super-agonist peptides for T-cell priming 79
3.2.7. Killing of melanoma cell lines by primed healthy donor CD8 T-cells 82
3.2.8. Phenotypic analysis of EAAGIGILTV and **MTSA**IGIL**P**V primed CD8 T-cells 85
3.2.9. **MTSA**IGIL**P**V shows improved binding to HLA A2 compared to EAAGIGILTV 86
3.2.10. Clonotypic analysis of EAAGIGILTV and **MTSA**IGIL**P**V primed CD8 T-cells 87
3.2.11. EAAGIGILTV-specific CD8 T-cells bind to **MTSA**IGIL**P**V-tetramer with greater fluorescent intensity 90
3.2.12. **MTSA**IGIL**P**V is a structural mimic of EAAGIGILTV when bound to HLA A2 92
3.2.13. Increased killing of autologous tumour by **MTSA**IGIL**P**V -primed CD8 T-cells in a metastatic melanoma patient 95

3.3. Discussion 97
3.3.1. Designing super-agonist peptides using dominant CD8 T-cell clonotypes 98
3.3.2. **MTSA**IGIL**P**V exhibits improved binding to HLA A2 and is a close structural mimic of EAAGIGILTV 99
3.3.3. EAAGIGILTV primed T-cells bind **MTSA**IGIL**P**V with increased affinity 99
3.3.4. **MTSA**IGIL**P**V primed T-cells exhibit improved killing of melanoma cells 100
3.3.5. Summary 100

4. MTSAIGILPV peptide primes T-cells that recognise multiple cancer epitopes 101

4.1. Background 101
4.1.1. T-cell cross-reactivity 101
4.1.2. Aims 103

4.2. Results 104
4.2.1. **MTSA**IGIL**P**V-T-cell clone ST8.24 recognises multiple TAA epitopes 104
4.2.2. TIL 24 infusion product contains T-cells specific for melan-A, BST2 and IMP2 106
4.2.3. Tumour associated antigens BST2 and IMP2 are expressed in multiple cancer types 108
4.2.4. TCR clonotype ST8.24 is reactive to multiple tumour types 111
4.2.5. Multipronged T-cells can prevent melanoma tumour escape 113
4.2.6. Analysis of the peptide specificity and tumour reactivity of therapeutically unsuccessful TCR, DMF4 117
4.2.7. Peptide priming with super-agonist peptide **MTSA**IGIL**P**V elicits CD8 T-cells specific for LLLGIGILVL and NLSALGIFST from HLA A2+ healthy donors 119

4.3. Discussion 122
4.3.1. T-cell cross-reactivity 122
4.3.2. The ST8.24 T-cell recognises multiple melanoma-derived antigens 123
4.3.3. Tumour associated antigen BST2 and IMP2 are widely expressed 124
4.3.4. TCR clonotype ST8.24 recognises multiple tumour cell lines 125
4.3.5. Multipronged CD8 T-cells can prevent melanoma tumour escape from the immune response 125
4.3.6. The DMF4 TCR does not recognise BST2 or IMP2 126
4.3.7. **MTSA**IGIL**P**V peptide-induced CD8 T-cells that recognise multiple cancer antigens 127
4.3.8. Summary 128

5. Super-agonist peptide MTSAIGILPV as a pan-cancer vaccine candidate 130

5.1. Background 130
5.1.1. Renal cell carcinoma 130
5.1.2. Leukaemia 131
5.1.2.1. Chronic lymphocytic leukaemia (CLL) 132
5.1.2.2. Acute myeloid leukaemia (AML) 133
5.1.3. Aims 134

5.2. Results 135
5.2.1. Successful priming with super-agonist peptide **MTSA**IGIL**P**V of CD8 T-cells from renal cell carcinoma (RCC) patient samples 135
5.2.2. Expression of TAA BST2 and IMP2 in leukaemia tumour samples 137
5.2.3. Successful priming with super-agonist peptide **MTSA**IGIL**P**V of CD8 T-cells from CLL patient samples 139
5.2.4. **MTSA**IGIL**P**V-priming of CD8 T-cells from CLL patient samples kill autologous cancer cells 141
5.2.5. **MTSA**IGIL**P**V peptide primes CD8 T-cells specific for TAA epitopes from leukaemia patient samples 145
5.2.6. **MTSA**IGIL**P**V-priming of multipronged CD8 T-cells from a AML patient sample 146
5.2.7. **MTSA**IGIL**P**V peptide primed CD8 T-cells from AML patient CH160486 kill AML tumour cells 149
5.2.8. Isolation of a **MTSA**IGIL**P**V-reactive multi-pronged CD8 T-cell from CLL patient U386 153

5.2.9. T-cell clone CLL9 does not respond to healthy cells 157

5.3. Discussion 161

5.3.1. Successful **MTSA**IGIL**P**V peptide priming in renal cell carcinoma (RCC) patient samples 161

5.3.2. BST2 and IMP2 expression in leukaemia cell lines 162

5.3.3. Successful priming of **MTSA**IGIL**P**V-tetramer positive cells in leukaemia patient samples that kill autologous tumour 162

5.3.4. Super-agonist peptide **MTSA**IGIL**P**V elicits cross-reactive CD8 T-cells from CLL patient samples 164

5.3.5. A T-cell clone isolated from **MTSA**IGIL**P**V-primed population is multipronged 164

5.3.6. Summary 165

6. General discussion 166

6.1. Summary of work 166

6.1.1. Identification of super-agonist peptide **MTSA**IGIL**P**V 167

6.1.2. Biophysical analysis of the superiority of **MTSA**IGIL**P**V 168

6.1.3. Super-agonist peptide **MTSA**IGIL**P**V and T-cell cross-reactivity 169

6.1.4. Super-agonist peptide **MTSA**IGIL**P**V as a pan-cancer vaccine candidate 172

6.2. Future work 172

6.3. Future perspectives 174

6.3.1. Clinical application of **MTSA**IGIL**P**V peptide and multipronged TCRs 176

6.3.1.1. TCR-T therapy 176

6.3.1.2. Polyclonal adoptive cell transfer (ACT) therapy 177

6.3.1.3. Conventional vaccine technology 178

6.4. Summary 179

References 181

Appendix 209

List of Figures

Figure 1.1: CD4 and CD8 αβ T-cells 2
Figure 1.2: MHC class I antigen processing and presentation 5
Figure 1.3: Crystal structure of a αβ TCR 7
Figure 1.4: TCR gene rearrangement 9
Figure 1.5: Thymic development of T-cells 10
Figure 1.6: CD8 T-cells vs cancer 11
Figure 1.7: Cancer Immunoediting 13
Figure 1.8: Amino acid glycosylation 17
Figure 1.9: Conversion of arginine to citrulline 18
Figure 1.10: Immune checkpoints 23
Figure 1.11: Chimeric antigen receptor T-cells 26
Figure 1.12: Aim of oncolytic viral therapy 30
Figure 1.13: Successful peptide cancer vaccination 33
Figure 2.1: Gating strategy for functional and phenotypic analysis of T-cells 47
Figure 2.2: Gating strategy for pMHC-tetramer staining 50
Figure 2.3: Gating strategy for flow-based killing assay 52
Figure 3.1: Isolation and validation of metastatic melanoma derived CD8 T-cell clone ST8.24 69
Figure 3.2: Agonist peptide FATGIGIITV does not elicit a response from melanoma patient MM909.24 derived CD8 T-cell clone ST8.24 or TIL infusion product. 71
Figure 3.3: CPL screening of ST8.24. 73
Figure 3.4: Screening of agonist peptides with melan-A-restricted CD8 T-cell clones identifies ITSGIGVLPV as a candidate super-agonist peptide for most melan-A T-cell clones. 75
Figure 3.5: MTSAIGILPV is the optimal super-agonist peptide for priming T-cells across five different HLA A2+ donors. 78
Figure 3.6: Priming of EAAGIGILTV-specific T-cells with MTSAIGILPV super-agonist peptide in multiple donors. 80
Figure 3.7: MTSAIGILPV-primed CD8 T-cells proliferate more than EAAGIGILTV-primed cells 81
Figure 3.8: MTSAIGILPV elicits a greater magnitude of EAAGIGILTV-tetramer positive cells than ITSGIGVLPV in all donors tested 82

Figure 3.9: Heathy donor CD8 T-cells primed with MTSAIGILPV exhibit improved killing of melanoma cells compared to EAAGIGILTV-primed CD8 T-cells. 84
Figure 3.10: MTSAIGILPV and EAAGIGILTV-primed cells have a similar phenotype. 86
Figure 3.11: Super-agonist peptide MTSAIGILPV exhibits improved binding to HLA A2 compared to the EAAGIGILTV wild-type peptide sequence. 87
Figure 3.12: Priming CD8 T-cells with EAAGIGILTV and MTSAIGILPV elicits different T-cell clonotypes 89
Figure 3.13: EAAGIGILTV-specific T-cells bind HLA A2-MTSAIGILPV tetramers with increased florescent intensity. 91
Figure 3.14: MTSAIGILPV is a structural mimic of EAAGIGILTV when bound to HLA A2. 93
Figure 3.15: MTSAIGILPV primed EAAGIGILTV-tetramer positive cells from patient blood that exhibited improved killing of autologous melanoma cells. 96
Figure 4.1: Peptide hits from a combinatorial peptide library (CPL) of ST8.24..... 105
Figure 4.2: Validation of potential ST8.24 tumour associated epitopes..... 108
Figure 4.3: Ubiquitous expression of tumour associated antigens BST2 and IMP2 in cancer cell lines 110
Figure 4.4: CD8 T-cell clonotype ST8.24 reacts to multiple tumour types..... 112
Figure 4.5: Additive effect of multipronged TCR ST8.24..... 114
Figure 4.6: Multipronged T-cells exhibit improved recognition of melanoma 116
Figure 4.7: rCD2 and CD3 expression of DMF4 and MEL5 TCR transduced CD8 T-cells 118
Figure 4.8: Analysis of the peptide specificity and tumour reactivity of therapeutically unsuccessful TCR, DMF4 119
Figure 4.9: Super-agonist peptide MTSAIGILPV primes more EAAGIGILTV, LLLGIGILVL, NLSALGIFST CD8 T-cells than the WT peptides in 3 HLA A2+ healthy donors. 121
Figure 4.10: Multipronged TCRs can prevent tumour escape 124
Figure 5.1: Successful priming with super-agonist peptide MTSAIGILPV of CD8 T-cells from RCC patient 2 136
Figure 5.2: Successful priming of MTSAIGILPV-reactive CD8 T-cells capable of killing autologous tumour from RCC patient 12..... 137
Figure 5.3: Expression of BST2 and IMP2 in leukaemia tumour samples 139
Figure 5.4: Phenotype of CLL patient PBMC sample..... 140
Figure 5.5: Successful priming of MTSAIGILPV-reactive CD8 T-cells from chronic lymphocytic leukaemia (CLL)..... 141

Figure 5.6: MTSAIGILPV-primed CD8 T-cells from CLL patient A626 kill autologous tumour in a chromium release assay 142
Figure 5.7: CD19 staining of CLL U386 tumour 143
Figure 5.8: MTSAIGILPV-primed CD8 T-cells from CLL patient U386 kill autologous tumour in a flow cytometry assay 144
Figure 5.9: MTSAIGILPV peptide priming of EAAGIGILTV and NLSALGIFST reactive CD8 T-cells from CLL patient samples 146
Figure 5.10: Phenotyping of PBMC from AML patient CH160486 147
Figure 5.11: Successful priming of EAAGIGILTV, LLLGIGILVL and NLSALGIFST reactive CD8 T-cells in AML patient CH160486 samples by super-agonist peptide MTSAIGILPV 149
Figure 5.12: Improved killing of autologous AML tumour by MTSAIGILPV-primed T-cells from AML patient CH160486 151
Figure 5.13: Superior killing of AML cell line THP-1 by MTSAIGILPV-primed T-cells from AML patient CH160486 152
Figure 5.14: Schematic of CD8 T-cell clone isolation from MTSAIGILPV-primed polyclonal T-cell populations 154
Figure 5.15: CD8 T-cell clone, CLL 9, is multi-pronged and kills autologous tumour 156
Figure 5.16: HLA A2 staining of tumour cell lines MM909.24, PANC1, SKBR3 and C1R 157
Figure 5.17: Recognition and killing of non-autologous tumour cell lines by CLL 9 158
Figure 5.18: Multi-pronged CD8 T-cell clone, CLL 9, recognises tumours distinct from CLL but not healthy cells 159
Figure 5.19: Tumour-reactive multi-pronged CD8 T-cell clone, CLL 9, does not kill healthy cells 160
Figure 6.1: Combinatory peptide library (CPL) screen 167
Figure 6.2: TCR clonotype MEL5 interacts with cancer derived epitopes from melan-A, BST2 and IMP2, in a conserved manner. 171
Figure 6.3: Taking super-agonist peptide MTSAIGILPV to the clinic 175

List of appendix figures

Appendix Figure 1: Sequences cloned into lentiviral vectors 209
Appendix Figure 2: Associated flow cytometry plots super-agonist peptide test 210
Appendix Figure 3: Associated peptide priming flow cytometry plots after 14 days 211
Appendix Figure 4: Associated peptide priming flow cytometry plots after 28 days 212
Appendix Figure 5: Associated flow cytometry plots T2 binding assay 213
Appendix Figure 6: pELNS plasmid for TCR transduction 214
Appendix Figure 7: Associated flow cytometry plots of super-agonist peptide priming healthy donor 1 215
Appendix Figure 8: Associated flow cytometry plots of super-agonist peptide priming healthy donor 2 216
Appendix Figure 9: Associated flow cytometry plots of super-agonist peptide priming healthy donor 3 217

List of Tables

Table 2.1 Cell culture medias 36
Table 2.2: Buffers used in cell culture 37
Table 2.3: Information on cell lines used 38
Table 2.4: Antibodies used for this work 46
Table 2.5: Plasmids used for lentivirus generation 53
Table 2.6: Sequences of primers used for PCR and molecular cloning 53
Table 2.7: Media for lentivirus production 55
Table 2.8: Conditions for electroporation of cell lines 57
Table 2.9: Culture media and buffers for protein expression, refolding, purification and crystallisation 57
Table 3.1: Ten highest ranked agonist peptides from CPL. 74
Table 3.2: EC_{50} of melan-A specific CD8 T-clones. NR = No response 76
Table 3.3: Refinement statistics 94
Table 4.1: Potential number of peptide epitopes for αβ T-cells 102

1. Introduction

1.1. The Immune system

The immune system provides protection from external pathogens, and is composed of two arms, the innate and adaptive systems. The innate immune system provides the first line of defence. Physical barriers, such as the skin and the mucosa, provide initial protection from foreign pathogens by blocking their entry. Pathogens that breach these barriers can be rapidly identified and removed by the cells of the innate immune system. Cells such as macrophages and dendritic cells phagocytose pathogens resulting in their destruction whilst granulocytes such as neutrophils release potent, toxic antimicrobial agents upon encountering invading pathogens. If the innate immune cells are unable to resolve the infection on their own the adaptive immune system is recruited, which is characterised by its ability to specifically identify individual pathogens. The cells that comprise the adaptive immune system are T and B lymphocytes. They possess highly diverse antigen recognition receptors on their surface and are activated when they recognise their cognate antigens. Upon initial encounter with antigen, the adaptive immune system is slower to act than the innate immune system. However, the development of memory T and B cells after antigen exposure, allows for rapid clearance of secondary infection. My studies focussed on T-cells, therefore B-cell mediated immunity will not be considered further here.

1.1.1. Conventional αβ T-cells

T-cells can be divided into conventional and unconventional T-cells based on the antigens they recognise and the TCRs they possess. Whilst unconventional T-cells are important in immunity, my thesis focussed on conventionally activated T-cells therefore unconventional T-cells will not be discussed. Conventionally activated T-cells possess an αβ T-cell receptor (TCR) and can be further subdivided based on the expression of co-receptor molecules CD8 and CD4 (**Figure 1.1**). CD8 T-cells recognise peptides presented by major histocompatibility (MHC) class I molecules through their TCR whilst CD4 T-cells recognise peptides presented on MHC class II molecules. MHC molecules and their role in peptide processing and presentation will be discussed further in **Section 1.2.** Generally speaking, CD8 T-cells can induce cell death of virally-infected or transformed cells through a variety of mechanisms and are known as cytotoxic T lymphocytes (CTL), whilst CD4+ T-cells facilitate the immune response and can be known as helper T-cells.

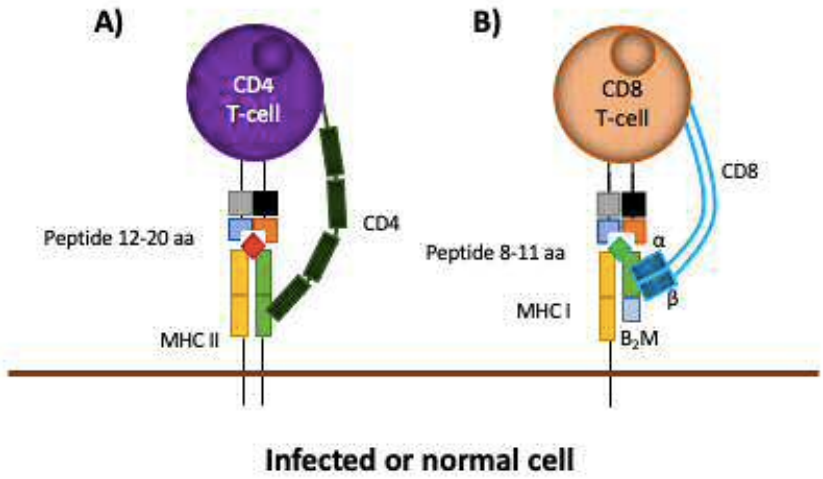


Figure 1.1: CD4 and CD8 αβ T-cells

(*A) CD4+ T-cells recognise peptides of 12-20 amino acids presented on MHC class II molecules. MHC class II is a heterodimer with two chains (α and β) and is expressed on antigen presenting cells. The CD4 co-receptor is comprised of four immunoglobulin domains. (****B****) CD8+ T-cells interact with MHC class I molecules which are expressed on the surface of all nucleated cells. MHC class I molecules present peptides of 8-12 amino acids in length and are composed of an α-chain and β$_2$-microglobulin (β2m). The glycoprotein CD8 serves as a co-receptor, facilitating TCR recognition of pMHC.*

1.2. MHC processing and presentation

1.2.1. Major histocompatibility complex

Conventional αβ T-cells recognise antigens in the form of peptides presented on major histocompatibility complex (MHC) molecules class I and class II molecules. The CD8 and CD4 'coreceptors' bind to MHC class I and class II respectively at a site that is distant from the TCR docking platform and can assist TCR-mediated signal transduction. These coreceptors are used to define T-cells that recognise MHC class I and class II. CD8 T-cells recognise peptide derived from endogenous proteins in the context of MHC class I whereas CD4 T-cells generally recognise peptides derived from exogenous proteins in the context of MHC class II. MHC class I molecules are expressed on all nucleated cells, whilst MHC class II are generally

only expressed by antigen presenting cells (APCs) of the immune system. As described below, interest from the cancer immunology standpoint has focussed on CD8 T-cells as these cells have the ability to directly sense and eliminate cancer cells. Despite the importance of CD4 T-cells in immunity, the remainder of this thesis will only focus on aspects pertaining to CD8 T-cells.

The MHC locus in humans is also known as human leukocyte antigen (HLA) and is located on chromosome 6. The human MHC locus contains genes encoding MHC class I and class II chains and many of the proteins associated with MHC processing and presentation. MHC class I α-chains are encoded by 3 distinct genes, HLA A, B and C. β_2-microglobulin (β_2m), with which HLA class I chains must associate to be expressed at the cell surface, is not encoded in this region and is located on chromosome 15. The HLA locus is the most polymorphic region in the human genome and there are over 18,000 different HLA alleles described to date. This huge variability in MHC molecules allows them to present a large variety of peptides and interact with diverse TCR molecules (Robinson *et al.*, 2015). Each HLA gene has many alleles and individuals are generally heterozygous for these alleles. This polygeny along with the polymorphisms found at each HLA gene accounts for the diversity in HLA expression seen in the population. The majority of polymorphisms in MHC class I molecules are found in the α-chain, specifically the TCR contact residues and the peptide binding groove (Eckle *et al.*, 2013; Rossjohn *et al.*, 2015; Dendrou *et al.*, 2018). Polymorphisms in the peptide binding groove can dictate the MHC class I molecules preference for antigens. For example, in the context of HLA A*02:01 the residues leucine (L) or methionine (M) are preferred anchor residues at position 2 of the peptide (Falk *et al.*, 1991). HLA A*02:01, is the most intensely studied HLA allele as it is the most frequent HLA A allele in humans (Hou *et al.*, 2019; Hurley *et al.*, 2020). Due to this high frequency, my studies focused on HLA A*02:01, as an example HLA molecule.

1.2.2. Peptide processing and presentation

Polypeptide chains found in the cytosol of an infected cell must first be broken down into smaller, more manageable peptides. The proteasome is a multi-catalytic complex residing in the cytosol, which routinely degrades proteins no longer required by the cell. In some cases, peptides from proteins degraded by the proteasome, are presented by MHC molecules, whilst others are removed from the cell as waste products. Proteins destined for proteasomal degradation are ubiquitin tagged by the ubiquitin proteasome system (UPS). The proteasome is responsible for cleavage at the carboxyl end of the peptide antigen whilst

final trimming of peptides, is undertaken by endoplasmic reticulum aminopeptidase associated with antigen processing 1 and 2 (ERAP 1 and 2) (Serwold *et al.*, 2002; Saveanu *et al.*, 2005). Knockdown of ERAAP, the murine equivalent of human ERAP1, mediated by RNA inference (RNAi), reduced peptide trimming and MHC class I surface expression (Serwold *et al.*, 2002).

Polypeptide chains of MHC class I molecules are assembled in the lumen of the endoplasmic reticulum (ER). Newly assembled MHC class I molecules are guided through development by an assortment of chaperone proteins, which keep the MHC molecules in a partially folded, peptide receptive state until they binds to an appropriate peptide (**Figure 1.2**). MHC molecules will not leave the lumen of the ER without being bound to a peptide. In order for antigenic peptides, processed by the proteasome in the cytosol, to load onto the peptide binding site of the MHC class I molecule they must be transported into the lumen. Transportation of peptides is facilitated by transporters associated with antigen processing 1 and 2 (TAP1 and TAP2), heterodimeric proteins associated with the ER membrane. An increase in interferon gamma (IFN-γ) production during inflammatory conditions causes changes in the composition of the proteasome, forming the immunoproteasome, and an upregulation of ERAP (Serwold *et al.*, 2002). This switch changes the enzymatic activity of the proteasome, causing an increase in cleavage of hydrophobic peptides and decreased cleavage of acidic residues generating peptides preferred by TAP and more suitable for binding to MHC class I. MHC class I molecules that have bound to a peptide antigen are released from the ER in vesicles and transported to the cell surface to present their antigen to CD8 T-cells. The vast array of different peptides that can be presented by the huge array of HLA across the human population must be recognised by the T-cell receptor (TCR). This requires that each individual must have a huge number of TCRs.

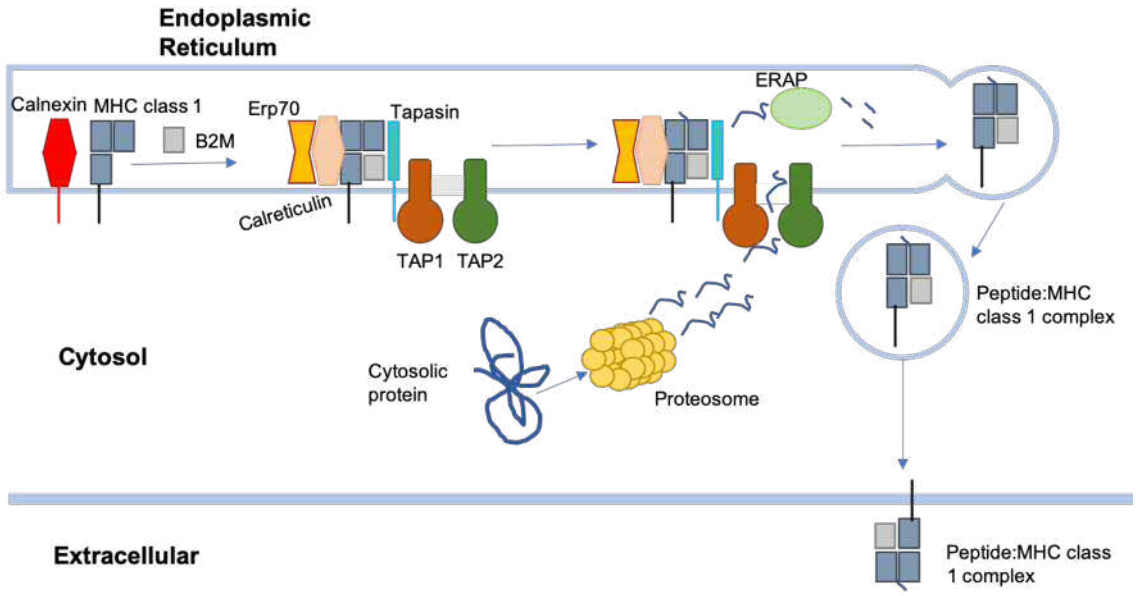


Figure 1.2: MHC class I antigen processing and presentation

MHC class I molecules are found on all nucleated cells and present endogenous peptide antigens to CD8 T-cells. MHC class I molecules are heterodimeric protein molecules composed of α- polypeptides and β_2M and they are only stable when bound to a peptide of 8-12 amino acids in length. MHC class I molecules are assembled in the endoplasmic reticulum (ER) and guided through the process of acquiring a peptide antigen by a selection of chaperone proteins. Peptides that have been processed in the cytosol by the proteasome, a multi-catalytic complex, can enter the ER through the heterodimeric transporters associated with antigen processing 1 and 2 (TAP1 and TAP2) complex. Prior to complexing with the MHC class I molecules, peptides are processed one final time by endoplasmic reticulum aminopeptidase associated with antigen processing (ERAP). A peptide-MHC class I complex can then be transported the surface of the cell and present the peptide to circulating CD8 T-cells.

1.3. Generation of TCR diversity

1.3.1. The αβ T-cell receptor

All conventional αβ T-cells possess a heterodimeric T-cell receptor (TCR) which is responsible for recognising peptides presented on MHC molecules. Engagement of the TCR by the pMHC complex, can result in T-cell activation through an intracellular signalling pathway. The TCR is extremely variable, and allows T-cells to recognise copious numbers of diverse peptides. TCRs are comprised of an α and a β chain, which pair up during gene rearrangement. TCR gene rearrangement, the process by which a TCR is generated will be discussed further in **Section 1.3.2**. Each chain consists of a germline encoded constant region which is anchored to the cell membrane. Here, cysteine residues form disulphide bonds which link the α and β

chains. Each TCR chain also has a variable domain which contains the antigen recognition site of a TCR, which is composed of six complementary determining region (CDR) loops (**Figure 1.3**). The majority of the TCR variability is found in CDR3, due to P and N nucleotide insertions and deletions acquired during gene rearrangement. The CDR3 loops are generally responsible for recognising and contacting the peptide within the MHC binding groove, whilst CDR1 and CDR2 typically interact with the MHC molecule itself.

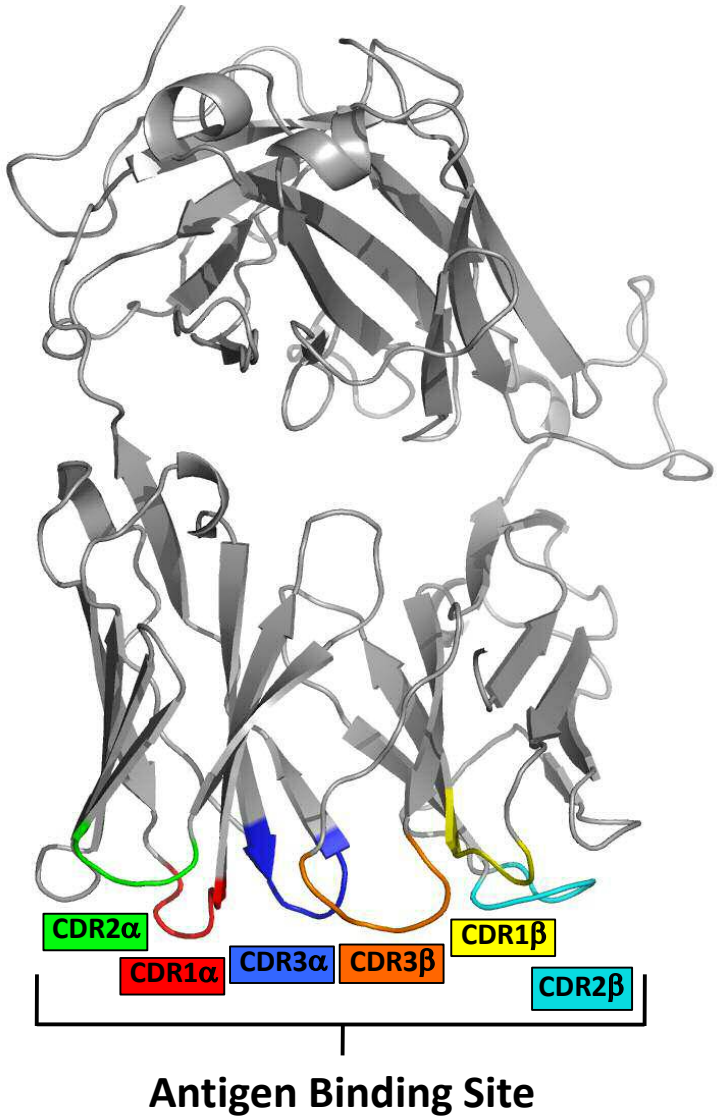


Figure 1.3: Crystal structure of a αβ TCR

The αβ TCR is composed of a constant (C) and a variable (V) domain. The C domain is germ line encoded. Within the V domain are the complementary determining regions (CDR) loops which comprise the antigen-recognition site of the TCR. They formed during TCR gene rearrangement from the V, D and J segments of the TCR chains. The CDR3 regions are the most hypervariable as they acquire P- and N- nucleotides during rearrangement, in most cases the CDR3 loops contact the peptide presented on MHC class I molecules. The CDR1 and 2 regions are less variable as they are germ line encoded, they interact with the MHC molecule itself, facilitating the TCR-pMHC interaction. Figure adapted from (Attaf *et al.*, 2015).

1.3.2. Gene rearrangement at the αβ TCR loci

TCR gene rearrangement begins with the β-chain, *trb*, which is located on chromosome 7. The β-chain locus is composed of two constant regions (Cβ1&2) and two clusters of diversity (Dβ1&2), 52 Vβ regions and 13 joining (Jβ) regions (**Figure 1.4**). Rearrangement begins with

one of the Dβ segments localising to one of the Jβ segments, this is quickly followed by the rearrangement of DJβ to a Vβ segment. The rearranged VDJ region is then spliced to the C segment creating a fully rearranged TCR β-chain. The splicing and recombination of gene segments during TCR gene rearrangement is an inaccurate process and leads to junctional diversity. Junctional diversity occurs due to the insertion and deletion of P and N nucleotides when the different gene segments combine to form a complete TCR chain. The presence of two distinct clusters of Jβ and Dβ segments permits rescuing of β-chain rearrangement if the initial attempt fails. This process begins in the DN3 stage of T-cell development and results in the expression of a pre-TCR (**Figure 1.5**). It is imperative that a T-cell successfully rearranges its β-chain at this stage, as without a pre-TCR the T-cell will not progress to DN4 and will die. TCR chains are guided though gene rearrangement by enzymes which form a multi-subunit complex, encoded by the recombination activating genes 1 and 2 (RAG1 and 2). Thymocytes expressing a fully rearranged β-chain acquire a pre-α-chain (pre-Tα) allowing rearrangement of its *tra* locus (**Figure 1.4**). The α-chain locus is located on chromosome 14 and lacks D gene segments but has many more J gene segments than the β-chain (**Figure 1.4**). Rearrangement stops when the T-cell is positively selected by interaction with a self-peptide presented on self MHC molecule or induced to die due to negative selection. The main region of TCR variability is located at the antigen-recognition site (**Figure 1.3**). In the centre of this region are the complimentary determining region (CDR) loops of the α and β chains, these are encoded by the V, D and J genes. The CDR3 region, which is located in the middle of the antigen-recognition site, is hypervariable due to the insertion of P- and N-nucleotides during TCR gene rearrangement. In most instances the CDR3 region makes contact with the immunogenic regions of a presented antigen. The hypervariability of CDR3 loops accounts for the broad diversity of recognition displayed by T-cells. The CDR1 and 2 regions are germline encoded and are not as diverse, they also tend to make contact with the MHC itself (Rudolph, Stanfield and Wilson, 2006; Cole *et al.*, 2014).

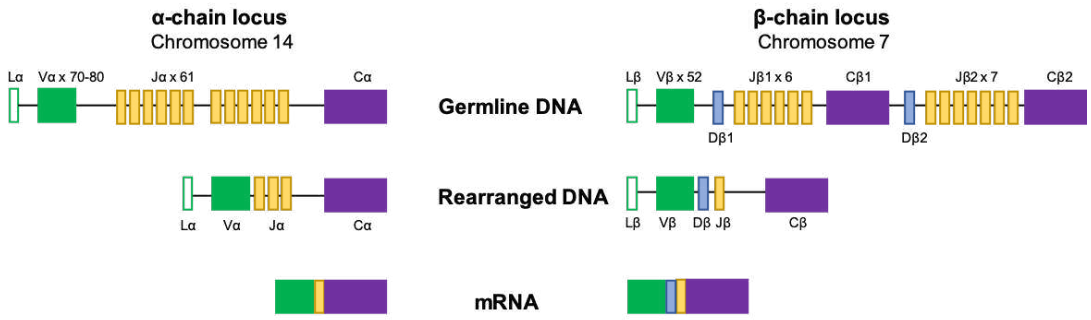


Figure 1.4: TCR gene rearrangement

Germline DNA of the α and β TCR chains located on chromosome 14 and 7, respectively. The α- chain locus consists of 70-80 variable (V) domains, 61 junction (J) domains and a constant domain. Whilst the β-chain consists of 52 V domains, and 2 distinct coding regions housing 6 or 7 J domains, a constant region and a diversity (D) domain. Upon rearrangement, guided by the RAG genes, DNA is translated to the mRNA which is expressed as a functional TCR. Rearrangement begins with the β-chain and the joining of one J segments to a D segment, following this the DJ segments then join to a V segment. After splicing of the rearranged VDJ regions to the C segment a fully rearranged β TCR chain is generated. Rearrangement of the α-chain only occurs after successful rearrangement of the β-chain.

1.3.3. Thymic selection of T-cells

Thymocytes that possess a fully rearranged TCR (both α and β chains) and possess either CD8 or CD4 co-receptors, i.e. are single positive (**Figure 1.5**), can now be screened for recognition of self-peptides presented by MHC molecules expressed in the thymus. MHC molecules expressed on thymic epithelial cells (TEC) present a diverse array of tissue-specific antigens, this unique property, termed promiscuous gene expression, facilitates the development of tolerance to self-antigens during T-cell development (Derbinski *et al.*, 2001). Transcription of tissue-specific antigens in the thymus is governed by the autoimmune regulator (AIRE) gene (Heino *et al.*, 1999), AIRE deficiency in humans, resulting in defective thymic selection, can cause severe autoimmunity. T-cells are positively or negatively selected based on their affinity to antigens presented on MHC. Positively selected T-cells do not recognise self-antigens with a high affinity. T-cells with TCRs that do not recognise a self-antigen weakly are not signalled and 'die by neglect' whilst T-cells with TCRs that bind relatively strongly to self-peptides have potential to be autoimmune and are eliminated. Overall, around 98% of thymocytes are eliminated at this stage (Zúñiga-Pflücker, 2004). This process of positive and negative selection is vital in generating central tolerance, negating the risk of the

development of autoimmunity. The elimination of self-reactive T-cells is not limited to the thymus, cells which escape central tolerance in the thymus are subjected to peripheral tolerance. This process is important in eliminating self-reactive T-cells capable of causing damage to healthy tissues and autoimmunity.

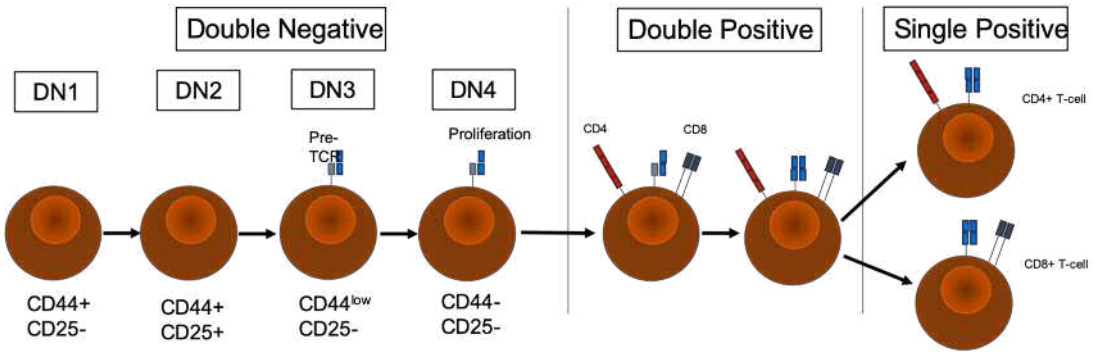


Figure 1.5: Thymic development of T-cells

Haemopoietic progenitors migrate from the bone marrow to the thymus where they commit to the T-cell lineage, beginning the process of differentiation. T-cell differentiation can be categorised by the expression of the co-receptors CD4 and CD8. Initially, immature thymocytes do not possess either of these molecules and are classified as being double negative (DN). In this first stage of development T-cells begin to rearrange their T-cell receptor. In the DN3 stage, thymocytes acquire a pre-TCR and gene rearrangement of the trb begins. T-cells cannot progress from the DN4 stage without a fully rearranged TCR β chain. T-cells then become double positive for CD8 and CD4, whist there TCR α chain undergoes rearrangement. Once T-cells possess a fully rearranged TCR they commit to the CD4 or CD8 lineage and progress through thymic section. 98% of thymocytes die during thymic selection.

1.4. CD8 T-cells and cancer

CD8 T-cells are critical mediators of anti-cancer immunity. They have the capacity to differentiate cancer cells from healthy cells through recognition of an altered array of peptides presented by MHC molecules on the surface of tumour cells. The canonical pathway of T-cell activation, tumour recognition and destruction by CD8 T-cells is depicted in **Figure 1.6**.

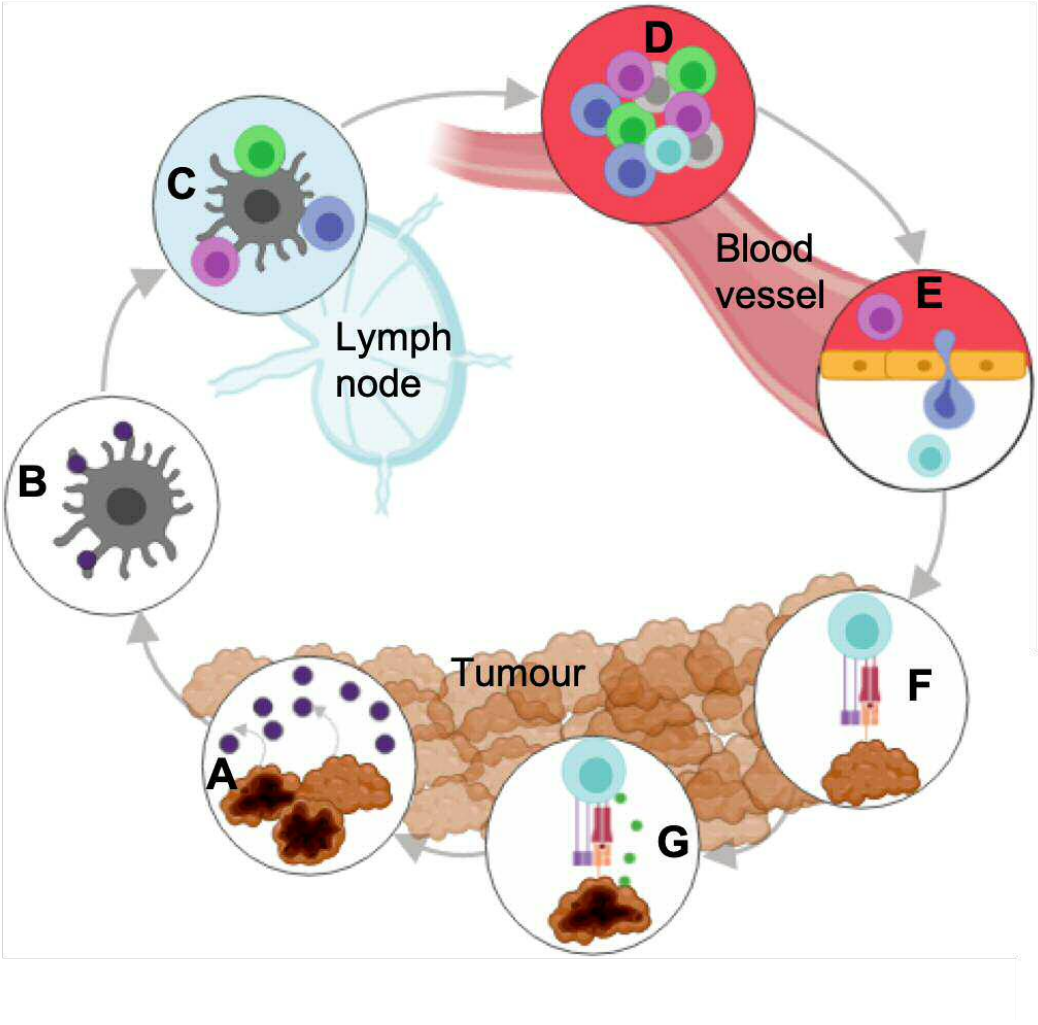


Figure 1.6: CD8 T-cells vs cancer

A) *The tumour mass contains stressed and necrotic tumour cells which provided an altered array of intracellular proteins from healthy tissue. These proteins contain CD8 T-cell antigens (conventionally peptides of 8-12 aa in length) which are displayed on the surface of tumour cells on MHC class I molecules.* ***B)*** *Surrounding antigen presenting cells, such as dendritic cells, phagocytose tumour antigens and proteins.* ***C)*** *DCs migrate to the lymph node where they encounter naïve CD8 T-cells which reside there.* ***D&E)*** *Presentation of processed tumour antigens on MHC class I molecules on DCs activate naïve CD8 T-cells which then leave the lymph node and migrate though the blood vessels to the tumour site.* ***F&G)*** *Antigen-specific CD8 T-cells recognise tumours with the corresponding peptide-MHC complex with their TCR. Recognition of the correct antigen initiates cytotoxicity and ultimately death of the tumour cell. Death of the tumour cell releases antigens and proteins into the tumour microenvironment, initiating the cycle once more.*

This process of T-cell activation and destruction of potentially transformed cells is termed immunosurveillance and can prevent the formation of malignant disease. The concept of immunosurveillance was originally proposed by Burnet and Thomas in 1957 and was widely

controversial as the importance of the immune system in protecting against the development of cancer was not fully appreciated (Burnet, 1957; Burnet, 1964). However, studies performed in the 1990's renewed confidence in this theory and began to unmask the link between the immune system and cancer. One such study demonstrated the importance of IFNγ in protecting BALE/C mice from carcinogen-induced Meth A fibrosarcoma (Dighe *et al.*, 1994) whilst another established how perforin produced by T-cells and NK cells could protect C57BL/6 mice from MCA-induced-tumour challenge (van den Broek *et al.*, 1996). However, arguably the most pivotal experiments came after the generation of mice lacking RAG1 and RAG2 genes. These genes are required for successful B, T and natural killer (NK) cell receptor rearrangement and mice lacking RAG1 and RAG2 are deficient in these cells (Shinkai *et al.*, 1992). Upon MCA tumour challenge mice lacking RAG genes developed MCA-induced-tumours more frequently than the matched wild type controls (Shankaran *et al.*, 2001). Dunn *et al* proposed a model to describe the relationship of the immune system and cancer, termed cancer immunoediting (Dunn *et al.*, 2002) (**Figure 1.7**). Their model comprises three stages of tumour development; elimination, equilibrium and escape, with cancer immunosurveillance included in the elimination phase. Tumours are heterogenetic masses of cells that develop in relation to the environment around them, the immunoediting models suggests that CD8 T-cells can sculpt the immunogenicity of a tumour through sustained pressure, altering the cellular composition of the tumour. Darwinian selection pressure results in immunogenic cells of the tumour being eradicated easily by the surrounding CD8 T-cells, leaving non-immunogenic cells behind. These tumour cells lacking the antigens required for immune cell mediated killing can then escape control by the immune system and develop into a clinically relevant tumour mass. In parallel to immune editing, the tumour microenvironment (TME) itself can affect CD8 T-cell responses to malignant disease. Tumours often promote an anti-inflammatory environment through the release of cytokines such as IL-10 and TGFβ and recruitment of regulatory T-cells (T-regs) and myeloid derived suppressor cells (MDSC) (**Figure 1.7**). In some cases, tumour cells downregulate expression of MHC class I, making it difficult for T-cells in the periphery to respond to malignant cells (Umansky and Sevko, 2012). Furthermore, tumours upregulate inhibitory molecules such as programme death receptor 1 (PD1) and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) which suppress CD8 T-cells (Walunas *et al.*, 1994; Freeman *et al.*, 2000). It has also been reported in malignancies such as acute myeloid leukaemia (AML) and chronic lymphocytic leukaemia (CLL) that the TME can affect the formation of the

immunological synapse by disrupting actin polymerisation (Ramsay *et al.*, 2008; Le Dieu *et al.*, 2009), leading to a defective CD8 T-cell response to tumour.

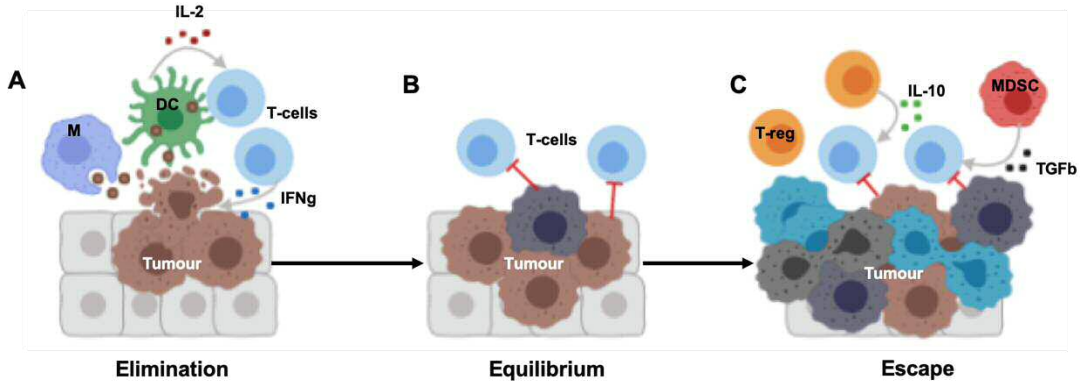


Figure 1.7: Cancer Immunoediting

The three stages of cancer immunoediting; elimination, equilibrium and escape. ***A****) tumour cells are recognised and destroyed by CD8 T-cells in the tumour microenvironment. CD8 T-cells are primed by macrophages (M) and dendritic cells (DC) expressing tumour associated antigens. Pro-inflammatory cytokines such as IL-2 and IFN*γ *creating a favourable environment for T-cell cytotoxicity.* ***B****) Cytotoxic T-cell killing eradicates immunogenic tumour cells, driving the propagation of mutated cells (shown in black). Furthermore, the tumour microenvironment becomes more anti-inflammatory with increased expression of checkpoint inhibitors, dampening the T-cell response.* ***C****) After sustained immunological pressure, the tumour mass grows and escapes immune surveillance. Non-immunogenic tumour cells (depicted in black and blue) expand. Regulatory T-cells (T-Regs) and myeloid derived suppressor cells (MDSC) create a hostile tumour microenvironment, emitting anti-inflammatory cytokines such as IL-10 and TGF*β*, preventing adequate T-cell activation and aiding the escape of the tumour cells.*

M: macrophage, DC: dendritic cell, MDSC: myeloid derived suppressor cell, T-reg: Regulatory T-cell

1.5. CD8 T-cell epitopes and cancer

1.5.1. Tumour antigens

A by-product of dysregulated protein expression associated with tumour development is the establishment of a unique profile of peptide antigens specific for the tumour type and the individual cancer patient. Tumour associate antigens (TAA) are expressed by healthy and cancer cells and can be shared across individuals whilst tumour specific antigens (TSA) are exclusively expressed by tumour cells. CD8 T-cells can recognise tumour antigens displayed on MHC class I molecules making them attractive targets for immunotherapy. The strong correlation of some TAA with certain cancers has permitted their use as biomarkers.

Prostate-specific antigen (PSA) and carcino-embryonic antigen (CEA) are well established TAA biomarkers in prostate (Schröder *et al.*, 2014) and colorectal cancer (Werner *et al.*, 2016) respectively, due to their increased levels in serum of individuals with developing tumours. Tumour antigens can be categorized based on their expression in healthy tissues into the broad sub-groups described below.

1.5.1.1. Overexpressed antigens

Some self-proteins are expressed in healthy cells at low copy numbers but overexpressed by cancer cells. Specific overexpressed antigens are strongly associated with certain cancer types such as; MUC1 which is commonly associated with epithelial cancers (Ansari *et al.*, 2014; Joshi *et al.*, 2014). Survivin, which is expressed ubiquitously in cancer cells but is rarely detected in healthy tissue has been demonstrated to be critical for the survival of cancer cells (Stauber, Mann and Knauer, 2007; Altieri, 2010). Survivin inhibits apoptosis (Altieri, 2003; Wheatley, 2015), and its downregulation supresses tumour growth (Khan *et al.*, 2017) making it a prominent clinical therapeutic target. Inhibition of survivin, has been demonstrated to induce apoptosis and increase the effectiveness of conventional therapeutics (Olie *et al.*, 2000; Khan *et al.*, 2010). Survivin-restricted CD8 T-cells have been isolated from cancer patients (Reker *et al.*, 2004), leading to the initiation of a phase I/2 clinical trial in renal cell carcinoma (RCC) using autologous dendritic cells loaded with a pool of HLA class I-restricted survivin peptides (Berntsen *et al.*, 2008).

1.5.1.2. Oncofetal antigens

Carcino-embryonic antigen (CEA) and alpha-fetoprotein are oncofetal antigens. Oncofetal antigens are only normally expressed during fetal development. CEA is expressed by a number of tumour types such as colorectal (Gold and Freedman, 1965) and gastric (Deng *et al.*, 2015) cancers, whilst alpha fetoprotein is commonly expressed in hepatocellular cancers (Bialecki and Di Bisceglie, 2005). The limited expression of oncofetal antigens in healthy tissues makes them attractive for targeted therapies due to the reduced risk of autoimmune side effects. There are several active clinical trials seeking to generate an effective immune response to CEA-expressing cancers. For example, the use of CEA-targeting chimeric antigen receptor T-cell (CAR-T) therapy in colorectal, gastric and pancreatic cancers is currently being explored in phase I trials (NCT03682744, NCT03818165). Additionally, the use of viral vector

vaccines encoding CEA are being investigated as mechanisms to induce anti-tumour CD8 T-cell responses (NCT01890213, NCT03050814).

1.5.1.3. Cancer-testis antigens

Similar to oncofetal antigens, cancer-testis (CT) antigens have a highly restricted expression pattern in healthy tissues as they are expressed exclusively in male germ-line cells. CT antigens include; the melanoma-associated antigen (MAGE) (Boon *et al.*, 1994), B melanoma antigen (BAGE) (Boël *et al.*, 1995) and G antigen 1 (GAGE) (Van den Eynde *et al.*, 1995) protein families and NY-ESO (Chen *et al.*, 1997) and are routinely over-expressed in cancers of different histological origins. The expression of CT antigens has been linked to uncontrolled growth, resistance to cell death and metastasis in cancer cells (Scanlan *et al.*, 2002; Gjerstorff, Andersen and Ditzel, 2015; Schooten *et al.*, 2018). CT antigens have been the focus of a number of clinical trials due to their restricted expression in healthy tissues. Clinical trials have sought to target cancers expressing CT antigens by using monoclonal antibodies (Bernardeau *et al.*, 2005; Saeed *et al.*, 2016), therapeutic vaccines targeting MAGE-A1 (van Baren *et al.*, 2005), MAGE-A3 (Kruit *et al.*, 2013) and engineered TCRs specific for peptide antigens derived from MAGE-A3 (Richard A Morgan *et al.*, 2013) or NY-ESO (Rapoport *et al.*, 2015).

1.5.1.4. Differentiation antigens

Proteins with restricted expression in healthy cells and associated with differentiation can become overexpressed in cancer cells. Melan-A/melanoma antigen recognised by T-cells (MART1) is exclusively expressed at low levels by terminally differentiated melanocytes (Kawakami *et al.*, 1994) but is commonly overexpressed in melanoma cancer cells. Melan-A is overexpressed in the majority of melanoma tumours (Coulie *et al.*, 1994; Kawakami *et al.*, 1994) and CD8 T-cells reactive to its immunogenic peptide antigens EAAGIGILTV and AAGIGILTV have been identified (van der Bruggen *et al.*, 1991). Due to the strong correlation between melanoma and melan-A, strategies to harness melan-A reactive cells have been a focus of clinical research, however to-date there has been little success in this field (Nestle *et al.*, 1998; Bins *et al.*, 2007). The differentiation antigen melan-A will be discussed in more detail in **Chapter 3**.

1.5.1.5. Heat shock proteins

Heat shock proteins (HSP) provide protection to healthy cells during stress conditions, so it is unsurprising that they are commonly upregulated in cancer cells. HSP can help cancer cells divide, metastasize and supress cellular apoptotic mechanisms (Ciocca, Arrigo and Calderwood, 2013). HSPs aid in the refolding and regulation of TAAs expression, such as survivin (Fortugno *et al.*, 2003; Plescia *et al.*, 2005). Murine studies targeting HSP90 with small molecule inhibitors have demonstrated inhibition of tumour cell growth and increased cell death (Plescia *et al.*, 2005; Kang, Neckers and Altieri, 2009). Moreover, inhibition of HSP90 increased the efficacy of chemotherapy and radiation in otherwise resistant pancreatic tumours (Nagaraju *et al.*, 2019).

1.5.1.6. Tumour specific antigens

Tumour specific antigens, otherwise known as neoantigens, are derived from exome mutations developed during tumorigenesis. The success of anti-PD1 and anti-CTLA-4 checkpoint inhibitor therapy has been correlated to the degree of mutational burden in a given tumour (Snyder *et al.*, 2014; Schumacher and Schreiber, 2015; Sharma and Allison, 2015). Checkpoint inhibitor therapy has been particularly successful in tumours such as melanoma (Snyder *et al.*, 2014), non-small cell lung cancer (Rizvi *et al.*, 2015), bladder cancer (Powles *et al.*, 2014) which all have a high rate of somatic mutations. Neoantigen-specific T-cells have been identified in the TIL infusion product and PBMC of metastatic melanoma patients who have undergone TIL therapy (Robbins *et al.*, 1996; Huang *et al.*, 2004; Zhou *et al.*, 2005; Lu *et al.*, 2013). In one case, the TIL infusion product reacted to ten neoantigens identified from exome sequencing of the patient's tumour. Moreover, 30% of the T-cells found in the patient post-cure were reactive to five neoantigens out of the original ten (Prickett *et al.*, 2016). In some rare cases 'public' neoantigens have been identified, for example, CD8 T-cells restricted to a handful of mutated calreticulin epitopes have been identified in multiple donors, mutations in exon 9 of calreticulin are common in patients with chronic myeloproliferative neoplasms (Holmström *et al.*, 2019). Neoantigen-based immunotherapy is complicated by the observation that not all mutations identified by exome sequencing are presented by MHC and recognised by patient-derived T-cells (Schumacher and Schreiber, 2015). Verdegaal et al. demonstrated that, through immunoediting, neoantigen specific patient T-cells can drive the negative selection and deletion of mutated antigens, providing further challenges for T-cell neoantigen-directed therapy (Verdegaal *et al.*, 2016).

1.5.2. Modified peptides

In addition to conventional peptides, CD8 T-cells can recognise peptides that have undergone post-translational modifications. Peptides can be post-translationally modified in several different ways, the most common will be discussed below.

1.5.2.1. Glycosylation

Glycosylation occurs when a carbohydrate is covalently attached to a protein, two forms of glycosylation exist, N and O-linked glycosides (**Figure 1.8**). Glycosylated peptide antigens are associated with autoimmune diseases and cancer. Studies have identified MHC class II presented glycosylated peptides which have been linked to the development of autoimmune diseases such as rheumatoid arthritis (Bäcklund *et al.*, 2002; Andersson *et al.*, 2011). Moreover, CD8 T-cells restricted to glycosylated MUC1 epitopes, a commonly overexpressed TAA, have been identified (Doménech, Henderson and Finn, 1995; Apostolopoulos *et al.*, 1997; Ninkovic *et al.*, 2009; Pejawar-Gaddy *et al.*, 2010). The resolution of a glycosylated peptide derived from MUC1 bound to the murine MHC class I molecule (H-2K^{b}) demonstrated that glycosylated amino acids can increase the binding affinity of the peptide by acting as anchor residues (Apostolopoulos *et al.*, 2003).

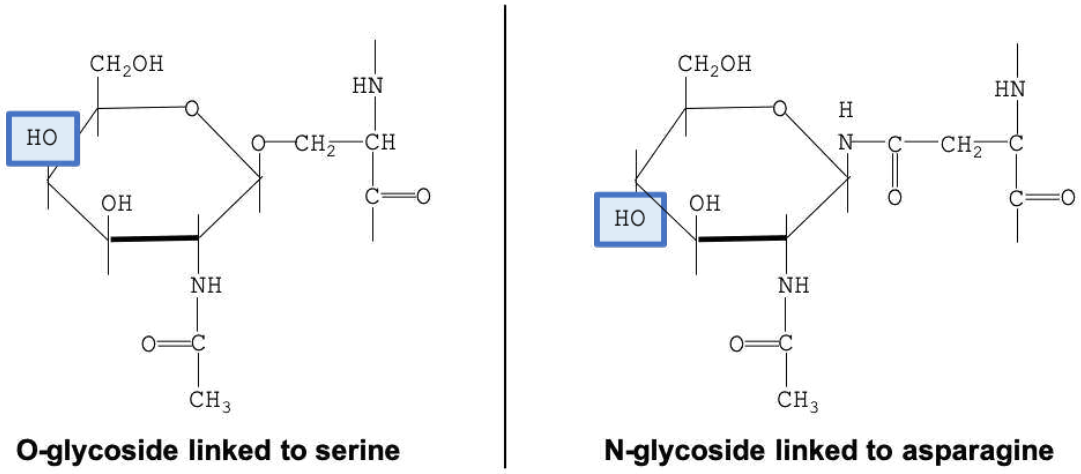


Figure 1.8: Amino acid glycosylation

Attaching a single sugar group to an amino acid, glycosylation, occurs naturally in two forms; O-linked and N-linked. O-linked glycosylation attaches the sugar group to the oxygen molecule of the carboxyl functional group. N-linked glycosylation occurs by attaching the sugar group the nitrogen of the amine functional group.

1.5.2.2. Citrullination

Citrullination occurs when the positively charged NH_2 group of an arginine is converted to a neutrally charged O (**Figure 1.9**). Autophagy is a mechanism by which cells breakdown unwanted cellular components, during this process peptidylarginine deiminase (PAD) citrullinates peptides for presentation by MHC molecules. Immune recognition of citrullinated peptides has been linked to autoimmune diseases such as rheumatoid arthritis and type 1 diabetes (McGinty *et al.*, 2014). Citrullinated peptides have been demonstrated to have a protective effect in anti-tumour immunity. A recent study by Brentville et al demonstrated that immunisation of epithelial tumour bearing mice with a citrullinated peptide, derived from the immediate filament protein vimentin, increased cytokine release and CD4 T-cell mediated tumour regression (Brentville *et al.*, 2016). Despite the accumulating evidence of the importance of MHC class II presented citrullinated peptides and CD4 T-cells, little is known about the interaction of CD8 T-cells and citrullinated peptides.

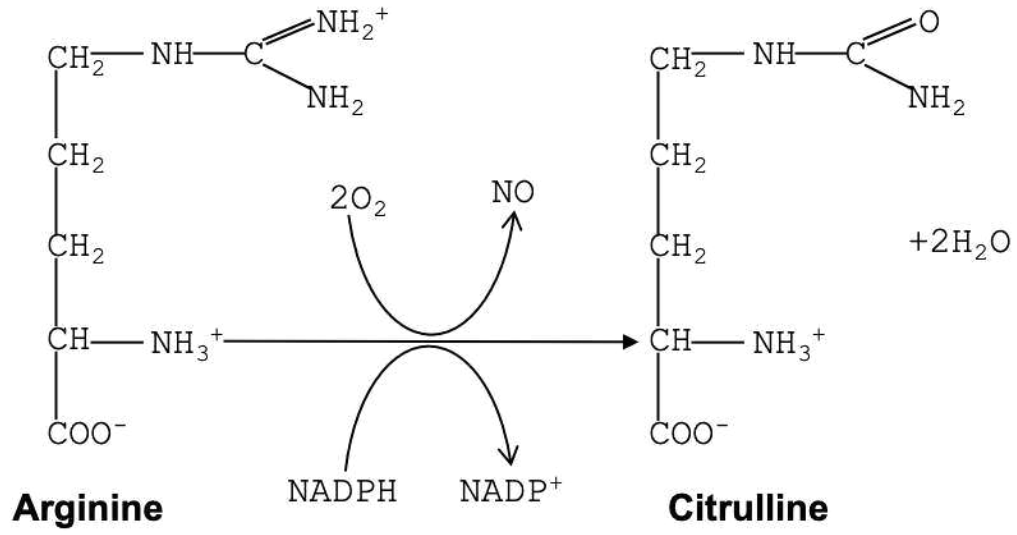


Figure 1.9: Conversion of arginine to citrulline

The conversion of arginine to citrulline is carried out by peptidylarginine deiminase (PAD). PAD removes the NH_2^+ group on arginine, converting it to a neutrally charged O group. This process generates two water molecules and a NO group and requires the reducing agent NAPDH which is converted to $NADP^+$. Generation of citrulline generally occurs during the intracellular process of autophagy.

1.6. Cancer Immunotherapy

Since the results of early studies demonstrated the capacity of the immune system to eradicate cancer in murine models, there has been increasing interest in developing novel cancer immunotherapies. The discovery of immunotherapeutic strategies targeting widely expressed molecules such as the checkpoint inhibitors programmed death receptor (PD-1) or cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) has led to the development of therapies that can be used in patients with different cancer types. However, with the complex nature of cancer development and subsequent protein expression on tumour cells, there is also a need for patient-specific immunotherapies, such as adoptive cell therapy (ACT). Current cancer immunotherapy strategies are discussed below.

1.6.1. Cytokine therapy

The immunosuppressive tumour microenvironment (TME) of many cancers is not conducive to an effective immune response. With the realisation of the importance of the immune system in treating cancer came a desire to modulate the TME, inducing a proinflammatory environment with the hope of enhancing cancer-specific immune responses. Early immunotherapeutic approaches consisted of recombinant, pro-inflammatory cytokines. IFNα is a proinflammatory cytokine which can inhibit tumour cell proliferation and can increase MHC class I expression leading to improved immune recognition and activation of CD8 and CD4 T-cells (Belardelli and Gresser, 1996). IFNα therapy was the first cytokine therapy to obtain FDA approval, with an early study demonstrating a complete remission rate of 30% with hairy cell leukaemia (Quesada *et al.*, 1986). Subsequently, IFNα has been used to treat renal cell carcinoma (Tsavaris *et al.*, 1995) and melanoma (Di Pucchio *et al.*, 2006).

Granulocyte-monocyte colony stimulating factor (GM-CSF) can also improve immune responses during cancer therapy. GM-CSF promotes the trafficking of T-cells and initiates the maturing of dendritic cells (DC) and macrophages which is critical for presentation of TAAs and subsequent activation of naïve T-cells. Despite promising murine studies, GM-CSF monotherapy has shown modest results clinically (Kaufman *et al.*, 2014). GM-CSF is now being explored as an adjuvant to improve the efficacy of peptide vaccines (NCT02510950), DC vaccines (NCT02465268) and Ipilimumab checkpoint therapy (NCT02275416).

Interleukins, which act directly to stimulate CD4 and CD8 T-cells, are an important class of cytokines being explored for immunotherapy. The discovery of interleukin 2 (IL-2) in 1976 (Morgan, Ruscetti and Gallo, 1976) permitted the growth of T-cells *in vitro* making it more feasible to study them. Treatment of melanoma and renal cell carcinoma patients with IL-2 showed clinical promise, with 20% of patients achieving durable, complete remission after therapy (Rosenberg *et al.*, 1998). The durability of clinical responses resulting from IL-2 therapy led to FDA approval of Aldeleukin, a recombinant IL-2 therapy for melanoma and renal cell carcinoma in 1998 (Rosenberg *et al.*, 1987; Wang *et al.*, 1989). However, due to the short half-life of IL-2, high-doses are required for treatment, and this can lead to extreme adverse effects in many patients, including cytokine release syndrome and vascular leakage (Alwan *et al.*, 2014). Despite the risk of side effects, IL-2 treatment has been used to increase the efficacy of other immunotherapeutic strategies such as tumour infiltrating lymphocyte (TIL) therapy (Rosenberg *et al.*, 1988; Dudley *et al.*, 2010a; Besser *et al.*, 2013; R. Andersen *et al.*, 2016).

1.6.2. Adoptive cell therapy

Adoptive cell therapy (ACT) utilised patient's own cells to target cancer by removing, culturing them *ex vivo* and reinfusing them back into the patient. Some forms of ACT involve modification of immune cells. The first successful use of ACT was seen in animal models whereby an autologous lymphocyte infusion eradicated fibrosarcoma in rats (Delorme and Alexander, 1964) and Maloney sarcoma-virus induced tumours in mice (Fefer, 1969). Early studies in humans used autologous lymphokine-activated killer (LAK) cells and high dose of recombinant IL-2 to treat metastatic melanoma, where objective responses were seen in 50% of patients and complete and durable regression of tumour in one patient (Rosenberg *et al.*, 1985).

A pertinent example of ACT is tumour infiltrating lymphocyte (TIL) therapy, whereby T-cells isolated from the tumour mass are rapidly expanded *ex vivo* and reinfused back into the patient. A high density of intratumoural lymphocytes is associated with improved outcomes in metastatic melanoma (Radvanyi *et al.*, 2012). Successful early murine studies using TIL therapy, IL-2 and cyclophosphamide to treat pulmonary and hepatic tumours (Rosenberg, Spiess and Lafreniere, 1986) coupled with the demonstration that cultured human-derived TIL could kill autologous tumour *in vitro* (Muul *et al.*, 1987) led to the first human TIL therapy clinical trial in metastatic melanoma (Rosenberg *et al.*, 1988). This trial combined the TIL

infusion product with administration of IL-2 and a dose of cyclophosphamide, 55% of patients achieved objective tumour regression (Rosenberg *et al.*, 1988), this seminal result led to many further TIL therapy clinical trials for metastatic melanoma (Itzhaki *et al.*, 2011; Radvanyi *et al.*, 2012; Besser *et al.*, 2013; Andersen *et al.*, 2016; Andersen *et al.*, 2018). The standardised treatment regime for TIL therapy includes nonmyeloablative lymphodepletion before administration of cultured autologous TILs (Klebanoff *et al.*, 2005). Lymphodepletion increases the persistence of the TILs and enhances tumour regression (Dudley *et al.*, 2002). Administration of TIL is followed by a high dose of IL-2. Initial trials used a bolus of IL-2 which had pronounced toxicities (grade 3 and 4) in some patients (Radvanyi *et al.*, 2012) however recent trials have used decrescendo intravenous administration of IL-2 which is generally better tolerated (Andersen *et al.*, 2016). Following the relative clinical success of TIL therapy in metastatic melanoma, attempts to grow large-scale TIL has followed in a range of other cancers including; ovarian cancer (Webb *et al.*, 2014), breast cancer (Wein *et al.*, 2017), renal cell carcinoma (RCC) (Andersen *et al.*, 2015) and gastrointestinal cancer (Turcotte *et al.*, 2013).

1.6.3. Checkpoint Inhibitor therapy

Immune checkpoints are an array of molecules that in ordinary circumstances keep the immune system in check, helping to prevent autoimmunity (**Figure 1.10**). Increased expression of immune checkpoints during malignancy can cause a dysfunctional immune response, facilitating rapid tumour cell growth.

The most widely studied immune checkpoints are cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and programme death receptor 1 (PD-1), both of which are negative regulators of T-cells. CTLA-4 was discovered by James Allison and colleagues in 1994 (Walunas *et al.*, 1994; Krummel and Allison, 1995). CTLA-4 has high homology to CD28 which binds to B7 (CD80/86) during TCR-MHC engagement, acting as the second signal for effective T-cell activation. CTLA-4 is upregulated upon T-cell activation and also binds B7 (CD80/86) therefore blocking this second signal. A monoclonal human antibody against CTLA-4, Ipilimumab, was developed as a blocking agent to stop the interaction of CTLA-4 and B7 (CD80/86). Early murine models demonstrated that administration of Ipilimumab induced tumour rejection and resulted in protection against a second exposure of 51BLim10 tumours in BALB/c mice (Leach, Krummel and Allison, 1996). Treatment of mice with a combination of Ipilimumab and granulocyte/monocyte colony stimulating factor (GM-CSF) in a B16 melanoma model led

to tumour rejection (Hurwitz *et al.*, 1998). Furthermore, a combination of Ipilimumab and cryoablation mediated rejection of prostate cancer in the TRAMP prostate cancer mouse model (Waitz, Fassò and Allison, 2012). The success of murine experiments led to human clinical trials using ipilimumab in combination with GP100 peptide, a melanoma-associated tumour antigen (Hodi *et al.*, 2010) or chemotherapy (Robert *et al.*, 2011). In both these largescale trials the overall survival rate of patients who receiving Ipilimumab was significantly higher than those received a placebo treatment, leading to FDA approval of Ipilimumab for the treatment of metastatic melanoma in 2011.

PD-1 was discovered in 2000 by *Freeman et al.*, it blocks T-cell responses by interfering with TCR-mediated signalling but does not interact with B7 (Freeman *et al.*, 2000). PD-1, is expressed by T-cells and interacts with its ligand PD-L1 on the opposing cell. PDL-1 is expressed at low levels on normal cells but is upregulated by tumour cells (Dong *et al.*, 2002). Moreover, IFNγ, which is produced by activated T-cells causes increased expression of PDL-1. (Dong *et al.*, 2002). Engagement of PD-1 and PDL-1 can lead to self-induced apoptosis by T-cells (Dong *et al.*, 2002). It is hypothesised that the interaction of PD-1 and PDL-1 is as a defence mechanism for healthy cells which is exploited by tumour cells, protecting them from T-cell mediated cytotoxicity. Anti-PD-1 monoclonal antibodies Nivolumab and Pembrolizumab were approved by the FDA for the treatment of metastatic melanoma in 2014, after clinical trials demonstrated success rates of up to 38% (Hamid *et al.*, 2013; Robert *et al.*, 2015). Subsequently, Nivolumab and Pembrolizumab have also been approved as second line therapies for the treatment of non-small cell lung carcinoma (NSCLC) (Gandhi *et al.*, 2018; Hellmann *et al.*, 2019).

Despite promising clinical results, CTLA-4 or PD-1 checkpoint blockade therapy does not work in all patients. Therapies targeting alternative immune checkpoints are currently being tested in clinical trials. One such example is a soluble fusion protein of lymphocyte activation gene 3 (LAG-3) and immunoglobulin G, IMP321, which binds MHC class II molecules. Studies have demonstrated that blockade of LAG-3 increased T-cell infiltration of tumour and activation of effector CD8 T-cells (Brignone *et al.*, 2009). A phase I/II trial with metastatic breast cancer patients treated with IMP321 demonstrated an objective response rate of 50% (Brignone *et al.*, 2010). Further examples of checkpoint inhibitors include monoclonal antibodies targeting T-cell immunoglobulin-3 (TIM-3) and T-cell immunoglobulin and ITIM domain (TIGIT) (Marin-Acevedo *et al.*, 2018), both of which have been demonstrated to be potent inhibitors of T-cell activation.

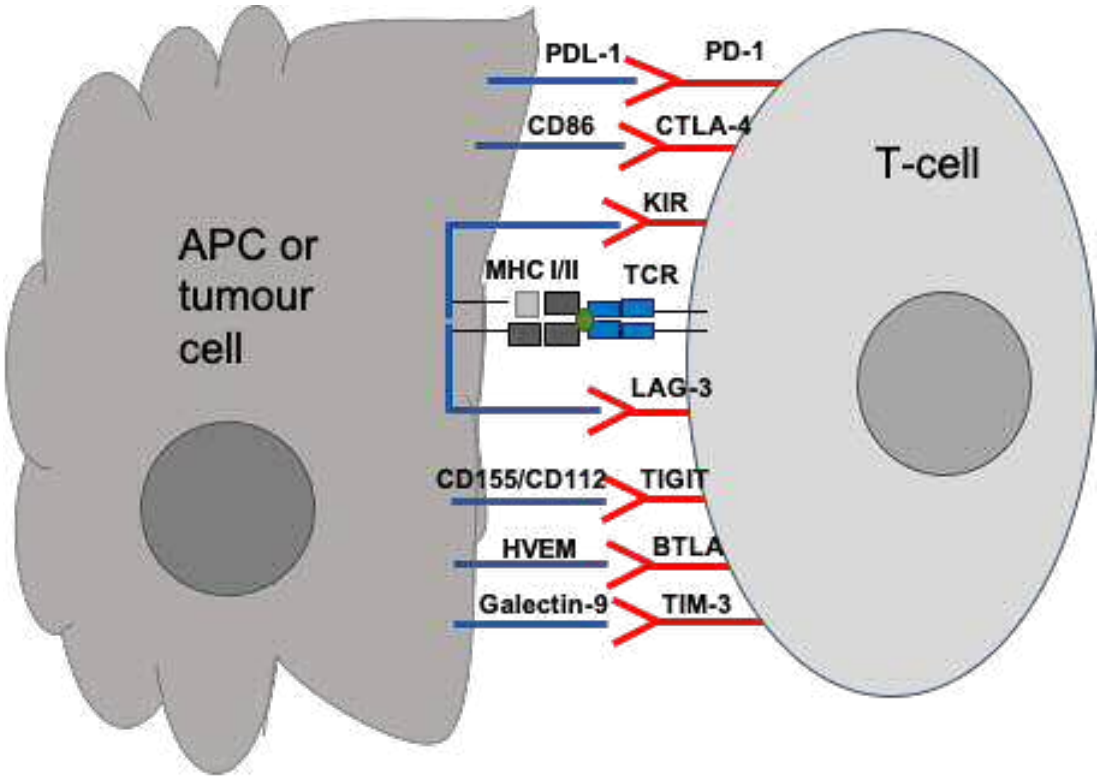


Figure 1.10: Immune checkpoints

In ordinary conditions, immune checkpoints function to control the T-cell response, preventing unnecessary cell damage and autoimmunity. During malignancy, tumour cells monopolise immune checkpoints, dampening the T-cell response and allowing rampant tumour growth and metastasis. Checkpoint inhibitors seek to block these inhibitory signals, activating the cytotoxic T-cell response in the tumour microenvironment with the hope of mediating tumour cell death.

1.6.4. Engineered T-cell therapy

The clear importance of T-cells, and specifically TCRs in cancer has led to many studies seeking to harness tumour-specific TCRs to treat malignant disease. High-throughput *in vitro* strategies are used to identify potent, tumour-antigen specific TCRs which are then transfected into autologous T-cells and reinfused into cancer patients. Adoptive cell therapy of the melan-A specific, DMF4 TCR to treat metastatic melanoma was the first human clinical example of engineered TCR therapy (Morgan *et al.*, 2006). Despite low response rates overall, T-cells expressing the DMF4 TCR persisted in most patients over a year after therapy and two patients demonstrated objective regression of their metastatic disease (Morgan *et al.*, 2006). The avidity of a TCR for its cognate peptide-MHC is a critical determent of the

outcome of the interaction. TCRs specific for self-peptides are generally low affinity for their cognate antigen which in normal circumstances helps to protect the host from autoimmunity. However in the cancer setting, this can result in sub-optimal clinical results and could present a stumbling block for engineered TCRs (Aleksic *et al.*, 2012). The affinity and avidity of T-cells for their cognate antigens is key when anti-cancer T-cells are the focus of an immunotherapeutic strategy. The affinity refers to the direct, bimolecular interaction of one TCR for one pMHC whilst avidity takes into account the accumulation of multiple protein to protein interactions. Therefore, when considering using a TCR for therapy the avidity, rather than the affinity of the interaction could be considered to be a more important pre-determinant to the success of any given immunotherapy. To improve upon the early studies using DMF4 TCR, which is a relatively low affinity TCR Johnson et al. sought to identify a high affinity TCR specific for melan-A. The DMF5 TCR, was identified from the tumour site of a melanoma patient (Johnson *et al.*, 2006). Adoptive cell transfer of the DMF5 TCR into metastatic melanoma patient resulted in improved clinical results, compared to the DMF4 TCR, with 30% of patients experiencing objective anti-tumour responses (Laura A. Johnson *et al.*, 2009). However, the majority of patients treated with DMF5 TCR experienced adverse effects after treatment including a widespread erythematous skin rash, uveitis and hearing loss (Laura A. Johnson *et al.*, 2009). These adverse side effects were due in part to the expression of melan-A by healthy melanocytes found in the skin, eye and ear. Cancer testis antigens are an attractive TAA due to their abundance expression on tumour cells but restricted expression patterns in healthy tissue. Two high affinity MAGE A3 restricted TCRs were designed to treat metastatic melanoma. Despite rigorous pre-clinical testing, both high affinity TCRs elicited fatal adverse effects in some treated patients (G. P. Linette *et al.*, 2013; Richard A. Morgan *et al.*, 2013). Post-therapy analyses demonstrated that one MAGE A3 TCR exhibited cross reactivity to MAGE A12 which is expressed in the brain and ultimately caused T-cell mediated brain damage and death of two patients (Richard A. Morgan *et al.*, 2013). Another MAGE A3 TCR was found to cross react with an epitope derived from titin, a component of beating cardiomyocytes and caused heart failure (Linette *et al.*, 2013). These studies highlighted that the cross reactivity of high affinity TCRs can be difficult to predict.

Chimeric antigen receptor (CAR) T-cells are another form of engineered T-cell therapy. CAR T-cells are generated by transduction of autologous, patient derived T-cells with a CAR molecule specific for a tumour associated protein. CAR molecules are composed of an antigen specific region and a T-cell receptor signalling domain (**Figure 1.11**). When a CAR T-cell comes into contact with its antigen it initiates T-cell cytotoxicity without the need for co-

stimulatory molecules or TCR-MHC engagement. CAR T-cells were first shown to have measurable effects on tumour cells in the late 1980's (Kuwana *et al.*, 1987; Gross, Waks and Eshhar, 1989). The most studied CAR T molecules are specific for CD19, a B-cell associated molecule widely expressed by all B-cells, healthy or cancerous. Studies have demonstrated remarkable clinical success using CAR T therapy with one study seeing a 50% response rate in advanced CLL patients (Porter *et al.*, 2015). Successful clinical trials lead to the licencing of two CAR T therapies by the FDA, Axicabtagene Ciloleucel for B-cell lymphoma (Neelapu *et al.*, 2017) and Tisagenlecleucel for acute lymphoblastic leukaemia (ALL) (Maude *et al.*, 2018). Treatment with CAR T-cells can result in severe side effects for some patients for example cytokine storms (Fitzgerald *et al.*, 2017) and B-cell aplasia, which can be mediated with intravenous immunoglobulin (Porter *et al.*, 2011). CAR T-cell therapy can also lead to antigen loss (Yu *et al.*, 2017) or resistance to the CAR T-cells in some cases, due to selection pressures applied by targeting one antigen. Despite relatively promising results in blood cancers, outcomes when using CAR T-cells against solid tumours have been disappointing. The discrepancy between solid and blood cancers has been put down to difficulty finding appropriate antigens, failure of CAR-T-cells to traffic to tumour sites and the suppressive tumour microenvironment commonly associated with solid tumours.

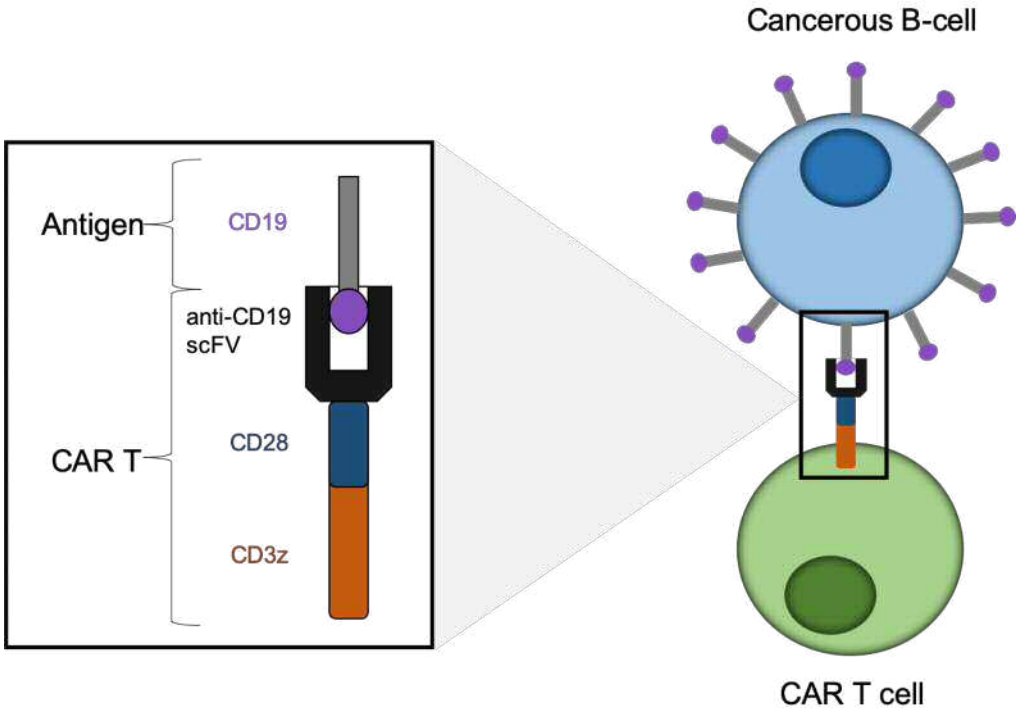


Figure 1.11: Chimeric antigen receptor T-cells

Chimeric antigen receptor (CAR) T-cells are composed of an antigen specific region (anti-CD19 scFV), CD28 domain and a CD3z domain. Engagement of the CAR-T with its antigen initiates internal T-cell signalling leading to the destruction of the CD19 expressing cancerous B-cell.

1.7. Cancer vaccines

Since the discovery of the smallpox vaccine by Edward Jenner, vaccines have been heralded as the biggest success story in modern medicine. Vaccination is currently considered the most effective way to prevent infectious disease (Hellstrom and Hellstrom, 2003). Population-wide vaccination strategies, implemented at a country and worldwide level culminated in smallpox being considered eradicated by the Worldwide Health Organisation (WHO) in 1980 (Fenner *et al.*, 1988). With the realisation that the immune system is paramount in eradicating cancerous cells multiple studies have sought to develop anti-cancer vaccines as a form of immunotherapy. The aim of cancer vaccines is to elicit the immune system, in particular CD8 T-cells, to mediate tumour regression whilst also generating immunological memory to prevent the return of malignant disease. The identification of cancer specific markers, such as TAAs and neoantigens, has provided ample anti-cancer vaccine targets. Many different vaccine strategies have been developed to elicit

cytotoxic T-cell responses in patients, these include; dendritic cells (DCs), peptide antigens, tumour lysates, viral vectors and DNA/RNA. These vaccine strategies and their clinical efficacy to-date will now be discussed in more detail.

1.7.1. Dendritic cell vaccines

Dendritic cell (DC) vaccines involve removing autologous DCs from cancer patients, maturing them *ex vivo* and loading them with a 'payload', such as antigenic peptides, mRNA or tumour lysate, before reinfusing them back into patients. In theory, DCs process and present the antigenic regions of the payload to T-cells, initiating an anti-tumour response. Peptides administered to DCs can be of MHC-I or II restricted length or some studies have utilised synthetic long peptide (SLP) containing known MHC-I and MHC-II peptide antigens of different lengths (Rosalia *et al.*, 2013). Since the discovery of DCs in 1975 by Ralph Steinman, the potential of DC therapy has been much anticipated (Steinman, Adams and Cohn, 1975). DCs are a subset of cells that bridge the gap between the innate and adaptive immune systems. They are efficient antigen presenting cells, requiring very little antigen to stimulate an effective T-cell response (Bhardwaj *et al.*, 1993). Moreover, DCs can cross-present antigens to CD8 T-cells whereby they process and present exogenous antigens through MHC class I molecules (Heath *et al.*, 2004; Amigorena and Savina, 2010). The source and maturity of the DCs used for vaccination differs between the evolving generations of DC vaccines. First generation DC vaccines which used less mature, monocyte-derived DCs. These studies were largely ineffective clinically but did provide evidence that DC vaccines were therapeutically safe. The early DC vaccines included DCs pulsed with antigenic peptides for known immunogenic melanoma associated antigens such as melan-A and GP100 (Mukherji *et al.*, 1995; Nestle *et al.*, 1998; Butterfield *et al.*, 2003). With the evolving knowledge of different DC subsets, second generation vaccines used *ex vivo* generated monocyte derived DCs which were fully matured using a cocktail of cytokines. The use of fully matured DCs increased the clinical efficacy of DC vaccines, with 8-15% of patients experiencing a complete response to treatment (Anguille *et al.*, 2014). However, this rate of efficacy is still low compared to other forms of immunotherapy. The only FDA approved DC vaccine, Provenge, licenced as a treatment for prostate cancer falls in between first and second generation vaccines as it utilises GM-CSF activated APCs to deliver a recombinant fusion protein containing a prostate cancer antigen (Kantoff *et al.*, 2010; Beer *et al.*, 2011). Patients treated with Provenge experienced a slight increase in overall survival but the treatment had limited effects on disease progression (Kantoff *et al.*, 2010). Next generation DC vaccines which are currently

being tested, isolate differentiated patient DC using magnetic beads. Recent studies using next generation vaccines have shown enhanced T-cell activation compared to previous vaccines (Tel *et al.*, 2013). One next generation vaccine study using CD1c+ DCs, 28% of metastatic melanoma patients experienced long, progression free survival after treatment (Schreibelt *et al.*, 2016). Due to the personalised nature of DC vaccines, they are labour intensive and expensive. However, a recent study has demonstrated that it is not always necessary to remove DCs to harness them for presentation of tumour antigens. Kranz et al demonstrated that DCs can capture RNA-lipoplexes causing DC maturation, efficient presentation to T-cells and tumour rejection in murine models (Kranz *et al.*, 2016). A recent phase I clinical trial using RNA-lipoplexes encoding four tumour associated antigens (MAGE A3, GP100, tyrosinase and TPTE) administered intravenously to three metastatic melanoma patients demonstrated an increase in antigen specific T-cell responses and reduction in tumour size and burden in all three patients (Kranz *et al.*, 2016). Unlike other immunotherapies the mutational load of the tumours does not seem to play a role in the effectiveness of DC vaccine therapy. Garg et al noted that the cancers in which DC vaccine therapy has been most successful have a very low mutational burden (Garg *et al.*, 2017). Tumours with low mutational burdens often have a low TIL infiltration, which in turn means they fail to respond to checkpoint inhibitor treatment. DC vaccine therapy has been shown to increase tumour infiltration by T-cells, recent clinical trials have sought to harness this by combining DC vaccines alongside anti-PD-1 or anti-CTLA-4 therapies (Wilgenhof *et al.*, 2016)(NCT03406715).

1.7.2. Viral vaccines

Viruses have the innate ability to infect human cells and initiate an immune response. Immunotherapeutic strategies have sought to exploit this inherent feature of viruses to treat malignant disease. Viruses have been used as vectors which are replication deficient to deliver vaccine payload. A clinical trial conducted in Cardiff University utilised a replication-deficient modified vaccinia ankara (MVA) virus expressing 5T4 alongside low-dose cyclophimide to treat metastatic colorectal cancer patients (Scurr *et al.*, 2017). This study demonstrated the safety of MVA viral vectors as no grade 3 or 4 toxicity was seen. Furthermore, anti-5T4 immune responses were significantly increased in the majority of patients. Viruses can also be oncolytic and genetically modified to specifically target malignant cells, avoiding infection of healthy host cells (**Figure 1.12**). Oncolytic viruses seek to enter a tumour cell, taking over the cellular machinery causing disruption to proliferation

and ultimately cell lysis. Lysis releases TAAs into the microenvironment activating pre-existing T-cells and priming naïve T-cells. The first oncoviral therapy to achieve FDA approval for the treatment of metastatic melanoma was a herpes simplex virus (HSV) derived oncovirus, Talimogene laherparevpec (T-VEC). T-VEC is genetically engineered to be less virulent in non-malignant cells and to over-express GM-CSF to improve T-cell priming at the tumour site (Kohlhapp and Kaufman, 2016). Complete response to viral therapy was achieved in 17% of patients treated with T-VEC as reported in the final stage IIIB clinical trial (Andtbacka *et al.*, 2019). An alternative approach to oncolytic viral therapy involves the addition of tumour associated proteins to the virus, allowing for a two pronged therapy; the first arm is T-cell priming from the lysed tumour cells whilst the second involves peptide specific priming from the attached tumour associated peptide (Boscheinen *et al.*, 2019).

Adenoviruses are also being explored as viral species for oncoviral therapy. Oncolytic adenoviruses have been genetically modified to selectively replicate within host tumour cells, avoiding infection of healthy cells (Pesonen, Kangasniemi and Hemminki, 2011). As with HSV oncoviruses, adenoviruses seek to infect tumour cells disrupting the internal machinery. Modified adenoviruses have been developed to improve immunogenicity at the tumour site, these include adenoviruses with MHC class I TAA covalently attached to its capsid (Capasso *et al.*, 2016) and with antioxidants such as L-carnosine, which can halt proliferative capacity of cancer cells (Garofalo *et al.*, 2016). Furthermore, adenoviruses have been manipulated to express immunomodulatory molecules such as PD-1 (Zhang *et al.*, 2019), CD40L (Gomes *et al.*, 2009), IL-12 (Wang *et al.*, 2017) and GM-CSF (Chang *et al.*, 2009; Cerullo *et al.*, 2010).

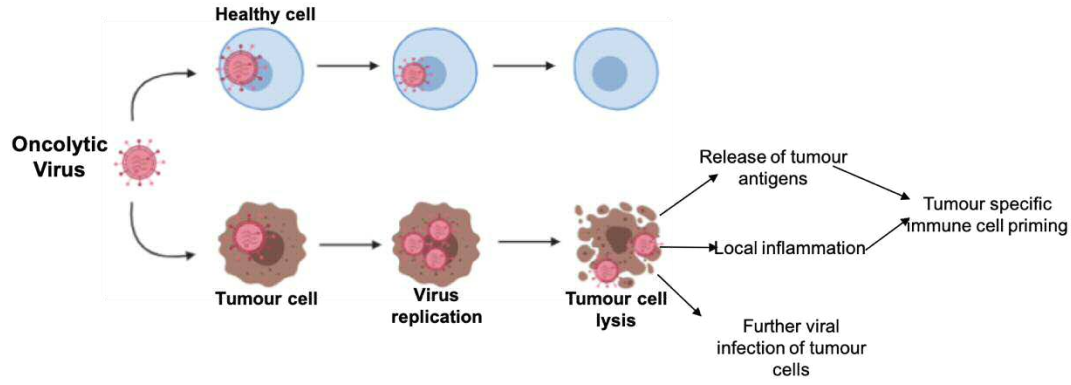


Figure 1.12: Aim of oncolytic viral therapy

Viruses innate ability to infect human cells has been exploited for cancer treatments using oncolytic viral therapy. The ultimate aim of oncolytic viral therapy is to engineer viruses that can specifically infect cancerous cells, whilst leaving healthy cells uninfected. Once the virus has infected the tumour cells, it replicates and eventually causes tumour cell lysis. Lysis of the tumour cell releases TAAs and other inflammatory compounds into the tumour microenvironment causing local inflammation and priming of tumour specific immune cells. Upon tumour cell lysis viral particles are also released and can go onto infect more tumour cells, perpetuating the cycle of tumour cell lysis and immune stimulation.

1.7.3. Whole tumour or subunit vaccines

Transformed cells display a number of antigens on their surface. An effective anti-tumour response is likely to involve T-cells reactive to many of these antigens. A technique to attempt to harness these cells is to vaccinate patients with tumour lysates or irradiated whole tumour cells. Incorporation of tumour fragments into autologous, patient-derived DCs allows presentation of multiple tumour antigens, initiating an expansive immune response. Typically, tumour material is delivered using autologous DCs, as with a DC vaccine discussed previously. The benefit of using a heterogenous source of tumour material is that it will contain a vast array of tumour associated antigens and danger signals. Moreover, as opposed to single peptide vaccines, expression of a diverse range of antigens could prevent selection driven tumour escape. Tumour matter used for vaccination can be autologous, allowing for expression of patient specific neoantigens on autologous DCs or initiation of an immune response to peptides presented by uncommon or poorly studied HLAs (Nagayama *et al.*, 2003; O'Rourke *et al.*, 2003). Alternatively, allogenic cancer cell lines can be used, allowing for a more 'off the shelf' approach (López *et al.*, 2009; Rojas-Sepúlveda *et al.*, 2018). Furthermore, vaccines using cancer cell lines can be engineered to express cytokines such as GM-CSF, to further promote a robust *in vivo* immune response (Small *et al.*, 2007). Despite early promise, vaccines using whole tumour lysates have not provided lasting success, this

could be put down to complicated manufacturing processes and an inability to routinely initiate a potent anti-cancer immune response.

1.7.4. Nucleic acid vaccines

Nucleic acid vaccines rely upon the delivery of nucleic acids, typically DNA or mRNA to antigen presenting cells. Nucleic acids can then be translated by APCs and the associated tumour associated antigens presented to the patients T-cells.

mRNA can be administered to patients directly or electroporated into autologous DCs *in vitro* as part of a DC-based vaccine. Since DC vaccines have been covered in **section 1.7.1**, this section will focus on direct injection of mRNA. Until recently, subcutaneous injection of naked mRNA has not been feasible due to rapid RNA degradation in the bloodstream, however, novel delivery technologies which provide protection to the nucleic acid, are facilitating systemic delivery of mRNA molecules, these include the use of lipid nanoparticles (Reichmuth *et al.*, 2016). Moreover, studies have tried a wide variety of delivery sites for mRNA vaccines, in an attempt to initiate an immune response, including; intranodal (Kreiter *et al.*, 2010), intratumoural (Scheel *et al.*, 2006), intranasal (Phua *et al.*, 2015) and subdermal (Johansson *et al.*, 2012). Injected mRNA can also initiate a generic pro-inflammatory immune response through activation of a host of innate immune receptors such as TLR7 (Zhang *et al.*, 2016) and TLR8 (Tanji *et al.*, 2015).

DNA plasmids have also been used to induce an immune response to a patient tumour. DNA plasmids typically encode antigen(s) of interest along with proinflammatory immune mediators. Once injected into the host, DNA vaccines replicate inside host cells facilitating expression of the antigen and initiating an adaptive immune response (Yang *et al.*, 2014). DNA vaccines, can also initiate an innate immune response through engagement of TLR9, which induces a cascade of proinflammatory signalling resulting in the activation of NFκB and the production of IL-1 (Kobiyama *et al.*, 2013). Early clinical studies indicated low risk of side effects when using DNA vaccines allowing them to be administered multiple times (MacGregor *et al.*, 1998; Rinaldi *et al.*, 2006). DNA vaccines have not lived up to initial clinical promise failing to induce adequate immune responses to tumour, most likely due to tolerance to self-antigens and the low uptake of DNA by antigen presenting cells (Yang *et al.*, 2014). DNA vaccines have performed better in cancers with viral ontology, such as HPV induced cervical cancer (Kim *et al.*, 2014), potentially due to the viral source of the antigens encoded by the vaccine. In order to increase the immunogenicity of DNA vaccines alternative

delivery strategies have been employed, such as gold nanoparticles. Gold nanoparticles facilitated effective transport of a MART-1 encoding DNA vaccine to *in vivo* DCs in a mouse model, successfully immunising the mice against melanoma challenge (Gulla *et al.*, 2019).

1.7.5. Peptide vaccines

During the course of tumorigenesis cancer cells develop aberrant protein expression and acquire mutations causing the expression of neoantigens. This altered antigen expression differentiates cancer cells from healthy cells and can provide targets for CD8 T-cells. Antigenic peptides which stimulate cytotoxic T-cells are an attractive vaccination tool and coupled with increasing knowledge of antigen recognition by T-cells designing peptide vaccines has become possible. Early murine studies vaccinating with CD8 T-cell epitope RAHYNIVTF, derived from human papillomavirus virus (HPV) oncogene E7, provided mice protection from transplanted HPV-virus induced tumours (Feltkamp *et al.*, 1993). Furthermore, vaccination with MUC1 antigenic peptides in mice with lung carcinoma tumours established before vaccination led to decreased tumour burden and reduction in tumour metastasis (Mandelboim *et al.*, 1995). In contrast to early murine studies, initial human clinical trials with peptide vaccines were largely unsuccessful, with some studies reporting that vaccination with the antigen sequence peptide alone can cause T-cell tolerance and eventual loss of anti-tumour immunity (Toes *et al.*, 1996). Moreover, some studies have demonstrated that peptide vaccines fail to elicit memory CD8 T-cells, which are critical in mediating long-lived anti-tumour protection (Bijker *et al.*, 2007). An important challenge when administering peptide vaccines is ensuring that they are successfully presented to CD8 T-cells on MHC class I molecules. For this to occur the peptides, once injected, must be processed by antigen presenting cells. Delivery mechanisms including poly lactic acid (PLA) nanoparticles (Noh *et al.*, 2011; Heo and Lim, 2014), gold nanoparticles (Lee *et al.*, 2012) and amphiphile micelles (Black *et al.*, 2012) have sought to improve the immunogenicity and trafficking of peptide vaccines. Murine studies have demonstrated protective immunity in mice vaccinated with nanoparticles containing the tumour-expressed OVA peptide antigen. One such study mice vaccinated with an OVA-polylactic acid (PLA) nanoparticle were protected from subsequent tumour challenge. (Heo and Lim, 2014). Investigation of the nanoparticles after administration demonstrated that they entered DCs and cross-presented OVA antigens to CD8 T-cells (Heo and Lim, 2014). An important step in the success of peptide vaccines is facilitating trafficking of the antigen to antigen presenting cells, such as DCs. DCs can then present antigen to naïve T-cells in the thymus, initiating a robust immune response. A study tracked the trafficking of nanoparticles after oral

administration to mice, the nanoparticles were able to move through the mucosa and were taken up by B-cells and DCs in secondary lymphoid organs (Primard *et al.*, 2010). Studies have also sought to use peptides with modified anchor residues resulting in a greater capacity to bind to HLA A2 and subsequently greater CD8 T-cell expansion to TAA epitopes (Valmori, Fonteneau, Lizana, Gervois, Liénard, Rimoldi, Jongeneel, Jotereau, Cerottini, Romero, *et al.*, 1998; Alves *et al.*, 2007; Smith, Rekoske and McNeel, 2014). These peptides, termed altered peptide ligands (APLs), will be discussed further in **Chapter 3**.

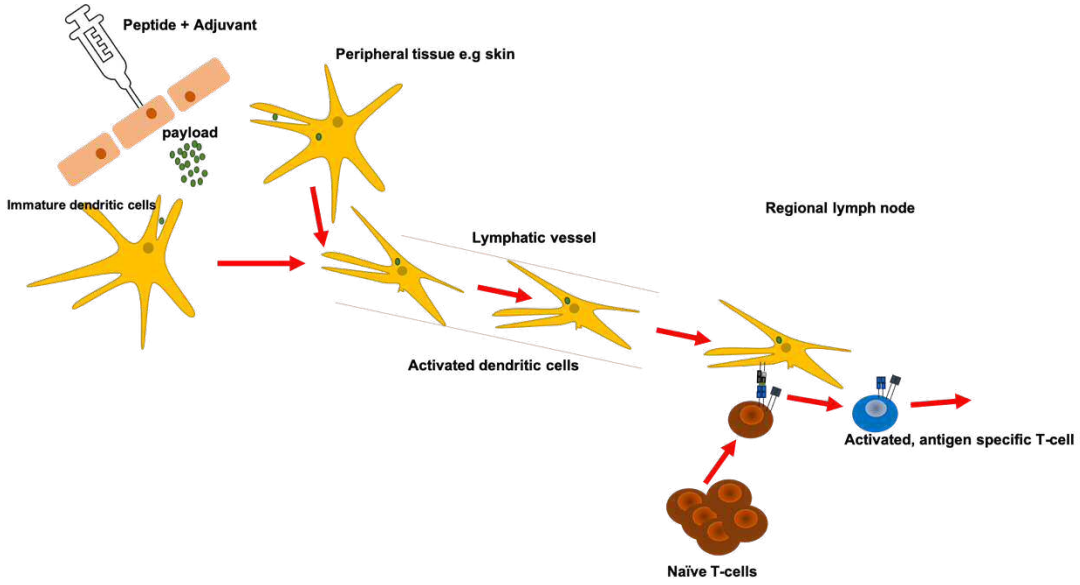


Figure 1.13: Successful peptide cancer vaccination

Successful vaccination first requires the correct administration of the payload alongside an adjuvant, which can stimulate the immune response. Identification of the correct route of administration and adjuvant are vital to the success of a vaccination. In order to activate a CD8 T-cell response, the payload must be phagocytosed by an immature dendritic cell (DC) which then becomes activated and migrates to a regional lymph node. The activated DC can then prime naïve T-cells which reside in the lymph node, these activated, payload-specific T-cells egress from the lymph node and seek out the target.

1.8. Project Aims

Tumour infiltrating lymphocyte (TIL) therapy has demonstrated remarkable success in some end stage metastatic melanoma patients. Peptide vaccines for cancer have been largely ineffective to-date. This has been attributed to poor peptide choice and the inherent low affinity of TCRs for self-antigens, a category to which many TAAs belong. Through a collaboration with The National Centre for Cancer Immune Therapy (CCIT), Copenhagen Denmark, we have had access to long-term (7+ years) survivors following TIL therapy for stage IV melanoma. To identify why only some patients responded well to treatment, detailed investigations have been carried out into the T-cell specificities and TCR repertoire from responding patients by previous PhD students. As part of these investigations, dominant and persistent TCR clonotypes were isolated from the PBMC of patient MM909.24. One such clonotype, ST8.24, was identified in the TIL infusion product used to treat MM909.24. This clonotype was also present in the PBMC post-cure. ST8.24 is recognises the melan-A antigen EAAGIGILTV and effectively kills the autologous MM909.24 tumour line. The overall aim of my study was to utilise ST8.24 to design peptide super-agonists to improve the efficacy of peptide vaccines for cancer immunotherapy. Specifically, in **chapter 3**, I aimed to use combinatorial peptide library (CPL) screening technology to identify candidate super-agonist peptides which are capable of priming melan-A reactive CD8 T-cells. I did this with this hope that these super-agonist peptides would prime EAAGIGLTV-specific CD8 T-cells more effectively than the WT melan-A derived epitope EAAGIGILTV. Based on the well-established principle of T-cell cross reactivity, the aim of **Chapter 4** was to interrogate the hypothesis that the superiority of super-agonist peptide **MTSA**IGIL**P**V could be attributed, in part, to its ability to elicit T-cells specific for multiple TAA epitopes. Finally, I sought to explore the use of super-agonist peptide **MTSA**IGIL**P**V as a pan-cancer vaccine candidate. This involved priming matched CD8 T-cell and tumour samples from renal cell carcinoma (RCC), chronic lymphocytic leukaemia (CLL) and acute myeloid leukaemia (AML) patients with **MTSA**IGIL**P**V, this work is discussed in **Chapter 5.**

2. Materials and Methods

2.1 Patient and healthy donor samples

2.1.1 Patient samples

Metastatic melanoma patient samples (MM909.24, MM909.37 and MM1413.12) and renal cell carcinoma (RCC) patient samples (RCC-2 and RCC-12) were kindly obtained from The National Centre for Cancer Immune Therapy (CCIT), Herlev, Denmark. Patients were all enrolled in tumour infiltrating lymphocyte (TIL) therapy clinical trials (Andersen *et al.*, 2015; R. Andersen *et al.*, 2016; R Andersen *et al.*, 2018) at the CCIT. Appropriate consent and material transfer agreements were in place.

Chronic lymphocytic leukaemia (CLL) patient samples were obtained through a collaboration with Professor Steve Man, Dr Chris Pepper and PhD student Peter Henley at the University Hospital of Wales (UHW), Cardiff. Patients were part of a screening programme which monitors the progression of their disease.

Acute myeloid leukaemia (AML) patient samples were obtained through a collaboration with Drs Caroline Alvares and Joanna Zabkiewicz and Professor Oliver Ottmann at the University Hospital of Wales (UHW), Cardiff. Samples were obtained when patients first presented with disease at the A&E department of the UHW. Appropriate ethical and consent was taken under the National Health Service (NHS) Research Ethics Committee (REC) approved project 17/LO/1566.

2.1.2 Healthy donor samples

Human 'buffy coats' were obtained from the Welsh Blood service (WBS), Talbot Green, Wales. Pooled PBMC isolated from 3 donors was used to expand CD8 T-cells every 2 – 3 weeks. Individual HLA A2 positive donors, identified by fluorescent antibody staining as documented in **section 2.7.1**, were used for peptide priming experiments as documented in **Chapters 3 and 4**.

2.2 Cell Culture

2.2.1 Cell Culture Reagents

The composition of all culture medium and buffers used for cell culture are listed in tables **Table 2.1** and **Table 2.2** respectively.

Culture Medium	Composition
R0	RPMI-1640 (Thermo Fisher Scientific), 100 U/mL Penicillin (Life Technologies), 100 µg/mL Streptomycin (Life Technologies), 2 mM L-Glutamine (Life Technologies)
R5	R0 with 5% Heat-Inactivated Fetal Bovine serum (FBS) (Life Technologies)
R10	R0 with 10% FBS
D10	Dulbecco's Modified Eagles' medium (DMEM) (Thermo Fisher Scientific), 100 U/mL Penicillin, 100 µg/mL Streptomycin, 2 mM L-Glutamine, 10% FBS
D10 F12	DMEM/F-12 medium (Thermo Fisher), 100 U/mL Penicillin, 100 µg/mL Streptomycin, 2 mM L-Glutamine, 10% FBS, 10 mM HEPES (Life Technologies), 1 mM Sodium Pyruvate (Life Technologies) 1x MEM Non-essential amino acids (NEAA) solution (LifeTechnologies)
T-cell culture medium	R10, 10 mM HEPES, 1 mM Sodium Pyruvate, 1x MEM Non-essential amino acids (NEAA) solution, 25 ng/mL IL-15 (PeproTech), 200 IU/mL IL-2 (Aldesleukin, brand name Proleukin; Prometheus)
T-cell expansion medium	R10, 10 mM HEPES, 1 mM Sodium Pyruvate, 1x MEM Non-essential amino acids (NEAA) solution, 25 ng/mL IL-15, 20 IU/mL IL-2, 1 µg/mL purified PHA (Phytohemagglutinin) (Alere)
MS5 stromal medium Co-culture medium	α-Minimum Essential Medium Eagle (αMEM) (Sigma-Aldrich), 10% FBS, 100 U/mL Penicillin 100 µg/mL Streptomycin, 2 mM L-Glutamine
HS5 stromal medium	DMEM high glucose (Sigma-Aldrich), 10% FBS, 1% Glutamax (Thermo Fisher Scientific), 100 U/mL Penicillin, 100 µg/mL Streptomycin
CLL tumour medium	D10 supplemented with 5 ng/mL IL-4 (Miltenyi Biotech)
Smooth muscle cell medium	Smooth Muscle Cell Medium (SCMC 1101) (ScienCell), 100 U/mL Penicillin, 100 µg/mL Streptomycin, 2 mM L-Glutamine, 10% FBS
Epithelial cell medium	Human Renal Epithelial Cell Medium (EpiCM 4101) (ScienCell), 100 U/mL Penicillin, 100 µg/mL Streptomycin, 2 mM L-Glutamine, 10% FBS
Hepatocyte cell medium	Hepatocyte medium (HM 5201) (ScienCell), 100 U/mL Penicillin, 100 µg/mL Streptomycin, 2 mM L-Glutamine, 10% FBS
Stellate cell medium	Stellate cell medium, 100 U/mL Penicillin, 100 µg/mL Streptomycin, 2 mM L-Glutamine, 10% FBS
Alveolar cell medium	Alveolar Epithelial cell medium (AEpiCM), 100 U/mL Penicillin, 100 µg/mL Streptomycin, 2 mM L-Glutamine, 10% FBS

Table 2.1 Cell culture medias

Buffers	Composition	Uses
Freezing buffer	90% FBS 10% DMSO (Dimethyl sulfoxide) (Sigma Aldrich)	Cell culture
Red blood cell (RBC) lysis buffer	155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA (pH 7.2-7.4)	Cell culture
PBS-EDTA	PBS, 2 mM EDTA	Cell culture
FACS buffer	PBS, 2% FBS	Flow cytometry staining
MACS buffer	PBS (500 ml) 2.5 g BSA 2 ml EDTA	Cell isolation
Wash buffer	0.05% Tween-20 in PBS	ELISA
Reagent diluent	1% bovine serum albumin (BSA) in PBS	ELISA

Table 2.2: Buffers used in cell culture

All media and buffers used for tissue culture were filtered using 0.2 µM syringe or filter bottles (Stericup®, Merck Millipore, Massachusetts, USA).

2.2.2 Antigen Presenting Cells (APC)

All cell lines used were incubated at 37 °C, 5% CO2 in T75 flasks in R10, D10 or D10F12 medium, depending on the cell type. Cells were split using PBS-EDTA once a confluency of 80% was reached, care was taken not to allow cell lines to exceed this confluency. For adherent cell lines, the whole content of the tissue culture flask was transferred into a 50 mL centrifuge tube and the flask washed with PBS to remove any remaining medium. PBS-EDTA was then added to detach the cells from the inner surface of the flask and the flask incubated at 37 °C for 2 min. The bulk cell suspension was transferred to the 50 mL centrifuge tube, centrifuged at 400 g for 5 min and resuspended in medium for counting. Cells were plated into a new flask at the recommended seeding density and required medium. Heathy cell lines and the appropriate culture media were purchased from ScienCell (California, USA).

For suspension cultures, cells were resuspended thoroughly and counted as required. The majority of the cell suspension was then removed, and the flask topped up with fresh R10 medium.

All cell lines used in this study, their morphology and culture medium are listed in **Table 2.3**.

Cell lines were routinely tested for mycoplasma contamination (MycoAlert™ Mycoplasma Detection Kit, Lonza).

Cell Line	Origin	Culture Method
MM909.24, MM909.37, MM1413.12	Metastatic melanoma TIL therapy trial patient	R10, adherent
FM-79, FM-2, FM-56, MEL-526, MEL-624	Melanoma cell lines	R10, adherent
RCC 2, 12, 23	Renal cell carcinoma patient	D10 F12, adherent
SKBR3, MDA-MB,231, MCF-7,	Breast cancer cell lines	D10 F12, adherent
PANC1	Pancreatic cancer cell line	D10 F12, adherent
RCC17, ACHN	Kidney cancer cell lines	D10 F12, adherent
PC3, LnCaP	Prostate cancer cell lines	R10, adherent
Colo 205	Colon cancer cell line	D10, adherent
SIHA, MS751	Cervical cancer cell lines	R10, adherent
HEPG2	Liver cancer cell line	R10, adherent
H69	Lung cancer cell line	R10, adherent
SAOS	Bone cancer cell line	R10, adherent
C1r	B-cell lymphoma	R10, suspension
T2	Lymphoblast	R10, suspension
Molt	Acute lymphoblastic leukaemia cell line	R10, suspension
K562	Chronic myelogenous leukaemia cell line	R10, suspension
THP-1	Acute myeloid leukaemia cell line	R10, suspension
CD40L	Fibroblast cell line	D10, adherent
HS5	Human stromal line	HS5 medium, adherent
MS5	Mouse stromal line	MS5 medium, adherent
Pulmonary alveolar epithelia	Healthy cell line	Alveolar cell medium, adherent
Renal epithelia	Healthy cell line	Epithelial cell medium, adherent
SMC3	Smooth muscle healthy cell line	Smooth muscle cell medium, adherent
Lung fibroblast	Healthy cell line	Alveolar cell medium, adherent
Pancreatic stellate	Pancreatic healthy cell line	Stellate cell medium, adherent
CIL-1	Non-pigmented bronchial ciliary epithelial healthy cell line	Epithelial cell medium, adherent
HH	Liver healthy cell line	Hepatocyte medium, adherent

Table 2.3: Information on cell lines used

2.2.3 Primary tumour culture

Acute myeloid leukaemia (AML) primary tumour samples isolated from the peripheral blood of emergent AML patients at the University Hospital of Wales were cultured on a layer of mouse or human stromal cells (MS5 or HS5). 3 days prior to culture, 3 x10^3 stromal cells were plated in 96 flat-well plates in the appropriate medium to allow cells to adhere to the culture wells. On day 3, 5 x10^4 AML tumour cells per well were plated on top of the stromal cells in co-culture medium.

Chronic lymphocytic leukaemia (CLL) tumour cells isolated from the peripheral blood of CLL patients undergoing routine monitoring of their disease, were cultured on a bed of a fibroblast cell line expressing CD40 ligand. One day prior to CLL tumour culture, CD40L fibroblast cell line was irradiated at 750 cGy and plated at the appropriate density to allow confluence in the culture well in D10 medium supplemented with IL-4. The following day CLL tumour cells were plated at a ratio of 10 tumour cells to 1 fibroblast cell.

2.2.4 Cell counting

Cells were resuspended in the appropriate medium and 10 µL of the cell suspension was mixed with 10 µL of trypan blue (Sigma Aldrich). 10 µL of the resulting mixture was loaded onto a haemocytometer, and live cells counted based on trypan blue exclusion. Cells density was calculated per mL using the formula:

$$Cell\ number = Cell\ count\ x\ 2\ (dilution\ f\ \ ctor)\ x\ 10{,}000$$

2.2.5 Cryopreservation and thawing

For cryopreservation, cells were washed, counted and resuspended in freezing buffer and moved to internal thread cryovials in 1 mL aliquots. The cryovials were cooled to -80 °C at an incremental rate using a freezing pot (Mr Frosty®, Nalgene). After 24 h cells were moved to liquid nitrogen for long-term storage. For thawing, cells were removed from liquid nitrogen on dry ice and part-thawed in a water bath at 37 °C, thawing was finished using warm R10 medium. Cells were then washed in excess R10, counted and seeded at the appropriate densities in the correct culture flask.

2.3 Maintenance and expansion of T-cell cultures

2.3.1 Isolation of peripheral blood mononuclear cells (PBMC)

Blood samples were obtained as EDTA-treated buffy coats via the Welsh Blood Service (WBS, Talbot Green) in accordance with the appropriate ethical approval. Samples were confirmed seronegative for human immunodeficiency virus (HIV-1), hepatitis B virus (HBV) and hepatitis C virus (HCV). PBMCs were isolated from whole blood by Lymphoprep™ (Stemcell™ Technologies Inc.) density gradient separation using SepMate™ (Stemcell™ Technologies Inc). SepMate™ tubes were centrifuged at 1200 g for 10 minutes, the top layer was decanted into a fresh 50 mL falcon and washed using RPMI-1640 medium and centrifuged at 700 g for 10 min. The pellet was resuspended in 25 mL red blood cell (RBC) lysis buffer and incubated in a water bath at 37 ^{o}C for 10 min. A further wash was performed at 400 g for 5 min to remove platelets, if the pellet still contained RBCs the lysis step could be repeated. PBMC were resuspended in R10 medium, counted and kept on ice for further processing.

2.3.2 Expansion and culture of T-cells

Up to 1 x10^6 T-cells were co-incubated with 15 x10^6 irradiated (3100 cGy) PBMC feeders, from 3 pooled donors, in a T25 culture flask in 15 mL of T-cell expansion medium and 1 mg/mL PHA. The flask was incubated at 37 °C, 5% CO2 at a 45° angle for 7 days. On day 5, 50% of the medium was removed and replaced. Cells were harvested on day 7, washed in R0 medium, counted and plated in T-cell culture medium at 3-4 x10^6 cells/well in 24 well plates (2 mL/well) or 1-2 x10^6 cells/well in 48 well plates (1 mL/well). The plate was incubated at 37 °C, 5% CO_2 and left until the wells were confluent. Every 2 days half the medium was replaced with fresh T-cell culture medium. 14 days' post-expansion the T-cells could be used in experiments, maintained for up to 4 weeks in culture or frozen until required.

2.3.3 Single Cell Cloning

CD8 T-cells from a polyclonal population were seeded at a density of 0.5 cells per well in 100 µL of T-cell expansion medium alongside 5 x10^4 feeders (extracted from buffy coats supplied by the WBS and irradiated at 3100 cGy) and 1 µg/mL PHA in 96 U well plates. After 7 days 100 µL of U20 medium was added to the plates and after 14 days the plates were either fed by removing and replacing 100 µL of U20 medium or expanded using feeders and 2 µg/mL

PHA. Potential clones were spotted by obvious growth in the well and could be tested by ELISA or a TAPI-O assay after 14 days.

2.4 Functional Assays

2.4.1 Peptides

Peptides required for this work were ordered as lyophilised products, reconstituted in-house with DMSO to a working stock of 20 mg/mL and stored at -80 °C.

Crude peptides (purity 40-60%) were manufactured by GL Biochem Ltd (Shanghi, China) whilst pure peptides (>90% purity) were synthesised by Peptide Protein Research Ltd (Hampshire, UK). Crude peptides were used for initial mass peptide screens, reactivity was confirmed using pure peptides.

Combinatorial peptide library (CPL) screens in a positional scanning format were synthesised by Pepscan Presto Ltd (Leylstad, The Netherlands). CPL screens were reconstituted initially in DMSO to a concentration of 20 mg/mL and stored at - 80°C. A further dilution was done in R0 medium before use to a working stock of 1 mg/mL, this could be stored at 4°C.

2.4.2 Chromium release assay

1 x10^6 target cells were washed 2 x in PBS in a 15 mL falcon, after the second wash all PBS was aspirated in order to leave a dry pellet. Target cells were then labelled with ^{51}Cr (sodium chromate, PerkinElmer) at a concentration of 5 mCi and incubated at 37 ^{o}C for 1 h. Cells were then washed in R10 medium and incubated in 2 mL R10 medium for a further hour to allow excess ^{51}Cr to leach from the cells. T-cells were plated in a 96 U-well plate titrated by serial dilution from 50:1 to 0.31:1 (T-cells:target cells) in R10 medium alongside 2000 target cells per well and the assay was incubated at 37 ^{o}C for 4 h. Negative and positive controls were included, such as target cells alone and target cells with lysis buffer (5% Triton in H_2O) to give spontaneous and maximum release of ^{51}Cr respectively. After incubation, 15 µL of supernatant was harvested and plated with 150 µL of Opitphase Supermix scintillantion cocktail (PerkinElmer) in microbeta plates (Perkin Elmer). The release of ^{51}Cr was measured using a 1450 MicroBeta TriLux (PerkinElmer). The specific lysis of the target cells by T-cells was calculated by:

$$\left(\frac{experimental\ release - spontaneous\ release}{maximal - spontaneous\ release}\right) \text{x}100.$$

2.4.3 Enzyme-Linked Immunosorbent Assay (ELISA)

2.4.3.1 Activation Assay for ELISA

On day 0, T-cells required for the assay were counted and rested in R5 medium overnight. The following day (day 1), 3 x10^4 T-cells were plated alongside either 6 x10^4 target cells or a peptide of interest in R5 medium at a final volume of 100 µL. Wells containing PHA + T-cells or T-cells alone were set up as positive and negative controls. Plates were incubated overnight at 37 °C. On day 2, 50 µL of supernatants were harvested and diluted with 70 µL of R0 medium.

2.4.3.2 ELISA protocol

MIP-1β ELISAs were performed using DuoSet® human ELISA kit following the manufacturer's instructions (R&D Systems). All washes were performed with 190 µL of 0.05% Tween 20-PBS (wash buffer) using an automated microplate washer (Thermo Scientific). Briefly, a half-well flat bottom ELISA microplate was coated with 50 µL of mouse anti-human MIP-1β capture antibody (1.5 µg/mL) and incubated overnight at room temperature (RT). The following day the plate was washed 3 times and 150 µL of 1% BSA-PBS (reagent diluent) solution was added to each well and the plate was incubated for 1 h at RT. Following 3 washes, 50 µL of cell supernatant collected from an activation assay was added to each well alongside a recombinant human MIP-1β standard (R&D Systems). Standards were titrated from 2000 – 15.6 pg/mL in reagent diluent and 50 µL plated in duplicate to allow generation of a standard curve, the plate was then incubated for 1 h and 15 min at RT. After incubation, the plate was washed 3 times in wash buffer and coated with 50 µL biotinylated goat anti-human MIP-1β detection antibody (50 ng/mL). After 1 h and 15 min incubation at RT, the plate was washed 3 times and HRP-conjugated streptavidin (50 µL) was added to the wells, the plate was then incubated in the dark at RT for 20 min. Following 3 final washes, 50 µL of colour reagents A and B at a 1:1 ratio (R&D Systems) were added to each well and monitored for a colour change. Once an appropriate colour change was observed, the reaction was stopped by adding 25 µL of stop solution (R&D Systems). The OD_{450nm} of each well was read using an iMark™ microplate reader (Bio-Rad) with correction set to 570 nm.

2.4.4 Combinatorial Peptide Library (CPL)

A 10-mer combinatorial peptide library (CPL) in a positional scanning format (Pepscan Presto Ltd) was used to identify to optimal peptide sequence for chosen CD8 T-cell clones. The activation of T-cell clones to the peptide mixtures was measured using a MIP-1β ELISA (**section 2.4.3.1**). The 10-mer CPLs is divided into 200 peptide mixtures, whereby every peptide mixture has one position with a fixed L-amino acid residue and the rest of the positions are degenerate. The cysteine residue is not included in the degenerate peptide positions so to avoid the formation of disulphide bonds between peptides in the mixture.

In order to prepare a CPL screen, CD8 T-cells were washed in R0 medium and rested overnight in R5 medium. Antigen presenting cells were plated in a 96 U-well plate at a concentration of 6 x10^4 cells per 45 µL of R5 medium and pulsed with 5 µL of peptide mixture at a concentration of 100 µM for 2 hours at 37 °C. Following incubation, 3 x10^4 CD8 T-cells were added in a volume of 50 µL to each well, with the final volume of the reaction equalling 100 µL, plates were incubated overnight at 37 °C. The following day, plates were centrifuged at 400 g for 5 min and 50 µL of supernatant harvested for use in a MIP-1β ELISA according to the manufacturer's instructions (R&D Systems) (**section 2.4.3.2**).

2.4.4.1 Epitope Identification

Epitopes were identified using the raw data generated by the CPL screen. Data was entered into an online webtool (https://picpl.arcca.cf.ac.uk/loginform.php) held on the Warwick University server. The algorithm exposed the CPL data to the entire decamer peptide universe allowing the prediction of the best possible peptides for a given clone (Szomolay *et al.*, 2016).

2.5 Magnetic activated cell sorting

2.5.1 CD8 T-cell isolation

CD8 T-cells were extracted from PBMC isolated from buffy coats supplied by the WBS (protocol described in **section 2.3.1**). The number of PBMC used was determined by the desired number of CD8 T-cells as the final product, where it was reasoned that CD8 T-cells make up 10% of an average individuals PBMC. PBMC was centrifuged at 300 x g for 10 minutes at 4 °C, resuspended in 80 µL MACS buffer and 20 µL anti-CD8 microbeads (MIltenyi Biotech) and incubated in the fridge for 15 min. After incubation, the sample was washed in

MACS buffer and centrifuged for 10 min at 300 g, the supernatant aspirated and the pellet resuspended in 500 µL MACS buffer. CD8 T-cell separation was carried out using a MACS magnet (Miltenyi Biotech) and an appropriate column the size of which was dependent on the number of PBMC being used in the experiment. The column was prepared before use by flushing with cold MACS buffer before the sample could be added. Once the buffer has washed through the column the sample was added, and the column was then washed three times to ensure complete removal of CD8 negative cells. After washing, the CD8 positive cells were collected by removing the column from the magnet and plunging with 1 mL of priming medium. The CD8 positive cells were then washed and counted. The protocol was generated by the manufacturer (Miltenyi Biotech) for maximum 10^7 PBMC therefore was adjusted according to the number of PBMC used in each individual experiment.

2.5.2 Isolation of reactive CD8 T-cells using TNF and IFNγ

To isolate cells reactive to a target cell or peptide of interest, TNF/IFNγ isolation was used, according to the manufacturers' instructions (Miltenyi Biotech). CD8 T-cells were washed in R0 medium, counted and rested overnight in R5 medium. The following day T-cells were co-incubated with target cells at a ratio of 1 T-cell:2 target cells for 4 h at 37 °C. After incubation, cells were washed in 5 mL of MACS buffer and resuspended in 60 µL of cold medium and 20 µL of TNF and 20 µL IFNγ capture antibodies (Miltenyi Biotech) and incubated for 5 min at 4 °C. Cells were resuspended in 10 mL of warm R5 medium and rotated at 37 °C for 45 min using a MACSMix rotor (Miltenyi Biotech). Cells were washed in cold MACS buffer and resuspended in 60 µL of MACS buffer, 20 µL of TNF and 20 µL IFNγ detection antibodies added and incubated at 4 °C for 5 min. Cells were washed and resuspended in 80 µL MACS buffer alongside 20 µL of anti-PE microbeads (Miltenyi Biotech) and incubated in the fridge for 15 min. Finally, cells were washed and resuspended in 500 µL MACS buffer ready for magnetic cell separation as described in **section 2.5.1.**

2.5.3 Isolation of tetramer positive cells

To isolate CD8 T-cells specific for a tetramer of interest, polyclonal CD8 T-cell populations were stained with pMHC using PKI, as described in **section 2.7.4**. After staining with pMHC-tetramer, cells were washed in MACS buffer and centrifuged at 300 g for 10 min. Cells were then labelled with anti-PE microbeads (Miltenyi Biotech) and incubated in the fridge for 15 min. Cells were then washed in excess MACS buffer, centrifuged at 300 g for 10 min and

resuspended in 500 μL of MACS buffer, ready for magnetic separation as described in **section 2.5.1**. Isolated tetramer positive cells were either plated in a 96 U well and expanded as a line using allogenic feeder cells as described in **section 2.3.2** or single cell cloned as described in **section 2.3.3.**

2.6 CD8 T-cell Peptide Priming

CD8 T-cells were isolated as described in **section 2.5.1**, and plated at a density of 2 $x10^6$ cells per 48 well, or 4 $x10^6$ cells per 24 well in 0.5 mL priming medium. CD8 negative cells were washed in R0 medium and counted. The CD8 negative cells were pulsed with the peptide of interest at a concentration of 25 μM for 1 hour at 37 °C, resuspending every 15 min or rotating using a MACSMix rotor (Miltenyi Biotech) to ensure full exposure of the CD8 negative cells to the peptide. The CD8 negative cells were then irradiated at 3100 cGy, washed and plated with the CD8 T-cells in 0.5 mL priming medium at a density of 4 $x10^6$ or 8 $x10^6$ CD8 negative cells per 48 or 24 well plate alongside 2.5 μL of CD28 antibody (Beckman Coulter). Cells were fed every 2-3 days by removing and replacing half the medium in the well. After 10-14 days' cells were able to be tested for reactivity and specificity to the peptide of interest or fed with U20 medium and maintained in culture.

2.7 Flow Cytometry

2.7.1 Labelling cells

Cells were washed in FACS buffer, counted before staining and transferred to 5 mL FACS tubes or into 96 U well plates. Cells were then stained with VIVID LIVE/DEAD ultraviolet stain (Life Technologies) diluted 1:40 in PBS, for 5 mins at RT to allow the exclusion of dead cells. The samples were then stained with appropriate surface antibodies and incubated on ice for 20 min. Finally, samples were washed and resuspended in 100 μL PBS ready for acquisition. Antibodies used for this work are listed in **Table 2.4**.

Flow cytometry data was acquired using a BD FACS Canto™ II (BD Biosciences) and analysed on FlowJo Software (TreeStar, Inc.; Ashland, OR). Compensation was performed using single staining of an irrelevant T-cell clone or anti-mouse Ig compensation particles (BD Biosciences). Cells were analysed using the gating strategy depicted in **Figure 2.1**.

Target	Colour (clone)	Manufacturer
CD8	APC PE APC Vio 770 (BW135/80)	Miltenyi Biotech
CD3	PerCP (BW264/56)	Miltenyi Biotech
CD4	FITC (BIT4)	Miltenyi Biotech
CD19	Pacific Blue (PB) (HIB19) PE (HIB19)	BioLegend
CD14	Pacific Blue (PB) (M5E2) PE (REA599)	BioLegend Miltenyi Biotech
CD107a	FITC (H4A3) PE (REA792)	Miltenyi Biotech
TNF	PE Vio770 (cA2) Per-CP Cy5.5 (Mab 11)	Miltenyi Biotech BD Bioscience
IFNγ	APC (45-15)	Miltenyi Biotech
IL-2	APC (MQ1-17H12)	BD Bioscience
MIP-1β	PE (11A3)	BD Bioscience
HLA A2	FITC (BB7.2) PE (BB7.2)	BioLegend BioLegend
CD45 RO	FITC (REA611)	Miltenyi Biotech
CD45 RA	PE Vio770 (T6D11)	Miltenyi Biotech
CD27	APC (REA499)	Miltenyi Biotech
(CD197) CCR7	PerCP Vio770 (REA546)	Miltenyi Biotech

Table 2.4: Antibodies used for this work

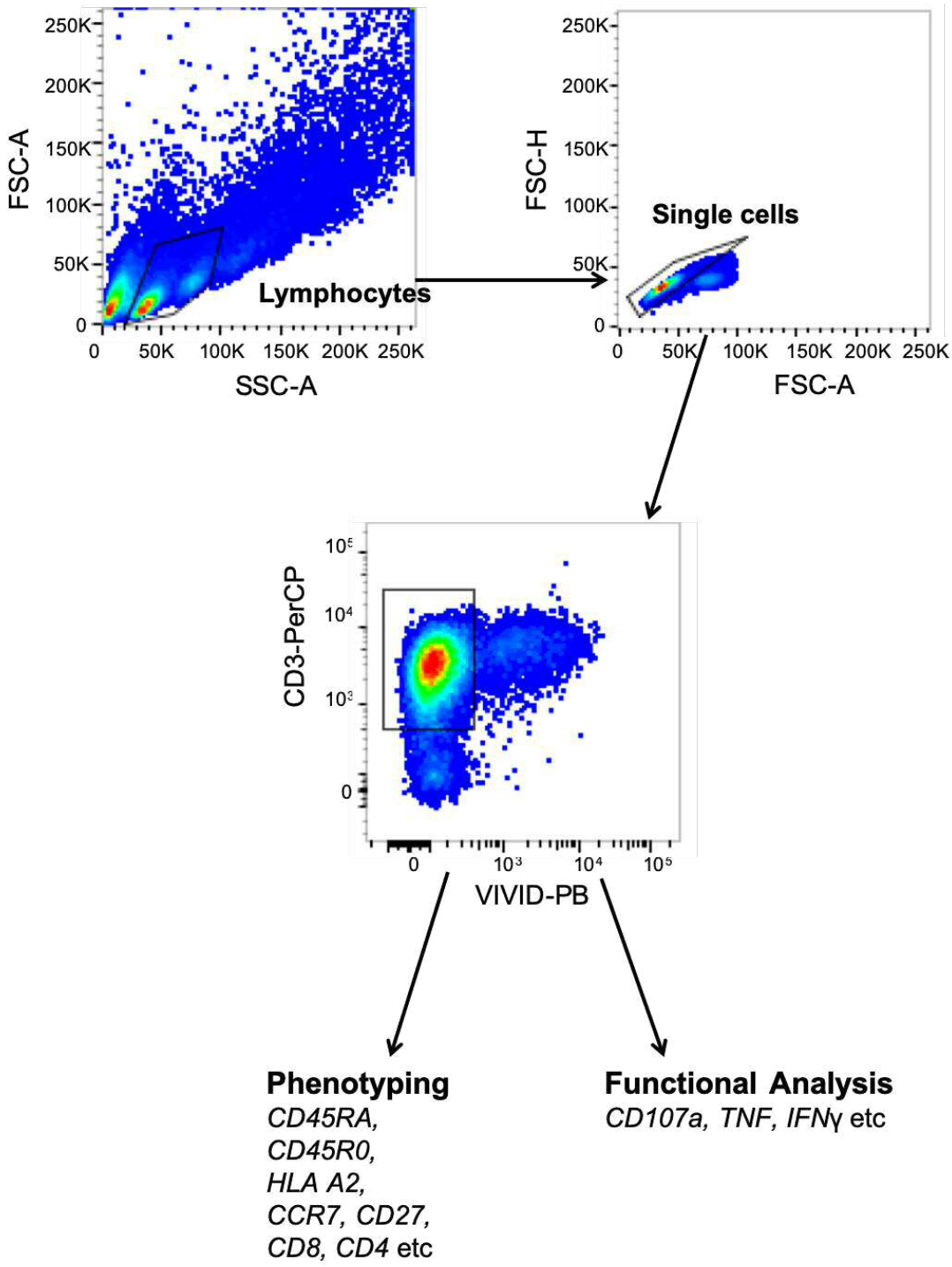


Figure 2.1: Gating strategy for functional and phenotypic analysis of T-cells

T-cells were gated based on well-defined characteristics of size and shape. Doublets and CD3 negative dead cells were then excluded allowing phenotypic and functional analysis of the CD8 positive T-lymphocytes. Representative plots are shown from a polyclonal population of CD8 isolated from healthy donor PBMC. FSC-A – forward scatter-area, FSC-H – forward scatter-height, SSC-A – side scatter-area.

2.7.2 Intracellular Cytokine Staining (ICS) assay

Cells were washed, counted and rested overnight in R5 medium prior to activation. Subsequently, cells were incubated at 37 °C, 5% CO_2 for 4 h, with and without target cells at a 1:2 T-cell to target cell ratio, or with a peptide of interest in R5 containing GolgiStop™, GolgiPlug™ (BD Bioscience) and 2 µL per well CD107a-PE antibody (BD Bioscience), according to the manufacturer's instructions. Cells were then stained with LIVE/DEAD® Violet and antibodies against desired cell surface markers as indicated in **section 2.7.1**. Samples were prepared for ICS by incubating with Cytofix/Cytoperm™ (BD Biosciences), according to manufacturer's instructions, before staining for 20 min on ice with mouse anti-human IFNγ-APC (Miltenyi Biotech) and TNF-PE Vio770 (Miltenyi Biotech). Cells were resuspended in PBS before flow cytometry and data analysis.

Polyfunctionality of T-cell clones was assessed using ICS, as described above, however IL-2 APC and MIP1-β PE (BD Bioscience) surface antibodies and TNF Per-CP (BD Bioscience) were included.

2.7.3 TNF Alpha Protease Inhibitor (TAPI) Assay

Cells were washed, counted and rested overnight in R5 medium before activation. Subsequently, cells were incubated in a 96 U-well plate at 37 °C for 4 h alongside APCs plus peptide or target cells at a ratio of 1:2 (T-cells: APC) in R5 medium supplemented with TNF-PE Vio 770 (Miltenyi Biotech), CD107a-FITC (BD Bioscience) and 30 µM TAPI-O (Sigma). After incubation, cells were then washed 3 times in 150 µL PBS and stained with LIVE/DEAD® Violet (Life Technologies) and desired cell surface markers antibodies, as described in **section 2.7.1**. Cells were washed and resuspended in PBS or fixed with 2% PFA and stored overnight at 4 °C in the dark before acquisition.

2.7.4 pMHC- Tetramer Staining

2.7.4.1 Manufacture of pMHC-tetramers

Soluble biotinylated peptide-MHC class I monomers were produced as previously described (Dolton *et al.*, 2014). Peptide-MHC-I tetramers were assembled over five separate 20 min steps with successive addition of streptavidin PE conjugates (Life technologies) to monomeric pMHC at a molar ratio of 1:4.

2.7.4.2 pMHC-tetramer staining

The desired number of cells, typically 5 x10^4 polyclonal T-cells and 3 x10^4 T-cell clone, were washed for 3 mins at 700 g and then treated with protein kinase inhibitor (PKI) (Dasatinib, Axon Medchem, Reston) at a final concentration of 50 nM for a minimum of 30 minutes at 37 °C. PKI treatment prevents TCR internalization (Dolton *et al.*, 2015). Without washing, cells were then stained with pMHC-tetramer at a concentration of 0.5 mg and incubated for 30 minutes on ice. Cells were washed in FACS buffer and stained with 1 µL anti-PE 1° antibody, to intensify the fluorescence of pMHC-tetramer staining, and incubated for 20 minutes on ice. In some cases, cells were washed in FACS buffer and stained with 0.5 µL of anti-PE 2° antibody for 20 minutes on ice in the dark (Tungatt *et al.*, 2015). Cells were washed in PBS before LIVE/DEAD® Violet stain (Life Technologies) and following a 5-minute incubation at room temperature in the dark, antibodies against cell surface markers were added directly without washing and incubated on ice for 20 min in the dark. Cells were then washed and resuspended in FACS buffer for immediate use or fixed with 2% PFA and stored at 4 °C to be acquired at a later time. Cells were analysed using the gating strategy depicted in **Figure 2.2.**

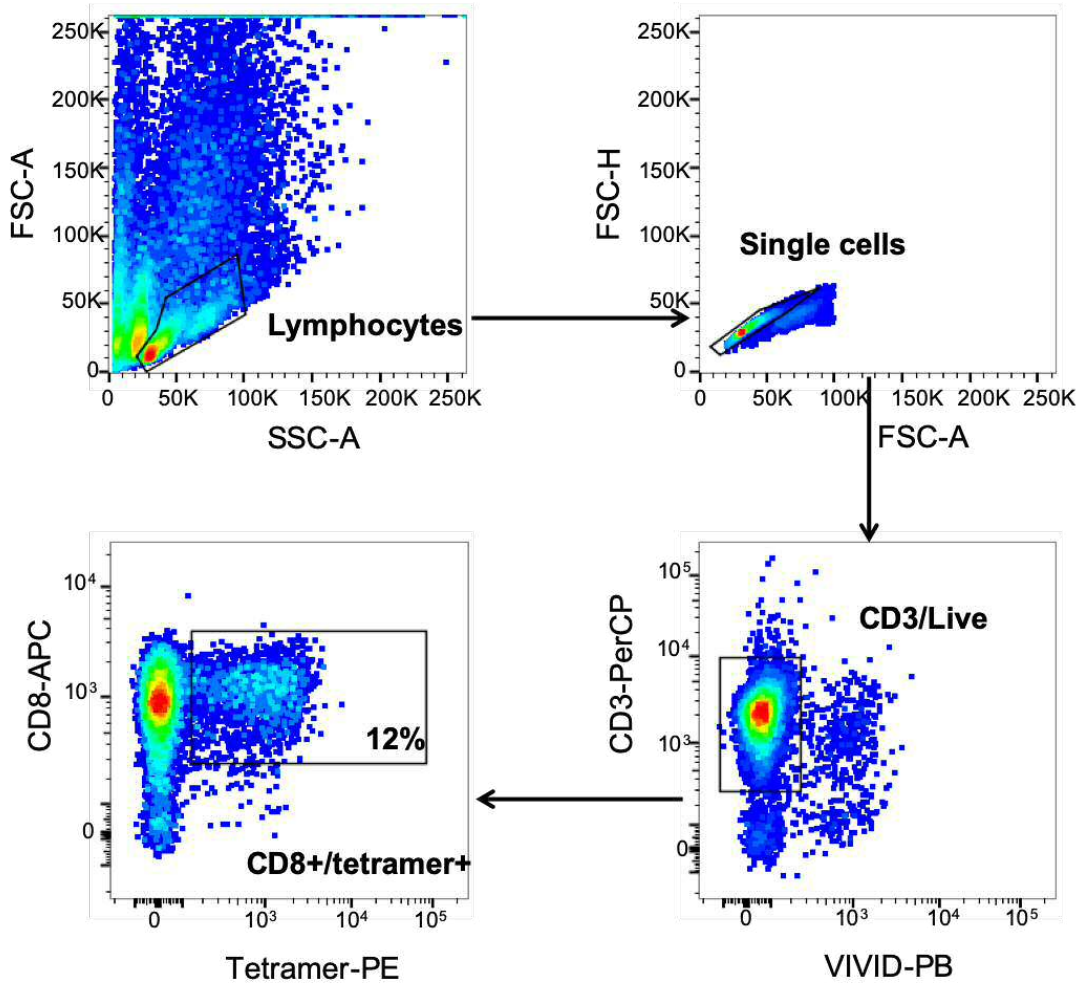


Figure 2.2: Gating strategy for pMHC-tetramer staining

Cells were first gated on FSC-A and SSC-A, followed by exclusion of doublet cells and dead/CD3- cells. Therefore, CD3+ cells could be analysed further, in this case for CD8 and tetramer co-positivity. Representative plots are shown from healthy donor 12 whose CD8+ cells were primed for 14 days with the agonist peptide ***MTSA****IGIL****P****V. FSC-A – forward scatter-area, FSC-H – forward scatter-height, SSC-A – side scatter-area.*

2.7.5 T2 Binding Assays

T2 A2 APCs were washed in serum free medium (AIM-V, Life Technologies), counted and 0.2 $x10^5$ – 10 $x10^5$ plated in a 96 well plates in serum-free medium. Peptides were added at concentration of 100 µM, 10 µM and 1 µM. To control for HLA A2 binding, a minimum of 1 known non-HLA A2 binding peptide and 1 known HLA A2 binding peptide were used. In addition, DMSO controls were added to equate for the volume of DMSO used to dissolve the peptides. The assay was incubated overnight at RT. The following day, cells were stained with 1 µL of HLA A2 FITC conjugated antibody (BioLegend) and incubated at 37 °C for 1 h. To allow

elimination of dead cells, a VIVID LIVE/DEAD stain was used, as described in **section 2.7.1**. Cells were then resuspended in PBS for acquisition.

2.7.6 Flow Cytometry Based Killing Assay

Flow cytometry based killing assays replaced the use of chromium release assays as a reduced number of T-cells (which were at a premium) could be used in each assay and the co-culture and potential killing of tumour cells could be assessed over a longer period of time. Furthermore, the use of flow cytometry allowed identification of tumour cells through surface markers, such as CD19, and quantification of the number of tumour cells remaining at the end of the assay.

2.7.6.1 Set up of flow cytometry based killing assay

Target cells and T-cells were harvested, washed in R0 medium and resuspended in T-cell expansion medium. Cells were then counted and plated a desired T-cell: target cell ratios, for example 1:1, in triplicate in 96 U-well plates. Wells containing target cells alone and T-cells alone were included as controls. The assay plate was then incubated at 37 °C for minimum 24 h, the incubation time varied depending on optimisation.

2.7.6.2 Running flow cytometry based killing assay

In order to determine the level of target cell death, carboxyfluorescein succinimidyl ester (CFSE) (Thermo Fisher Scientific) -labelled C1r APCs were used as a reference population. C1r cells were harvested, washed 2x in PBS and counted. Cells were then resuspended at a density of 1 x10^6 cells per mL in PBS and stored on ice. CFSE, at a stock concentration of 10 mM, was diluted 1:50,000 and 1 mL of diluted CFSE added per 1 x10^6 APCs. CFSE labelling occurred at 37 °C in the dark for 10 min. Cells were washed in excess R10 medium, in order to stop the reaction and resuspended in R10 medium. Prior to commencement of antibody staining and after sufficient incubation, 2 x10^4 CFSE labelled APCs were added per well to the flow cytometry based killing assay, including CFSE labelled cells alone as a control. The assay was then stained for live/dead VIVID and surface antibodies as previously described in **section 2.7.1.** Upon acquisition, target cell killing was analysed using the gating strategy depicted in **Figure *2.3*** and calculated using the following equation:

$$\% \text{ killing} = 100 - \left(\frac{\left(\frac{experimental\ cell\ events}{experimental\ CFSE\ C1R\ events}\right)}{\left(\frac{control\ target\ cell\ events}{control\ CFSE\ C1R\ events}\right)}\ x\ 100\right)$$

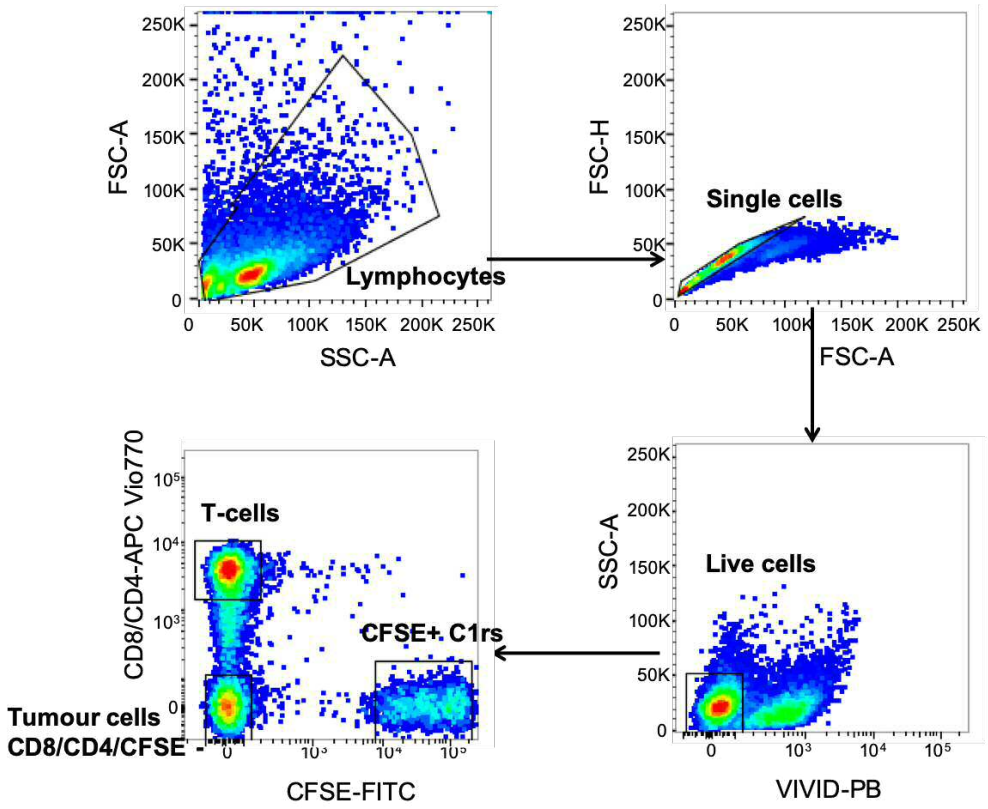


Figure 2.3: Gating strategy for flow-based killing assay

Lymphocytes, tumour cells and reference C1r population were isolated based on known size characteristics. Doublets and dead cells were excluded, and live cells gated. Live cells could then be identified based on fluorescent markers, expression of surface markers or neither i.e. CD8/CD4 for T-cells, FITC-CFSE for reference C1r population and no fluorescence for the tumour cells. Representative plots are from a CLL patient where killing of autologous tumour by CD8 T-cells cultured with no peptide for 28 days. FSC-A – forward scatter-area, FSC-H – forward scatter-height, SSC-A – side scatter-area.

2.8 Molecular Biology

2.8.1 Lentiviral transduction of cell lines

Lentivirus was made with the help of Dr Angharad Lloyd (HLA A2) and Dr Cristina Rius Rafael (BST2, melan-A and IMP2) using the following methodology.

2.8.1.1 Vectors

The vectors depicted in **Table 2.5** were used to insert codon-optimised mRNA transgenes of interest into 3rd generation transfer plasmid pELNS (kindly provided by Dr James Riley, University of Pennsylvania, PA) using the XbaI and XhoI restriction sites.

Plasmid name	Plasmid purpose	Provider
pELNS	Transfer plasmid	Dr James Riley (UPen)
pMD2.G	Envelope plasmid	Addgene (plasmid #12259)
pMDLg/pRRE	Packaging plasmid	Addgene (plasmid #12251)
pRSV-Rev	Packaging plasmid	Addgene (plasmid #12253)

Table 2.5: Plasmids used for lentivirus generation

2.8.1.2 Sequences and primers

Sequences from genes of interest were ordered from Genewiz. Constructs contained Xba1 and Xho1 restriction enzyme sequences at the 5' and 3' ends, respectively and a Kozak sequence (Kozak, 1986). Sequences can be found in **Appendix Figure 1**.

Primers used for the molecular cloning of sequences of interest and subsequent lentiviral transfection are detailed in **Table 2.6**.

Primer Name	Sequence (5' -> 3')	Application
pELNS F1	GAGTTTGGTVTTGGTTCATTC	pELNS colony PCR and pELNs sequencing
pELNS R3	AGAAACTTGCACCGCATATG	pELNS colony PCR

Table 2.6: Sequences of primers used for PCR and molecular cloning

2.8.1.3 Molecular cloning

In order to clone codon optimised inserts (purchased from GeneArt, ThermoFisher), vectors were first digested for 1 h at 37 °C. Digested products were then loaded onto a 1% agarose gel alongside the 1 kb ladder and run at 80 V for 1 h. Antigenic proteins were delivered in the pUC57 vector backbone, flanked by Xbal and Xhol restriction sites, inserts were cloned into the pELNS vector.

Reagent	Amount
pUC57 or pELNs plasmid	1 µg
10x FastDigest buffer (Thermo Scientific)	2 µL
Xbal (FastDigest, Thermo Scientific)	1 µL
Xhol (FastDigest, Thermo Scientific)	1 µL
Nuclease-free H_2O	up to 20 µL

Digested DNA was extracted from agarose gel using Wizard® SV Gel and PCR Clean up System (Promega), according to the manufacturer's instructions. DNA was then eluted in 20 µL nuclease-free H_2O and the concentration measured using the NanoDrop™ (Thermo Scientific). DNA product was then ligated to the pELNS backbone at RT for 2 h.

Reagent	Amount
pELNs vector	30 fmol
Insert	150 fmol
10x T4 DNA ligase buffer (Thermo Scientific)	2 µL
T4 ligase	1 µL
Nuclease-free H_2O	up to 20 µL

5 µL of the ligation product was transformed into 50 µL recombination-deficient XL10-Gold® cells (Agilent Technologies) as per the manufacturer's instructions. Briefly, ligation mixture and bacterial cells were co-incubated for 30 min on ice, heat shocked for 30 s at 42 °C before returning to ice. Cells were then incubated for 1 h at 37 °C in 250 µL of pre-warmed Super Optimal Broth (SOC, Clontech) to aid cell recovery. 100 µL of cells were then grown on Ampicillin-containing (100 µg/mL) LB agar plates at 37 °C overnight. The following day, bacterial colonies were screened by colony PCR, to assess the presence of insert, using the following mastermix:

Reagent	Vol
pELNs F1 (10 µM)	1 µL
pELNs R3 (10 µM)	1 µL
GreenTaq 2x Mastermix	12.5 µL
Nuclease-free H_2O	10.5 µL
Bacterial colony	1 colony

The following PCR conditions were used:

Temperature (°C)	Time (min:sec)	
94	10:00	
94	0:20	30 cycles
60	0:20	
72	2:00	
72	5:00	

PCR products were run on a 1% agarose gel. Positive colonies, containing an insert, were used to inoculate 5 mL of LB medium supplemented with 100 µg/mL carbenicillin overnight at 220 rpm and 37 °C. Plasmid DNA was then extracted using the PureLink® Quick Miniprep Kit (Invitrogen) as per the manufacturer's instructions. Extracted DNA was sequenced using

the Mix2Seq service (Eurofins Genomics) and confirmed using the ApE tool (A plasmid Editor, M. Wayne Davis).

In order to obtain enough DNA for efficient lentivirus preparation, miniprep products were subjected to a maxiprep, as per the manufacturer's instructions (PureLink® HiPure Plasmid Filter Maxiprep Kit, Invitrogen). Briefly, 250 mL of LB medium supplemented with 100 µg/mL ampicillin was inoculated with XL10-Gold® colonies containing verified inserts and incubated overnight at 220 rpm and 37 °C. Bacterial cultures were then centrifuged at 4,000 g for 10 min and the pellet resuspended in R3 buffer containing RNAse A. Cells were lysed with L7 buffer, followed by neutralisation with N4 buffer. Solution was then transferred to an equilibrated HiPure Filter column. The column was washed with W8 buffer and flow through allowed to drain by gravity flow. The plasmid, which was bound to the column, was then eluted into a falcon containing isopropanol and centrifuged at 4,000 g for 1 h at 4 °C. Supernatant was then removed, and the pellet washed 3x with ethanol. The pellet was then allowed to air dry before being resuspended in TE buffer, the presence of the correct sequence was confirmed by Sanger sequencing using pELNS F1 primer.

2.8.1.4 Lentivirus production

Medium	Composition	Uses
Transfection medium	DMEM 100 U/mL Penicillin, 100 µg/mL Streptomycin 2 mM L-Glutamine 10% FBS	Culture of 293T prior to transfection
pH 7.1 medium	DMEM 25 mL HEPES	Co-transfection

Table 2.7: Media for lentivirus production

In preparation for transfection, 2 x10^7 293T cells were plated in T175 flasks in 50 mL of transfection medium (**Table 2.7**) and allowed to reach 80% confluency.

The pELNS transfer plasmid (15 µg) carrying the appropriate inserts was co-transfected with the packaging plasmids pRSV-Rev (18 µg) and pMDLg/pRRE (18 µg) and the envelope plasmid pMD2.G (7 µg) in a 15 mL tube in pH 7.1 medium. Next, 150 µL of 1 M $CaCL_2$ solution was added to the DNA transfection mix and incubated at RT, vortexed and added drop wise to the 293T flask. Cells were incubated overnight, with the supernatant replaced after 16 h. Supernatant was collected from the flask after 48 and 72 h, filtered using a 0.45 µm filter and stored at 4 °C. The harvested supernatants were pooled and concentrated by

ultracentrifugation at 140,000 g for 2 h at 4 °C (Optima™ L-100 XP with SW28 rotor, Beckman Coulter). The lentivirus pellet was harvested, resuspended in R10 medium and stored at -80 °C.

2.8.1.5 Lentiviral transduction of tumour lines

Antigen presenting cells or tumour cell lines that were to be transduced were washed in R0 medium, counted and plated at a density of 5 x10^4 per 500 µL of R10 medium in 1 well of a 24 well plate one day prior to transduction. Immediately before transduction, medium was aspirated, and 1 mL of lentivirus was added to the cells along with polybrene (Santa Cruz Biotech) at a concentration of 8 µg/mL. The plate was then centrifuged at 500 g for 2 h at 37 °C. The following day all virus was harvested from the wells and 1 mL of R10 medium added, cells were tested for protein expression after 72 h.

2.8.2 Gene-silencing using CRISPR/Cas 9 system

In order to knock-out endogenously expressed proteins, such as HLA A2, the Neon® Transfection System was used (Life Technologies). Guide RNAs (gRNA) were ordered as DNA oligonucleotides and were prepared for transfection by annealing to tracrRNA using PCR according to the manufacturer's instructions (GeneArt Precision gRNA Synthesis Kit, Thermo Fisher). Annealed gRNA: tracrRNA duplex was kept at -20 °C until required.

APC or tumour lines were counted, washed 2 x in PBS and resuspended in resuspension buffer (Life Technologies) at the appropriate concentration based on the cell type being used, details of cell numbers used for each cell type can be found in **Table 2.8**. Guide RNA and cas-9 (GeneArt Platinum Cas9 Nuclease) were combined and incubated on ice for 10 min, concentration of each was dependent on the cell type being used however in most cases this was 2 µg of cas-9 and 12 pmol of gRNA. After incubation cells and the cas-9/gRNA mixture were combined and electroporated using the neon transfection system, according to the manufacturer's instructions (Life Technologies). Details of voltages, pulse duration and pulse number for each cell type used can be found in **Table 2.8**. Cells were plated in a 24 well in 2 mL of medium and allowed to recover for 7 days before analysis by flow cytometry.

Cell type	Cell number (10^5)	Voltage (v)	Pulse duration (ms)	Pulse number	Cas-9/gRNA
RCC tumour line	1.2	1200	20	4	2 μg/ 12 pmol
PANC 1	2	1400	15	4	2 μg/ 12 pmol
THP-1	2	1700	20	1	2 μg/ 12 pmol

Table 2.8: Conditions for electroporation of cell lines

2.9 Protein expression, refolding, purification and crystallisation

Refolding and crystallisation of peptide-MHC was carried out with the help of Aaron Wall and Anna Fuller. Data was collected at the Diamond Light Source Facility, Oxford UK. Protein expression, refolding and crystallisation was carried out using the buffers and medias indicated in **Table 2.9**.

Buffer	Composition
LB medium	10 g/L tryptone (Fisher Scientific), 5 g/L yeast extract (Fisher Scientific), 5 g/L NaCl (Fisher Scientific), supplemented with 50 mg/L carbenicillin (Carbenicillin Direct)
LB agar plate medium	15 g/L agar bacteriological (Oxoid), 10 g/L tryptone (Fisher Scientific), 5 g/L yeast extract (Fisher Scientific), 5 g/L NaCl (Fisher Scientific), Supplemented with 50 mg/L carbenicillin (Carbenicillin Direct)
TYP medium	16 g/L tryptone, 16 g/L yeast extract, 5 g/L potassium phosphate dibasic (Acros Organics)
Lysis buffer	10 mM Tris pH 8.1, 10 mM $MgCl_2$, 150 mM NaCl, 10% glycerol
Triton wash buffer	0.5% Triton X, 50 mM Tris pH 8.1, 100 mM NaCl, 2 mM EDTA (Fisher Scientific)
Guanidine buffer	6 M guanidine, 50 mM Tris pH 8.1, 100 mM NaCl, 2 mM EDTA
Non-reducing sample buffer	125 mM Tris pH 6.8, 4% SDS, 20% glycerol, 20 μg/mL bromophenol blue
Reducing sample buffer	125 mM Tris pH 6.8, 4% SDS, 20% glycerol, 20 μg/mL bromophenol blue, 10% DTT
pMHC I refold buffer	50 mM Tris pH 8, 2 mM EDTA, 400 mM L-arginine (SAFC), 0.74 g/L cysteamine,0.83 g/L cystamine
Ion exchange buffer A	10 mM Tris
Ion exchange buffer B	10 mM Tris, 1 M NaCl
Crystal buffer	10 mM Tris pH 8.1, 10 mM NaCl
Screen solution	20%w/v polyethylene glycol 3350, 0.2M sodium nitrate, 0.1M BIS-TRIS propane pH6.5

Table 2.9: Culture media and buffers for protein expression, refolding, purification and crystallisation

2.9.1 Vectors and protein sequences

2.9.1.1 pGMT7 Vector

The carbenicillin resistant pGMT7 expression plasmid (Studier *et al.*, 1990; Banham and Smith, 1993) was used as a vector for bacterial protein expression of peptide-MHC class I molecules. The vector contained a sequence encoding the protein of interesting which is inducible by IPTG (Isopropyl β-D-1-thiogalactopyranoside), controlled by the T7 RNA polymerase promoter. The sequence of interest is flanked by BamHI and EcoRI restriction sites.

2.9.1.2 Sequences

HLA A2 heavy chain:

MGSHSMRYFFTSVSRPGRGEPRFIAVGYVDDTQFVRFDSDAASQRMEPRAPWIEQEGPEYWD
GETRKVKAHSQTHRVDLGTLRGYYNQSEAGSHTVQRMYGCDVGSDWRFLRGYHQYAYDGKDY
IALKEDLRSWTAADMAAQTTKHKWEAAHVAEQLRAYLEGTCVEWLRRYLENGKETLQRTDAP
KTHMTHHAVSDHEATLRCWALSFYPAEITLTWQRDGEDQTQDTELVETRPAGDGTFQKWAAV
VVPSGQEQRYTCHVQHEGLPKPLTLRWEPGLNDIFEAQKIEWHE

β2-microglobulin:

MIQRTPKIQVYSRHPAENGKSNFLNCYVSGFHPSDIEVDLLKNGERIEKVEHSDLSFSKDWS
FYLLYYTEFTPTEKDEYACRVNHVTLSQPKIVKWDRDM

Peptide Sequences:

MTSAIGIL**P**V, EAAGIGILTV, LLLGIGILVL and NLSALGIFST

2.9.2 Transformation of E. coli cells

In order to produce MHC class I and B_2M inclusion bodies, competent Rosetta (DE3) pLysS E. coli cells (Invitrogen) were transformed with 50 – 100 ng of plasmid DNA. Transformation took place at 5 min on ice, cells were then heat-shocked for 2 min at 42 °C and then allowed to recover for a further 5 min on ice. Cells were then grown overnight on a LB agar medium plate (supplemented with 50 μg/mL carbenicillin).

2.9.3 Expression of inclusion bodies in Rosetta *E. coli* cells

To ascertain expression of desired peptide-MHC class I proteins a single colony from **Section 2.9.2** was grown in 30 mL of TYP medium (supplemented with 50 μg/mL carbenicillin), at 37 °C and 220 rpm until the optical density reached 0.5 (OD_{600nm}). To induce protein expression 0.5 mL of 1 M IPTG was added and the culture incubated at 37 °C for 3 h at 220 rpm. To check

the level of protein expression a 0.5 mL sample of culture was taken before and after the addition of IPTG and run on a SDS-PAGE gel, as per **Section 2.9.4.** After incubation, cells were harvested and centrifuged at 3450 g for 20 min and the pellet then resuspended in 40 mL of lysis buffer. Cells were then sonicated on ice at 20% power for 20 min using 2 sec intervals (MS73 probe, Bandelin). Following sonication, the pellet was incubated with 200 μL of 20 mg/mL DNAse (Sigma) for minimum 30 min at RT. Following incubation cells were resuspended in 100 mL of triton wash buffer and centrifuged at 15,180 g for 20 min, and the pellet resuspended in 100 mL of triton wash buffer following homogenization (VWR VD25, 17,000/min). Cells were then resuspended in 10 mL resuspension buffer and centrifuged for 20 min at 15,180 g and the pellet dissolved in 5 – 10 mL of guanidine buffer and stored at -20 °C.

2.9.3.1 Spectrophotometry to estimate protein concentration

The concentration of peptide-MHC class I proteins was measured using a spectrophotometer (NanoPhotometer®, Geneflow). Protein samples were diluted 1/100 in PBS or appropriate buffer. The spectrophotometer was blanked using buffer before use and readings measured at 280 nm wavelength. Protein concentration was calculated using the dilution factor and extinction co-efficient (calculated using ProtParam tool) following Beer's Law formula:

$$A_{280nm} = \varepsilon CL$$

2.9.4 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as per the manufacturer's instructions, in order to determining the purity and quantity of each protein (Life Technologies X-Cell SureLock™ system). 30 μL of sample supernatant was harvested and combined with 3 μL DTT and 11 μL of reducing loading buffer and incubated for 5 min at 95 °C. 20 μL of each sample was then loaded onto a pre-cast 10% Bis/Tris gel (NuPAGE, Invitrogen) with 1x running buffer (NuPAGE, Invitrogen) alongside BLU Wide Range (10 to 245 kDa) protein ladder (Geneflow Ltd) which acted as a reference. Gels were run at 90 V for 1.5 h. After completing the running time, gels were stained with Quick Coomassie stain (Generon) and de-stained with dH_2O to allow for band visualisation.

2.9.5 Soluble peptide-MHC class I molecules

A 1 L peptide-MHC class I refold was carried out by mixing 30 mg of HLA A2 α-chain inclusion bodies, 30 mg of B_2M and 4 mg of synthetic peptide (**MTSA**IGIL**P**V, EAAGIGILTV, LLLGIGILVL or NLSALGIFST) at 37 °C for 15 min before adding to cold refold buffer for a minimum of 1 h at 4 °C. Dialysis was undertaken in 10 mM Tris pH 8.1 until the conductivity of the refolds was <2 mS/cm. Refolds were then filtered using a 0.22 μM and then a 0.45 μM filter (Fisher Scientific) before fast protein liquid chromatography (FPLC).

2.9.6 Fast protein liquid chromatography (FPLC)

An anion exchange column (POROS® 50HQ, Life Technologies) was equilibrated with buffer A and washed with buffer B before being used to purify peptide-MHC class I refolded proteins. Peptide-MHC class I protein samples were loaded at a flow rate of 20 mL/min (5 MPa pressure) and eluted using buffer A to FPLC tubes (Grenier Bio-one). Eluted fractions were analysed for purity by SDS-PAGE, then pooled and concentrated at 3000 g in order to obtain a sample volume <1 mL using a 4 mL Vivaspin® concentrator tube (Sartorius). A Superdex™ HR 200 size-exclusion column (Amershame Pharmacia) was used for buffer exchange and removal of aggregated by gel filtration, samples were eluted into FPLC tubes. Fractions with correctly folded peptide MHC class I proteins were identified using SDS-PAGE and concentrated as previously described. Samples could be stored at -20 °C until required.

2.9.7 Crystallisation, diffraction data collection and model refinement

Protein crystals of HLA A2- **MTSA**IGIL**P**V and HLA A2-EAAGIGILTV were grown at 18°C by vapour diffusion via hanging drop technique. 1 μL of HLA A2- **MTSA**IGIL**P**V (10 mg/ml) in crystallisation buffer was added to 1 μL of screen solution. HLA A2-EAAGIGILTV crystals grown in the same conditions were crushed until no visible crystal remained using a MicroBead seed kit (Molecular Dimensions) and added to the solution.

Crystallisation screens were conducted by hand, and data were collected at 100 K at the Diamond Light Source (DLA), Oxfordshire, UK at a wavelength of 0.98 Å using an ADSC Q315 CCD detector. Reflection intensities were estimated using XIA2 (Winter, et al. 2013) and the data were analysed with SCALA and the CCP4 package (Collaborative Computational Project, Number 4, 1994). Sequences were adjusted with COOT (Emsley and Cowtan, 2004) and the models refined with REFMAC5. Crystal structures were solved with molecular replacement

using PHASER (McCoy *et al.*, 2007) and visualised using PyMOL software (The PyMOL Molecular Graphics System, Version 1.8 Schrödinger, LLC). The reflection data and final model coordinates were deposited in the Protein Databank (PDB) database (HLA A2-**MTSA**IGIL**P**V, PDB: 6G3J).

2.10 TCR clonotyping

T-cells were sorted using a BD FACS Aria (Becton Dickson) with the help or Dr Catherine Naseriyan and Dr Ann Kift-Morgan (Central Biotechnology Services, CBS) based on HLA A2-EAAGIGILTV tetramer positivity. TCR clonotyping was carried out with the help of Dr Meriem Attaf and Dr Cristina Rius Rafael.

2.10.1 mRNA extraction from T-cells

Cells isolated by cell sorting were lysed in 350 μL of RLT lysis buffer and the RNA extracted using RNeasy Plus Mini Kit (Qiagen) as per the manufacturer's instructions. Briefly, lysed cell samples were added to a gDNA Eliminator Spin column placed in a 2 mL collection tube and centrifuged for 30 sec at 8000 g. The flow through was then mixed thoroughly with 350 μL of 70% ethanol. Sample was then transferred to a RNeasy spin column in a collection tube and spun for 30 s at 8000 g, 500 μL of buffer RPE was then added and the column centrifuged at 8000 g for 2 min. The column was then transferred to a new collection tube and spun dry in the centrifuge for 1 min at full speed. RNA was then eluted from the column by adding 50 μL of RNAse free water to the column and centrifuging for 8000 g for 1 min. RNA can be used immediately or stored at -80 °C.

2.10.2 cDNA synthesis

The RNA extracted from sorted T-cells, as per **section 2.10.1**, was converted to cDNA using the SMARTer 5'/3'RACE kit (Takara Bio, ClonTech). Initially, 10 μL of RNA was added to 1 μL of 5' CDS primer, placed in a thermal cycler and incubated for 3 min at 72 °C and 2 min at 42 °C. Tubes were spun briefly after incubation and then added to the following mastermix which was made up for the appropriate number of reactions:

Reagent	Vol
5X First Strand buffer	4 µL
DTT (100 mM)	0.5 µL
dNTPs (20 mM)	1 µL
RNase inhibitor (20U)	0.5 µL
SMARTScribe reverse transcriptase	2 µL
SMARTer IIA Oglionucleotide (12 µM)	1 µL

The samples were then incubated at 42 °C for 90 min and 70 °C for 10 min then stored at -20 °C.

2.10.3 Step-out and Nested PCR

In order to carry out a step-out PCR the following mastermix was generated and volumes adjusted depending on the number of conditions:

Reagent	Vol
5X HF Buffer	10 µL
dNTPs	1 µL
10x Universal Primer A	5 µL
Cα-R1 or Cβ-R1	1 µL
DMSO	0.5 µL
HF Taq	0.25 µL
H_2O	29.75 µL
Sample cDNA	2.5 µL

The constant region reverse primers (Cα-R1 or Cβ-R1) were used depending on which TCR chain was being sequenced. Along with the universal primer A, specific for the universal anchor on cDNA molecules. The PCR cycle was carried out as follows:

Temperature (°C)	Time (min:sec)	
94	5:00	
94	0:30	30 cycles
Cα @ 63 or Cβ @ 66	0:30	
72	1:30	
72	7:00	

A nested PCR was then carried out using 2.5 µL of the step-out product and the same thermal conditions. The following mastermix was made up for the appropriate number of reactions:

Reagent	Vol
5X HF Buffer (green)	10 µL
dNTPs (10 mM)	1 µL
Primer short	1 µL
Cα-R2 or Cβ-R2	1 µL
DMSO	0.5 µL
Phusion Tag	0.25 µL
H_2O	33.75 µL
Sample cDNA	2.5 µL

2.10.4 Agarose gel electrophoresis

Following amplification, the nested PCR products were purified using gel electrophoresis. A 1% agarose gel was cast by dissolving 0.5 g of agarose powder (Invitrogen) in 50 mL of TAE buffer at a gentle heat in the microwave. After slight cooling, 2.5 µL of Mildori DNA stain (GeneFlow) was added before casting the gel. Once the gel was set, samples were added alongside 5 µL of DNA hyperladder (100 bp DNA ladder, Bioline) and the gel run at 80 V for 40 min. Upon completion of the run, gel was visualised using a LED illuminator (FastGene) and the DNA bands excised from the gel. DNA was purified from agarose slices using the QIAEX II Gel Extraction Kit (Qiagen) as per the manufacturer's instructions. Briefly, agarose slice was weighed and 100 µL of buffer QG added per 100 mg of gel and the sample incubated at 50 °C for 10 min. If the DNA was known to be <500 bp or >4 kb, then 100 µL of isopropanol was added to the sample per 100 mg of gel. To acquire the DNA, the dissolved gel sample was spun for 1 min in a QIAquick column with a collection tube, the DNA binds to the column. To wash the column 750 µL of buffer PE was added and the QIAquick column centrifuged for 1 min, and after discarding the flow-through the column was spun once more to remove residual ethanol. To elute the DNA from the column 50 µL of buffer EB or nuclease-free water was added to the column and centrifuged for 1 min. The acquired DNA sample was sequenced using an Illumina MiSeq instrument.

2.11 Data Analysis

Unless otherwise stated, plots were generated using GraphPad prism software (GraphPad Software, Version 6.01). Crystal structures were solved with molecular replacement using PHASER (McCoy *et al.*, 2007) and visualised using PyMOL software (The PyMOL Molecular Graphics System, Version 1.8 Schrödinger, LLC) with the help of Aaron Wall. Flow cytometry data was analysed using FlowJo Software (Tree Star Inc). Clonotyping results were displayed using Circos plots (Krzywinski *et al.*, 2009).

Where possible statistics were used to show significance and error. Error bars depict the standard deviation (SD), and although it is not optimal, were used where N > 2. Due to a low number of replicates (N=2 or N=3) it was not possible to do further statistics on the majority of the data, where significance is shown the number of data points exceeded N=4.

3. Peptide super-agonist enhances T-cell responses to melanoma

3.1. Background

3.1.1. Altered peptide ligands (APL) for treatment of melanoma

Peptide cancer vaccines are an attractive form of immunotherapy due to their ease of administration, potential to work in many donors of the same HLA type and relatively low cost (Finn, 2018). Despite their benefits, progression in this field has been disappointing (Finn, 2003).

There are many potential targets for peptide cancer vaccines including neoantigens, onco-feotal proteins, cancer testis antigens and over-expressed self-proteins. Melanoma has provided a dominant model system for the study and development of peptide vaccines for cancer due to the dominant expression of one over-expressed protein, melan-A. Melan-A is over-expressed in the majority of melanoma (Valmori *et al.,* 1998) and despite being expressed in healthy tissue, it is the most researched target for immunotherapy to treat melanoma (Pardoll, 1999; Finn, 2017). In HLA A2 positive individuals the dominant tumour-associated antigen derived from melan-A is the peptide EAAGIGILTV (Kawakami *et al.*, 1994). As melan-A is a self-protein that is expressed at low levels in healthy melanocytes, the EAAGIGILTV peptide is poorly immunogenic and fails to induce an adequate CD8 T-cell response in melanoma patients (Kawakami *et al.*, 1994). Several strategies have attempted to improve the immunogenicity of the EAAGIGILTV peptide.

The position 2 alanine residue in EAAGIGILTV is suboptimal for binding to the HLA A2 so early studies used the altered peptide E**L**AGIGILTV where the alanine (A) amino acid at position 2 of the peptide was substituted to Leucine (L), the preferred anchor residue (Valmori *et al.*, 1998). The improved binding of E**L**AGIGILTV (bold underlined residues indicate non-germline amino acids) to HLA A2 induced increased expansion of EAAGIGILTV-specific CD8 T-cells when used for T-cell priming *in vitro* (Valmori *et al.*, 1998), promoting E**L**AGIGILTV as a promising candidate for clinical trials in metastatic melanoma. However the increase in magnitude of EAAGIGILTV-specific CD8 T-cells did not lead to any measurable decrease in tumour burden *in vivo* (Bins *et al.*, 2007). Subsequent studies demonstrated that EAAGIGILTV and E**L**AGIGILTV adopted different structures in the HLA A2 peptide binding

groove (Cole *et al.*, 2010). These differences can be sensed by melan-A-specific TCRs (Madura *et al.*, 2015). Thus TCR clonotypes induced by E**L**AGIGILTV could fail to cross-react with the natural peptide sequence as presented at the melanoma surface (Cole *et al.*, 1997; Wieckowski *et al.*, 2009). The failure of E**L**AGIGILTV as a candidate for an anti-melanoma peptide vaccine inferred that a new approach is needed when designing altered peptide ligands (APL) for cancer immunotherapy.

An alternative method to identify candidate APLs is to harness the cross-reactive nature of CD8 T-cells through combinatorial peptide library (CPL) screening, whereby a given CD8 T-cell clone is exposed to all possible combinations of amino acids in a peptide of a given length (Linnemann *et al.*, 2001). CPL screening of the melan-A-restricted clone MEL5 identified a super-agonist peptide, **F**A**T**GIGI**I**TV (bold residues indicate non-germline amino acids) (Ekeruche-Makinde *et al.*, 2012). **F**A**T**GIGI**I**TV increased the magnitude of EAAGIGILTV-specific CD8 T-cells *in vitro* in 5/7 donors tested (Ekeruche-Makinde *et al.*, 2012). Moreover, CD8 T-cells isolated from healthy donors primed with **F**A**T**GIGI**I**TV showed superior killing of melanoma tumour compared to EAAGIGILTV-primed cells. Whilst the results of this study were promising, the lack of efficacy in all donors left room for improvement.

To build on previous work, I sought to improve upon previous attempts to design altered peptide ligands capable of eliciting EAAGIGILTV-specific CD8 T-cells and improve targeting of melanoma tumour in all HLA A2+ donors. The main difference between my own study and the earlier study undertaken by my laboratory was that I had access to the TIL used to successfully treat an HLA A*0201 homozygous melanoma patient. This then allowed me to design and test super-agonist peptides for a T-cell clone from the TIL that persisted in patient blood during and after complete cancer remission *in vivo*

3.1.2. Aims

Previous studies have demonstrated the importance of the individual TCR clonotypes induced by peptide vaccines. I therefore started with a CD8 T-cell clonotype, isolated from the TIL infusion product used to successfully induce a complete, durable remission in an HLA A2 homozygous metastatic melanoma patient. Importantly, this clonotype was seen to persist in patient blood long after 'cure'.

Specifically, this study sought to:

- Use a combinatorial peptide library screen of a dominant TCR clonotype to identify novel HLA A2 restricted super-agonist peptides.
- Validate newly identified super-agonist peptides using individual TCR clonotypes and polyclonal CD8 T-cell populations.
- Determine whether any newly identified super-agonist peptides are superior to previously identified agonist peptides E**L**AGIGILTV and **F**A**T**GIGI**I**TV.
- Determine whether super-agonist peptides induce functionally superior CD8 T-cells compared to the wild type, melan-A antigen EAAGIGILTV.

3.2. Results

3.2.1. Isolation and validation of ST8.24 T-cell clone

Through an ongoing collaboration with the National Centre for Cancer Immune Therapy (CCIT) we have been fortunate enough to obtain samples from metastatic melanoma patients who have been treated using tumour infiltrating lymphocyte (TIL) therapy (Andersen *et al.*, 2016). Work completed by Dr Valentina Bianchi during her PhD (Bianchi, 2016a), identified dominant and persistent cancer-reactive CD8 T-cell clonotypes in the TIL infusion product given to patient MM909.24, who successfully cleared their tumour after undergoing TIL therapy and remains cancer-free 7 years post treatment. Interestingly, these clonotypes were also found in the patients' peripheral blood 6 months after complete remission (**Figure 3.1A**). It was hypothesised that the few TIL infusion product-derived cancer-reactive T-cells that were expanded and persisted in patient blood after complete remission were pivotal in cancer clearance and in keeping the patient tumour-free. The TIL infusion product from patient MM909.24 was found to be highly reactive to autologous tumour with 44% of the CD8 T-cells producing TNF and CD107a compared to 0.7% in the unstimulated control, some of this reactivity could still be seen in the PBMC 6 months post cure where 3.5% of the CD8 T-cells produced TNFα and CD107a. (**Figure 3.1B**)**.** One of the CD8 T-cell persistent clonotypes found in the TIL and PBMC, ST8.24, was isolated by single cell cloning of EAAGIGILTV-tetramer positive CD8 T-cells isolated from the TIL infusion product. ST8.24 was highly effective at killing autologous tumour (**Figure 3.1C**).

Due to the potency and apparent dominance of ST8.24, it was proposed that this clone could be used to investigate and improve peptide vaccination for metastatic melanoma.

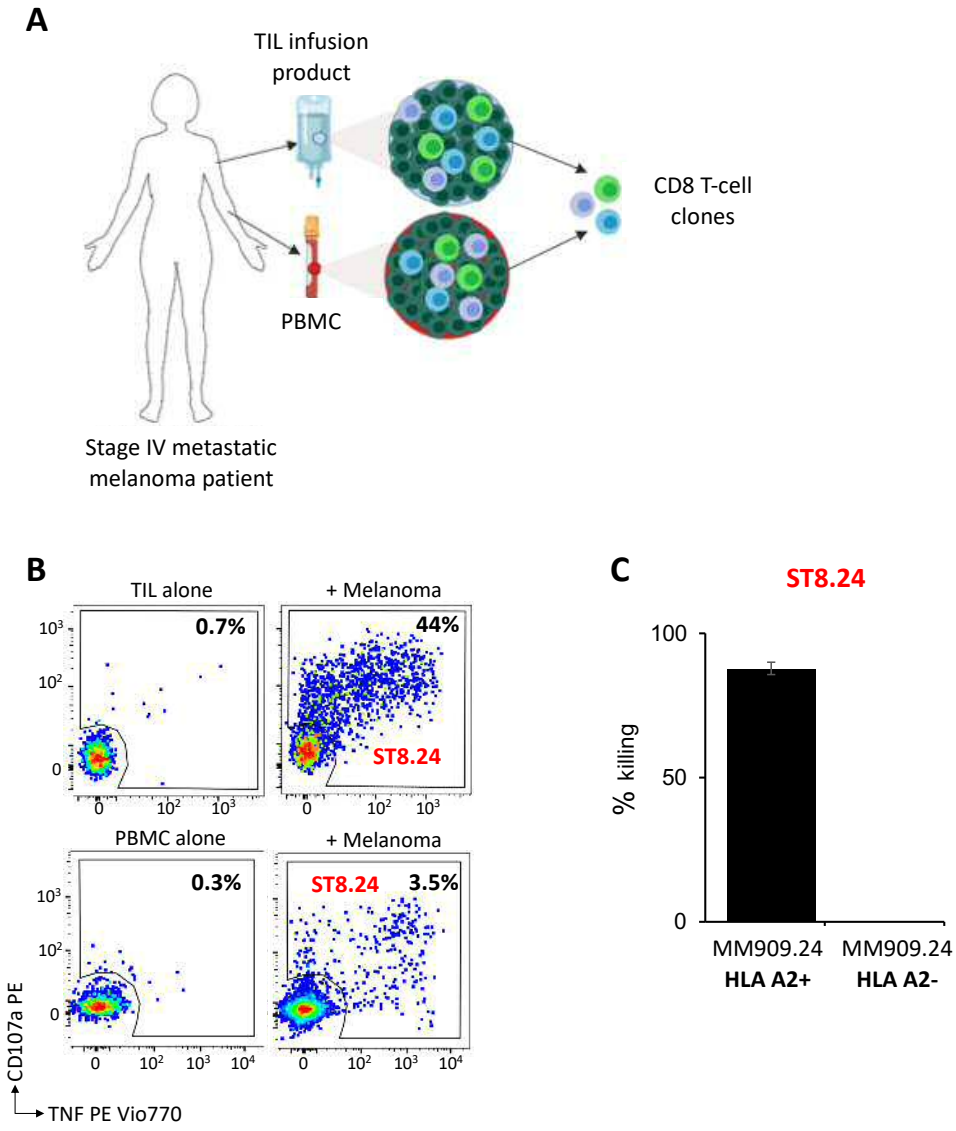


Figure 3.1: Isolation and validation of metastatic melanoma derived CD8 T-cell clone ST8.24

*(**A**) Tumour infiltrating lymphocyte (TIL) infusion product and PBMC samples 6 months' post-cure were obtained from patients who underwent TIL therapy through an ongoing collaboration with the CCIT, Herlev, Denmark. Previous studies in the Sewell lab identified dominant CD8 T-cell clonotypes found in the TIL infusion product and PBMC from the same patient, MM909.24. Patient MM909.24 successfully cleared their cancer following TIL therapy and remains cancer-free over 7 years after this therapy. Dominant clonotypes were isolated though single cell cloning of the TIL infusion product. (**B**) Activation of TIL infusion product and PBMC towards autologous tumour assessed via TAPI-O activation assay. Release of TNF and CD107a measured via flow cytometry analysis. Percentage of cytokine producing cells is displayed. Dominant CD8 T-cell clonotype ST8.24 was amongst the tumour-reactive population in both the TIL infusion product and in patient blood 6 months after complete remission. (**C**) Cytotoxicity of ST8.24 following 2 days of co-incubation with autologous tumour MM909.24 +/- HLA A2 measured in a long term killing assay. Percentage killing was normalised to the 'no T-cell' sample. Assay was carried out in triplicate. Error is depicted as standard deviation (SD).*

3.2.2. Previously identified agonist peptide **F**A**T**GIGI**I**TV fails elicit a response from the TIL used to successfully treat patient MM909.24

A previous study in the Sewell lab identified an agonist peptide **F**A**T**GIGI**I**TV using MEL13, a melan-A restricted T-cell clone isolated from a healthy donor. MEL13 had an identical TCR as the clone MEL5 that was isolated from the same donor at the same time. This TCR has been very well studied and there are published co-crystal structures of the MEL5 TCR with HLA A2-E**L**AGIGILTV and HLA A2-EAAGIGILTV (Cole *et al.*, 2009b; Madura *et al.*, 2015). T-cells bearing the MEL5 TCR were more sensitive to **F**A**T**GIGI**I**TV than the melan-A WT peptide EAAGIGILTV and the widely used heteroclitic variant E**L**AGIGILTV (**Figure 3.2A**).

Initial experiments in this study showed that whilst ST8.24 responded to EAAGIGILTV and E**L**AGIGILTV as expected, no response was seen to **F**A**T**GIGI**I**TV (**Figure 3.2A**). Analysis of the TIL infusion product given to patient MM909.24 showed no reactivity to **F**A**T**GIGI**I**TV above baseline activation (**Figure 3.2B**).

These results indicated that clonotype ST8.24 that persisted in a patient after complete remission of malignant melanoma had a TCR with a different specificity than the MEL13 TCR isolated from a healthy donor. They further show that the large melan A-specific T-cell population within the TIL used to treat patient MM909.24 did not recognise the **F**A**T**GIGI**I**TV peptide.

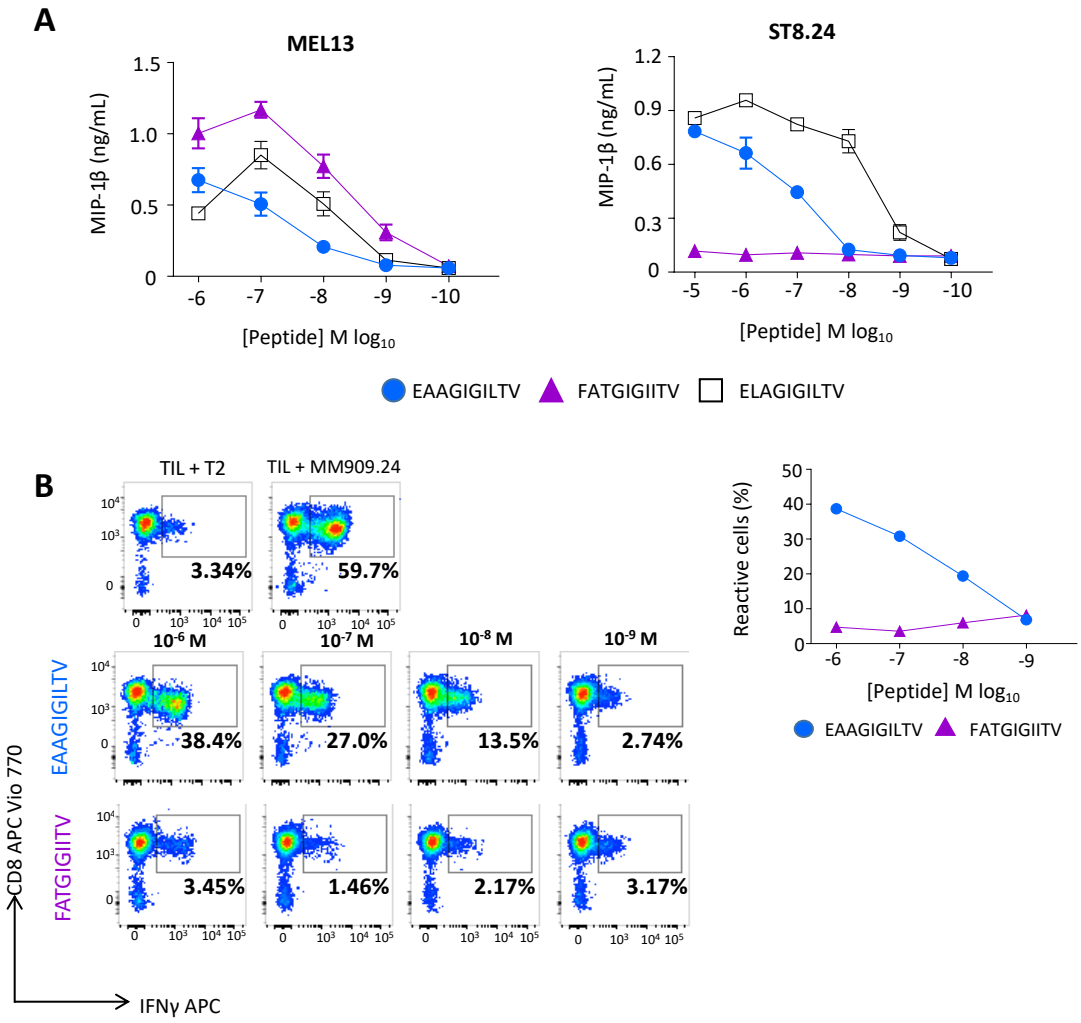


Figure 3.2: Agonist peptide FATGIGIITV does not elicit a response from melanoma patient MM909.24 derived CD8 T-cell clone ST8.24 or TIL infusion product.

*(**A**) 6 x10^4 T2 APCs and 3 x10^4 CD8 T-cells (clones ST8.24 and MEL13) were incubated at 37 °C overnight alongside either EAAGIGILTV, **F**A**T**GIGI**I**TV or E**L**AGIGILTV peptides at a concentration range from 10^{-5} to 10^{-10} M. The following day supernatants were harvested and the production of MIP-1ß assessed by an ELISA. Results are shown as an average of 2 replicates where the value of T2 + T-cells with no peptides was deducted, error is shown as SD from the mean. (**B**) Reactivity of TIL from metastatic melanoma patient MM909.24 to peptides EAAGIGILTV and **F**A**T**GIGI**I**TV and autologous tumour was measured through IFNγ release by flow cytometry.*

3.2.3. Designing candidate super-agonist peptides for T-cell clone ST8.24 using a combinatorial peptide library (CPL) screen

The combinatorial peptide library (CPL) screening data shown in **Figure 3.3** was used to design new candidate super-agonist peptides for ST8.24, the 'index' amino acid from the EAAGIGILTV sequence is highlighted in blue. Raw data from the CPL were entered into an online webtool available at (https://picpl.arcca.cf.ac.uk/loginform.php) where the algorithm was applied to the decamer peptide universe to determine and rank the optimal peptides for this clone (Szomolay *et al.*, 2016). Results are depicted in **Table 3.1**, where the amino acids differing from the index peptide are highlighted. The scope to design agonist peptides using a CPL screen is highlighted by the lack of conservation seen between the index peptide EAAGIGILTV and the peptides generated by the webtool. This result gives insight into the vast number of peptides that each CD8 T-cell can recognise through presentation on its corresponding HLA molecule.

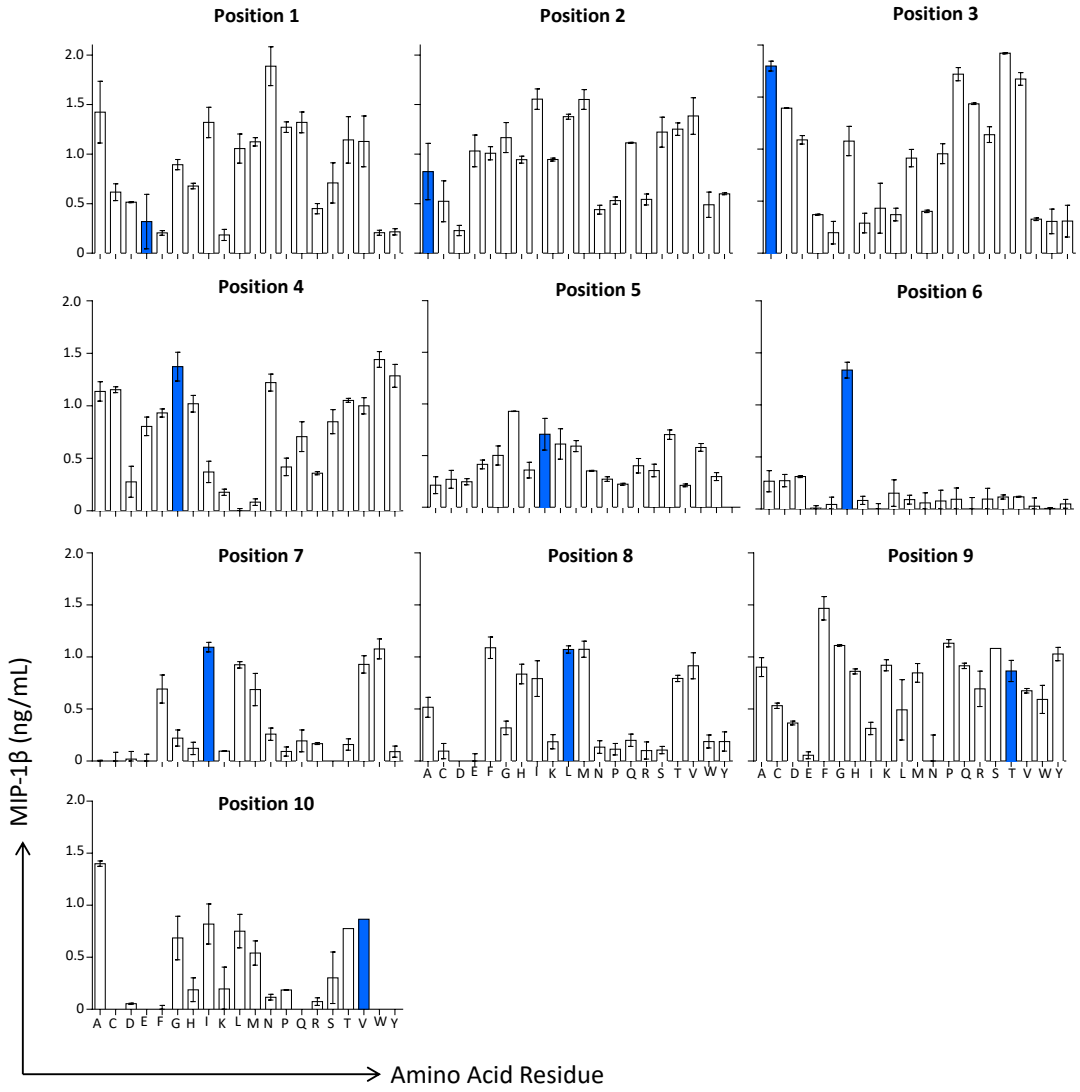


Figure 3.3: CPL screening of ST8.24.

6 x10^4 T2 APCs were pulsed for 2 hours with each mixture from a decamer CPL screen at 37 ^{o}C, after which 3 x10^4 T-cells were added and the assay incubated overnight. The following day, supernatants were harvested and assayed for MIP-1β production in an ELISA. Results are displayed as the average of 2 replicates, minus the value of T2s + T-cells with no peptide, error is shown as SD. The amino acid found in the index peptide EAAGIGILTV is highlighted in blue for each position. CPL screen carried out by Dr V Bianchi. Error is depicted as standard deviation (SD).

Rank	Amino Acid Sequence
Wild Type/Index	EAAGIGILTV
1	**ITSA**IG**V**L**P**V
2	**ITSA**IGIL**P**V
3	**MTSA**IG**V**L**P**V
4	**QTSA**IG**V**L**P**V
5	**MTSA**IGIL**P**V
6	**LTSA**IG**V**L**P**V
7	**ITS**GIG**V**L**P**V
8	**ITSA**IG**V**L**PI**
9	**QTSA**IGIL**P**V
10	**ITSA**IG**V**L**F**V

Table 3.1: Ten highest ranked agonist peptides from CPL.

3.2.4. Testing the activity of the top ten predicted super-agonist peptides

The top ten ranked agonists predicted from the ST8.24 CPL data shown in **Figure 3.3** were tested for T-cell activation by a MIP-1β ELISA. ST8.24 showed good sensitivity to all predicted agonist peptides (**Figure 3.4A**). In order to assess how other TCRs might respond to these peptides, further screening was undertaken using 3 additional melan-A restricted T-cell clones (**Figure 3.4B**). KTCR.32 and CR3 were single cell cloned from a polyclonal population isolated from healthy donor PBMC based on melan-A tetramer positivity. A hierarchy of recognition to the agonist peptides was identified based on the EC_{50} of the MIP-1β values from each clone (**Table 3.2**) and displayed as a heat bar graph where the darkest red spots indicate the greatest activation (**Figure 3.4C**). ST8.24 preferred peptide 4, **QTSA**IG**V**L**P**V whilst MEL13, KTCR.32 and CR3 preferred peptide 7 **ITS**GIG**V**L**P**V. Combining the EC_{50} of each clone for each peptide indicated that the preferred peptide overall was peptide 7, **ITS**GIG**V**L**P**V (**Figure 3.4C**).

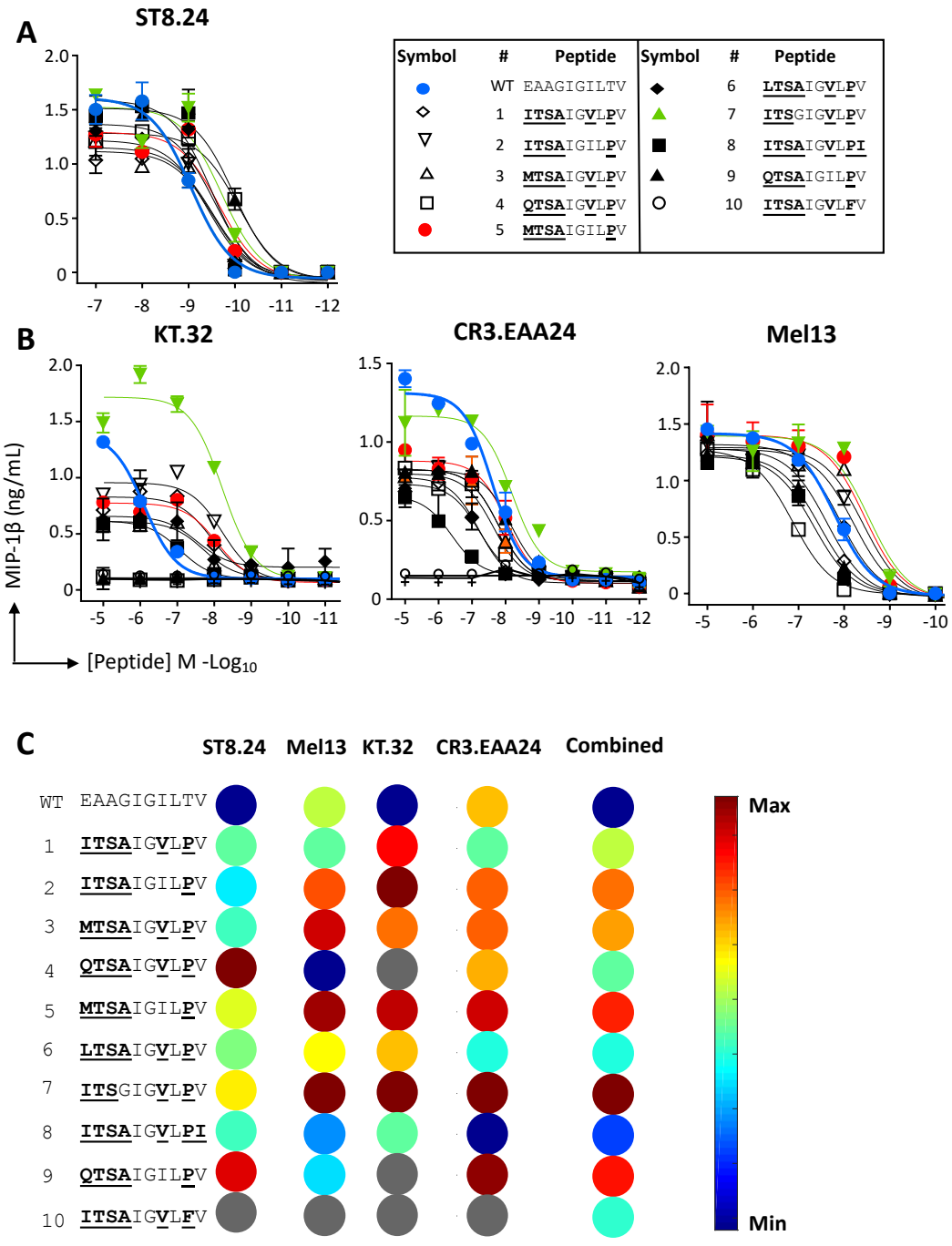


Figure 3.4: Screening of agonist peptides with melan-A-restricted CD8 T-cell clones identifies ITSGIGVLPV as a candidate super-agonist peptide for most melan-A T-cell clones.

*(**A**) 3 x10^4 T-cells (ST8.24) and 6 x10^4 T2 APCs were incubated overnight at 37 °C alongside candidate super-agonist peptides at a concentration range of 10^{-5} – 10^{-12} µM. The following day, supernatants were harvested and the MIP-1β production assessed via an ELISA. Results are shown as an average of 2 replicates with SD displayed. (**B**) Three additional clones (KT.32, CR3.EAA24, Mel13) were assessed for reactivity to the panel of candidate super-agonist peptides as above. For ELISA data, results are expressed as an average of 2 replicates. (**C**) EC$_{50}$ values of MIP-1β production in response to each candidate super-agonist peptide displayed as a heat map, where the darkest red colour indicates maximum response and grey indicates there was no response (EC$_{50}$ values can be found in **Table 3.2**).*

Peptide	ST8.24	CR3	CR32	MEL13
EAAGIGILTV	-9.06	-7.625	-6.111	-7.821
ITSAIG**V**L**P**V	-9.53	-7.22	-7.932	-7.657
ITSAIGIL**P**V	-9.42	-7.798	-8.221	-8.218
MTSAIG**V**L**P**V	-9.5	-7.798	-7.725	-8.411
QTSAIGVL**P**V	-10.08	-7.643	NR	-6.887
MTSAIGIL**P**V	-9.66	-8.042	-8.083	-8.488
LTSGIG**V**L**P**V	-9.56	-7.126	-7.547	-7.923
ITSGIG**V**L**P**V	-9.71	-8.2	-8.221	-8.563
ITSAIG**V**L**PI**	-9.5	-6.404	-7.08	-7.324
QTSAIGIL**P**V	-9.98	-8.17	NR	-7.454
ITSAIG**V**L**F**V	NR	NR	NR	NR

Table 3.2: EC_{50} of melan-A specific CD8 T-clones. *NR = No response*

3.2.5. Testing the potency of T-cell priming by ST8 super-agonist ligands

My ultimate aim was to design and test an optimal peptide for vaccination. Such a peptide would need to break tolerance to induce a robust melan A-specific T-cell response across all donors. The melan-A response was specifically chosen as a model system as it has been known for over 15 years that thymic selection in HLA A2+ individuals generates a large population of cells bearing TCRs that can response to this antigen in all individuals (Zippelius *et al.*, 2002a). The unusually high frequency of naïve T-cells that can respond to HLA A2-EAAGIGILTV is thought to arise due to a combination of recognition of this peptide is conferred by the germline-encoded CDR1 loop of the TRAV12-2 chain (Hsu *et al.*, 1996; Trautmann *et al.*, 2002; Dietrich *et al.*, 2003; Cole *et al.*, 2009b) and a failure to express melan-A full length mRNA in the thymus (Pinto *et al.*, 2014). As a consequence, VDJ rearrangements produce TCRs that can bind to HLA A2-EAAGIGILTV with a frequency hundreds of times higher than normal and central tolerance fails to delete these T-cells.

I next tested the ability of the ten peptides identified above in T-cell priming assays using cells from two healthy HLA A*0201 positive donors. Polyclonal CD8 T-cell populations isolated from both donors were primed for 14 days with the 10 candidate peptides, **FAT**GIGI**I**TV or the WT peptide in parallel. As the aim was to induce T-cells capable of recognising the natural EAAGIGILTV peptide presented by melanoma cells, priming success

was assessed using HLA A2-EAAGIGILTV tetramer staining (**Figure 3.5A**). Peptide 5, **MTSA**IGIL**P**V, elicited the most EAAGIGILTV-tetramer positive cells in both donors. Importantly, **MTSA**IGIL**P**V elicited more EAAGIGILTV specific cells than **FAT**GIGI**I**TV in both donors. I therefore decided to drop further study of the **FAT**GIGI**I**TV peptide. To confirm the superiority of **MTSA**IGIL**P**V, 3 additional donors were used. In all three donors **MTSA**IGIL**P**V elicited more EAAGIGILTV-tetramer positive cells compared to EAAGIGILTV (**Figure 3.5B**). In 4/5 donors **MTSA**IGIL**P**V primed the greatest magnitude of EAAGIGILTV-tetramer positive cells, in donor 3, **ITSA**IGIL**P**V elicited the most tetramer positive cells with **MTSA**IGIL**P**V second. Importantly, **MTSA**IGIL**P**V elicited more HLA A2-EAAGIGILTV-tetramer positive cells than **ITS**GIG**V**L**P**V in all donors. Taken together this data highlighted **MTSA**IGIL**P**V as the optimal super-agonist peptide for T-cell priming of EAAGIGILTV-specific T-cells across all donors tested.

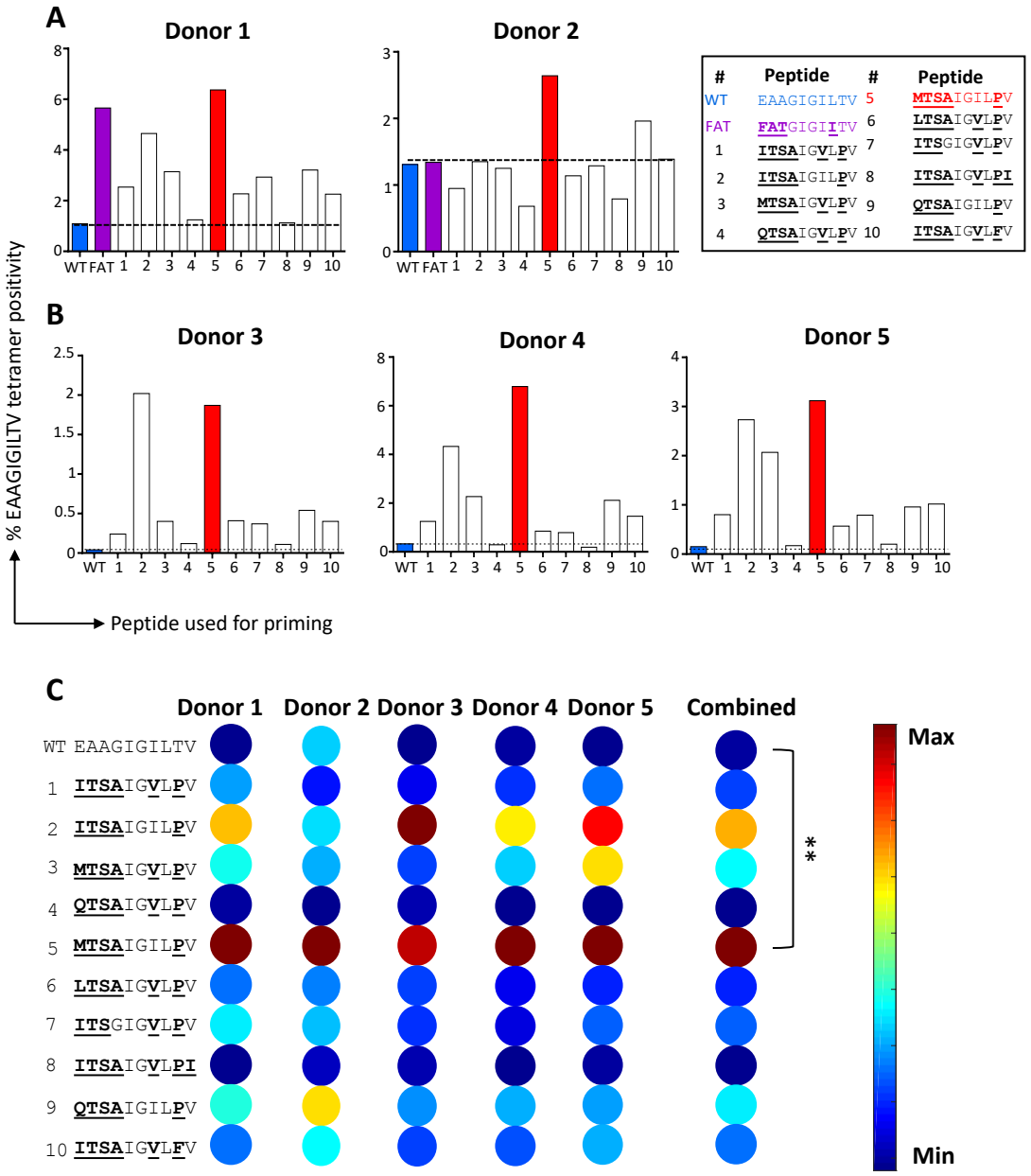


Figure 3.5: MTSAIGILPV is the optimal super-agonist peptide for priming T-cells across five different HLA A2+ donors.

*Polyclonal CD8 T-cells isolated from healthy donors were incubated for 14 days with their autologous irradiated PBMC pulsed for 1 h with each candidate peptide. (**A**) Initial screening with 2 healthy donors, peptides included EAAGIGILTV, **FA**T**G**II**T**V and the 10 candidate peptides. (**B**) A further 3 healthy donors screened with EAAGIGILTV and the 10 candidate peptides. For priming experiments 25 µM of peptide was used. Results are expressed as histograms of the percentage of EAAGIGILTV-tetramer positive cells elicited by each peptide. (**C**) Percentage of EAAGIGILTV-tetramer positive cells generated in each donor by each peptide collated and displayed as a heat map, where the darkest red spots indicate the greatest percentage of EAAGIGILTV-tetramer positive cells. (Paired T-test, ** ≤0.001).*

3.2.6. Validation of optimal super-agonist peptides for T-cell priming

Since previously identified agonist peptide **FAT**GIGI**I**TV failed to induce more EAAGIGILTV-tetramer positive cells in all donors (Ekeruche-Makinde et al., 2012) it was important to test **MTSA**IGIL**P**V with many different donors. CD8 T-cells isolated from seven additional healthy donor PBMC were primed with **MTSA**IGIL**P**V or EAAGIGILTV for 14 and 28 days and the magnitude of EAAGIGILTV-tetramer positive cells assessed. Representative flow plots are shown for Donor 12 in **Figure 3.6A**. Plots for all donors are shown in **Figure 3.6B**. After 14 days, **MTSA**IGIL**P**V primed polyclonal populations had a significantly greater magnitude of EAAGIGILTV-specific CD8 T-cells than the EAAGIGILTV-primed populations (** ≤ 0.001). The trend continued after 28 days, where the **MTSA**IGIL**P**V-primed populations had significantly more EAAGIGILTV-tetramer positive cells than the EAAGIGILTV-primed populations. Furthermore, after 28 days the number of tetramer positive cells in the **MTSA**IGIL**P**V-primed population significantly increased compared to the **MTSA**IGIL**P**V-primed population after 14 days (**Figure 3.6B**), corresponding flow plots can be found in **Appendix figure 3 and 4**. Collectively, these data and data from **section 3.2.5** demonstrate that **MTSA**IGIL**P**V primed more EAAGIGILTV-tetramer positive cells than EAAGIGILTV in 12/12 donors tested. It was not possible to undertake experiments for time points longer than 28 days as T-cells began to die in the absence of further stimulation.

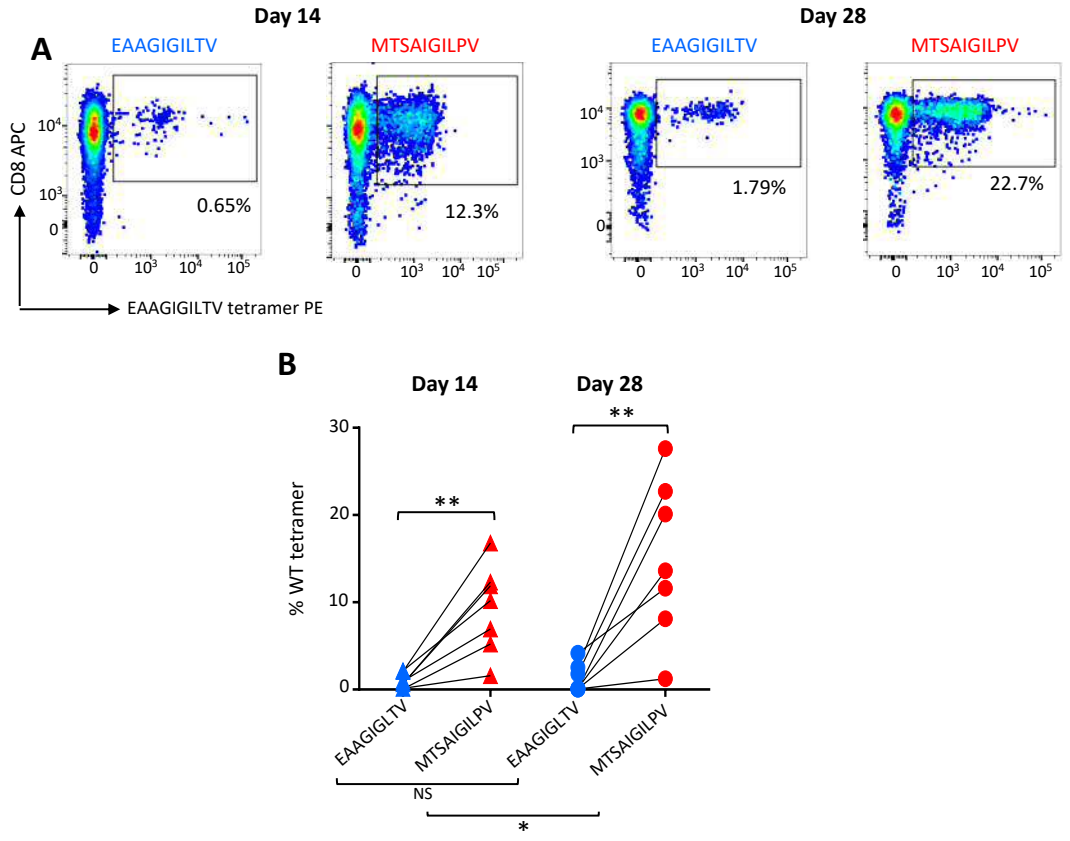


Figure 3.6: Priming of EAAGIGILTV-specific T-cells with MTSAIGILPV super-agonist peptide in multiple donors.

*(**A**). Representative flow cytometry plots showing tetramer staining of polyclonal CD8 T-cells isolated from healthy donor PBMC primed with EAAGIGILTV or **MTSA**IGIL**P**V for 28 days. The percentage of EAAGIGILTV-tetramer positive cells is shown. (**B**) Polyclonal T-cells from 7 additional donors were primed with EAAGIGILTV or **MTSA**IGIL**P**V peptides for 28 days. EAAGIGILTV-tetramer positivity was assessed on day 14 and day 28, using an optimised tetramer staining protocol (protein kinase inhibitor (PKI) + tetramer + 1^{o} PE antibody* (Tungatt *et al.*, 2015)*). Associated flow cytometry plots in **appendix figure 3 and 4**. (** ≤ 0.001 – paired T-test).*

The results in **Figure 3.6** suggest that either the **MTSA**IGIL**P**V peptide primed EAAGIGILTV-specific T-cells that cannot be primed with the natural sequence or that **MTSA**IGIL**P**V-primed cells exhibit higher levels of proliferation. In order to formally test for the latter possibility, proliferation of **MTSA**IGIL**P**V-, EAAGIGILTV- and E**L**AGIGILTV-primed CD8 T-cells from healthy donors was assessed using carboxyfluorescein succinimidyl ester (CFSE) staining. The heteroclitic peptide E**L**AGIGILTV was included as it was designed to be a good binder to HLA A2 and induced more EAAGIGILTV-tetramer positive cells than EAAGIGILTV *in vitro* (Valmori, et al., 1998). A population of CD8 T-cells was also not primed with peptide as a control, the

values for this population were subtracted from the peptide primed populations. **MTSA**IGIL**P**V-primed populations showed greater proliferation over 28 days in comparison to EAAGIGILTV and E**L**AGIGILTV -primed populations in 4 healthy donors tested (**Figure 3.7**).

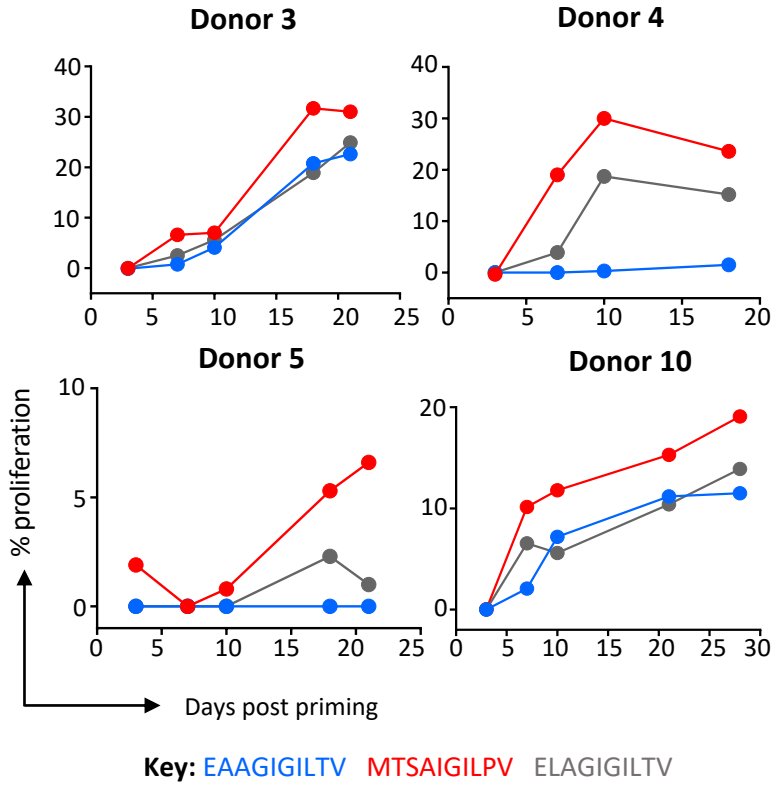


Figure 3.7: MTSAIGILPV-primed CD8 T-cells proliferate more than EAAGIGILTV-primed cells

*CFSE labelling was used to assess proliferation of CD8 T-cells from healthy donors 3,4, 5 and 10 primed with either EAAGIGILTV, EL**A**GIGILTV or **MTSA**IGIL**P**V peptides for a maximum of 28 days. Proliferation was assessed at multiple time points though flow cytometric staining (LIVE/DEAD VIVID + surface markers CD8 APC and CD3 PerCP). Proliferation from a DMSO primed control (no peptide) was subtracted to give the final values.*

Finally, in order to be confident that **MTSA**IGIL**P**V was the optimal super-agonist peptide from my panel of ten super-agonists shown in table 2.2, the percentage of EAAGIGILTV-tetramer positive cells elicited by **MTSA**IGIL**P**V in healthy donors was compared to that induced by the best candidate identified for activation of T-cell clones, **ITS**GIG**V**L**P**V (**Figure 3.8**). The same healthy donors used in **Figure 3.6** were primed with **ITS**GIG**V**L**P**V and the percentage of EAAGIGILTV-tetramer positive cells assessed. In all seven donors tested the super-agonist peptide **MTSA**IGIL**P**V elicited more EAAGIGILTV-tetramer positive cells than EAAGIGILTV or **ITS**GIG**V**L**P**V peptides (**Figure 3.8**). The corresponding flow cytometry plots can be found in **Appendix figure 2**.

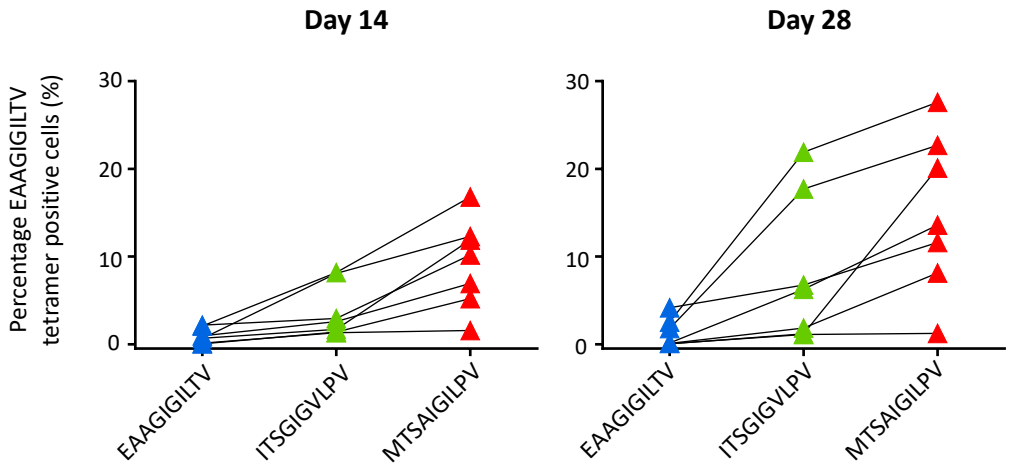


Figure 3.8: MTSAIGILPV elicits a greater magnitude of EAAGIGILTV-tetramer positive cells than ITSGIGVLPV in all donors tested

*CD8 T-cells isolated from seven healthy donors were primed for 28 days with EAAGIGILTV, **MTSA**IGIL**P**V or **ITS**GIG**V**L**P**V peptides. At day 14 and day 28 post-priming, CD8 T-cell populations were stained with EAAGIGILTV-tetramer using an optimised protocol (PKI + tetramer + 1° PE antibody), and the number of EAAGIGILTV-tetramer positive cells ascertained. Results are expressed as the percentage of HLA A2-EAAGIGILTV tetramer positive cells. Samples from the same donor are linked by a line. Associated flow cytometry plots can be found in **appendix figure 2**.*

Overall, there is comprehensive evidence to suggest that **MTSA**IGIL**P**V is the superior super-agonist peptide from the panel identified through the CPL screen of CD8 T-cell clone ST8.24.

3.2.7. Killing of melanoma cell lines by primed healthy donor CD8 T-cells

It was important at this stage to investigate whether **MTSA**IGIL**P**V not only elicits a greater magnitude of EAAGIGILTV-tetramer positive CD8 T-cells compared the EAAGIGILTV but whether these cells had an equal or increased capacity to eliminate melanoma cells. EAAGIGILTV-specific CD8 T-cells are often poor at lysing melanoma. This may be because melanocytes express low levels of melan-A so it is possible that tolerance mechanisms operate for this antigen when expressed at normal levels.

CD8 T-cells from healthy donors, 11 and 14, were isolated from PBMC, primed with EAAGIGILTV or **MTSA**IGIL**P**V peptides and stained with EAAGIGILTV-tetramer, as previously described. The frequencies of EAAGIGILTV-tetramer positive cells in each primed population are indicated as a percentage in **Figure 3.9**. In healthy donor 11 **MTSA**IGIL**P**V primed 4.96% EAAGIGILTV-tetramer positive cells compared to 0.046% primed by EAAGIGILTV peptide. In

healthy donor 14, **MTSA**IGIL**P**V primed 8.57% EAAGIGILTV-tetramer positive cells compared to 4.83% in the EAAGIGILTV primed population (**Figure 3.9A**).

Lysis of melanoma tumour MM909.24 by CD8 T-cells from donor 11 was measured through a chromium release assay, **MTSA**IGIL**P**V-primed cells killed a greater percentage of melanoma tumour than the EAAGIGILTV-primed CD8 T-cells (**Figure 3.9B**). Killing of melanoma tumours FM79 and MM909.24 by CD8 T-cells primed from healthy donor 14 was assessed by a flow cytometry-based killing assay, in this case killing of HLA A2 negative control FM79 and MM909.24 tumour cells was subtracted to give the final percentage killing. **MTSA**IGIL**P**V-primed cells from donor 14 killed more MM909.24 and FM79 tumour cells than EAAGIGILTV-primed cells and CD8 T-cells primed with no peptide (**Figure 3.9C**).

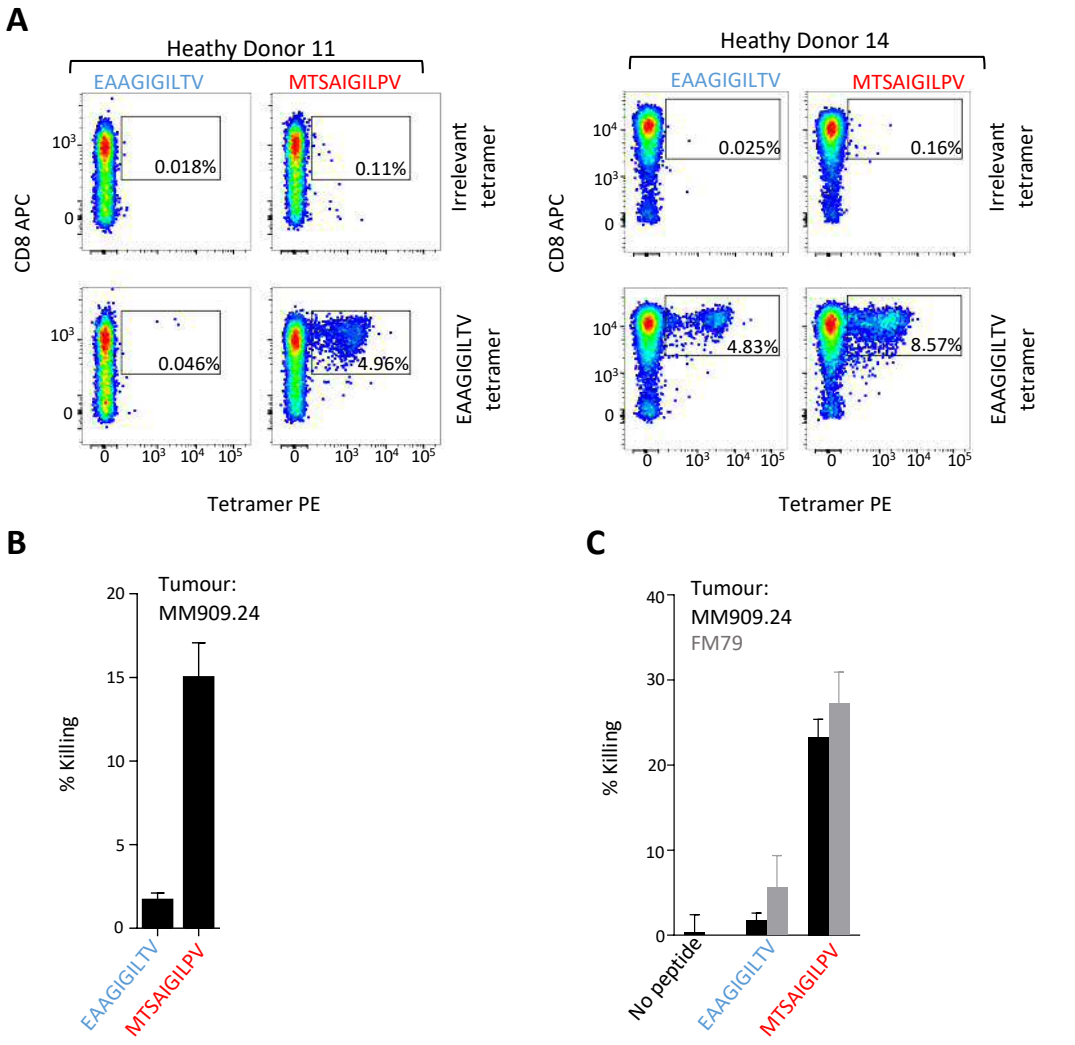


Figure 3.9: Heathy donor CD8 T-cells primed with MTSAIGILPV exhibit improved killing of melanoma cells compared to EAAGIGILTV-primed CD8 T-cells.

*(**A**) CD8 T-cells isolated from healthy donors 11 and 14, primed with EAAGIGILTV or **MTSA**IGIL**P**V peptides for 28 days and subsequently stained with EAAGIGLTV tetramer or ALWGPDPAAA tetramer (used here as an irrelevant, derived from pre proinsulin) using an optimised protocol (PKI + tetramer + 1^{o} PE antibody). Percentage of tetramer positive cells are shown. (**B**) CD8 T-cells from donor 11 were co-incubated with chromium labelled-MM909.24 tumour cell line overnight. Chromium-release was measured the following day from the harvested supernatants. Results are expressed as percentage killing relative to maximum chromium release. Results are an average of 2 values with the standard deviation from the mean shown. (**C**) CD8 T-cells from donor 14 were incubated with MM909.24 (A2+/) (black bars) and FM79 (A2+/-) (grey bars) melanoma tumour cell lines overnight at 37 ^{o}C. The following day, CFSE-labelled cells were added, and all cells stained with LIVE/DEAD VIVID (to allow exclusion of dead cells), CD8 APC and CD3 PerCP (to identify the CD8 T-cells) and samples were acquired by flow cytometry. The absolute number of tumour cells remaining in the assay was quantified using the CFSE-labelled cells as a constant. Killing of HLA A2- tumour cells was subtracted from the HLA A2+ killing to give a final percentage killing value. The assay was carried out in triplicate and results expressed as an average, with the SD shown.*

3.2.8. Phenotypic analysis of EAAGIGILTV and MTSAIGILPV primed CD8 T-cells

The phenotype of EAAGIGILTV and **MTSA**IGIL**P**V primed cells was assessed, to see if priming polyclonal CD8 T-cells with **MTSA**IGIL**P**V induced a different subset of CD8 T-cell which could explain the improved functional capacity of these cells. Polyclonal CD8 T-cells were primed for 28 days and the percentage of EAAGIGILTV-tetramer positive cells determined (**Figure 3.10**). The phenotype of the tetramer positive cells from each peptide primed population was evaluated with the conventional CD8 T-cell phenotyping markers; CD45RO, CD45RA, CCR7 and CD27. There was no difference in the phenotype of the cells primed by either peptide with a large proportion of the EAAGIGILTV and **MTSA**IGIL**P**V primed populations having a CD45RA+, CD45RO-, CCR7+, CD27+ phenotype (**Figure 3.10**). The notable features of **MTSA**IGIL**P**V-primed cells, increased proliferative capacity and superior anti-tumour responses, are indicative of stem cell memory (T_{SCM}) cells (Gattinoni *et al.*, 2011). T_{SCM} cells have a similar phenotype to naïve cells but express large amounts of CD95, IL-2Rβ, CXCR3 and LFA-1 (Gattinoni *et al.*, 2011). Further investigation would be required to determine whether **MTSA**IGIL**P**V-primed cells have this distinct phenotype.

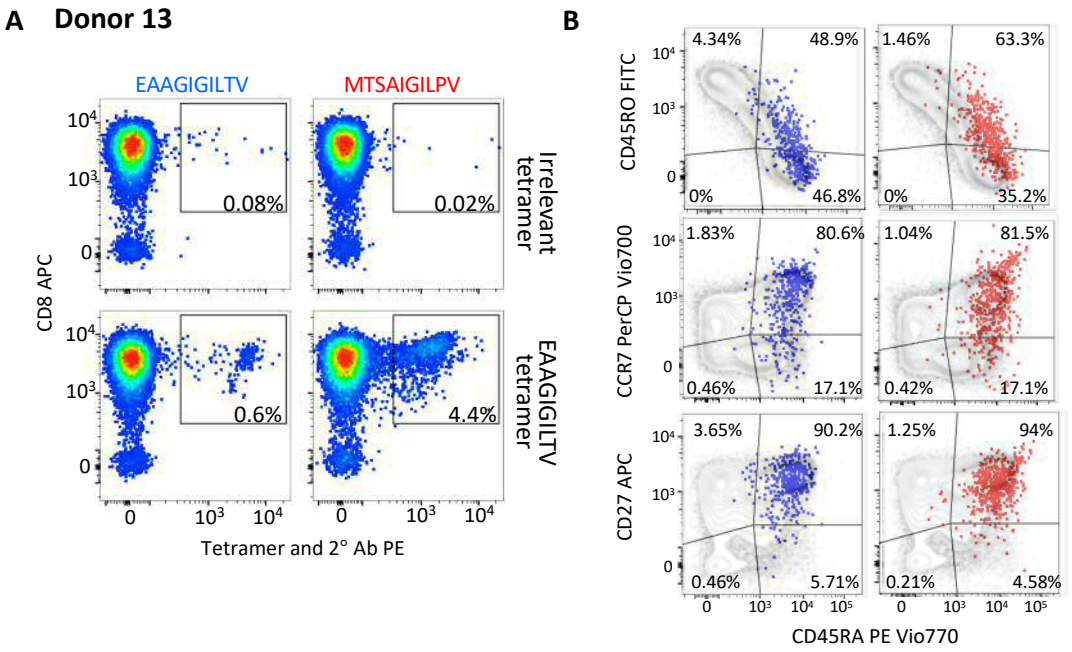


Figure 3.10: MTSAIGILPV and EAAGIGILTV-primed cells have a similar phenotype.

*CD8 T-cells from healthy donor #13 were primed with EAAGIGILTV (blue) or **MTSA**IGIL**P**V (red) peptides for 28 days. (**A**) Primed CD8 T-cells were assessed for melan-A specificity through EAAGIGILTV-tetramer staining using an optimised protocol (PKI + tetramer + 1° anti-PE antibody + 2° PE antibody), irrelevant tetramer ALWGPDPAAA (peptide derived from preproinsulin) was included as a control. Results are expressed as the percentage of EAAGIGILTV-tetramer positive CD8 T-cells. (**B**) CD8 T-cells from the same donor primed with EAAGIGILTV or **MTSA**IGIL**P**V used for phenotypic analysis. Cells shown are gated on EAAGIGILTV-tetramer positivity. Fluorescence minus one controls were used to set the gates. CD8 T-cells isolated from healthy PBMC were used as a control and can be seen in grey. The number of cells residing in each quadrant is displayed as a percentage.*

3.2.9. MTSAIGILPV shows improved binding to HLA A2 compared to EAAGIGILTV

The next question was why does the **MTSA**IGIL**P**V peptide exhibit a superior ability to induce EAAGIGILTV-specific T-cells? The obvious possibilities are that the **MTSA**IGIL**P**V peptide exhibits an enhanced interaction with HLA A2, EAAGIGILTV-specific TCRs or both. Peptide binding was tested using HLA A2+ T2 cells which are deficient in transporter associated with antigen processing (TAP) and therefore fail to process and present most endogenous peptides (Hosken and Bevan, 1990). As a result of the TAP deficiency, the HLA A2 expressed at the surface of T2 cells is largely unstable and rapidly turned over resulting in low levels of HLA A2 at the cell surface. Addition of peptides that bind to HLA A2 stabilizes the HLA at the T2 cell surface allowing peptide binding to the assessed using an fluorochrome-conjugated HLA A2-specific antibody in conjunction with flow cytometry. This assay was used to show

that **MTSA**IGIL**P**V exhibited improved binding to HLA A2 compared to EAAGIGILTV and bound similarly to E**L**AGIGILTV, a peptide designed to be an optimal binder to HLA A2 (Valmori *et al.*, 1998) (**Figure 3.11**).

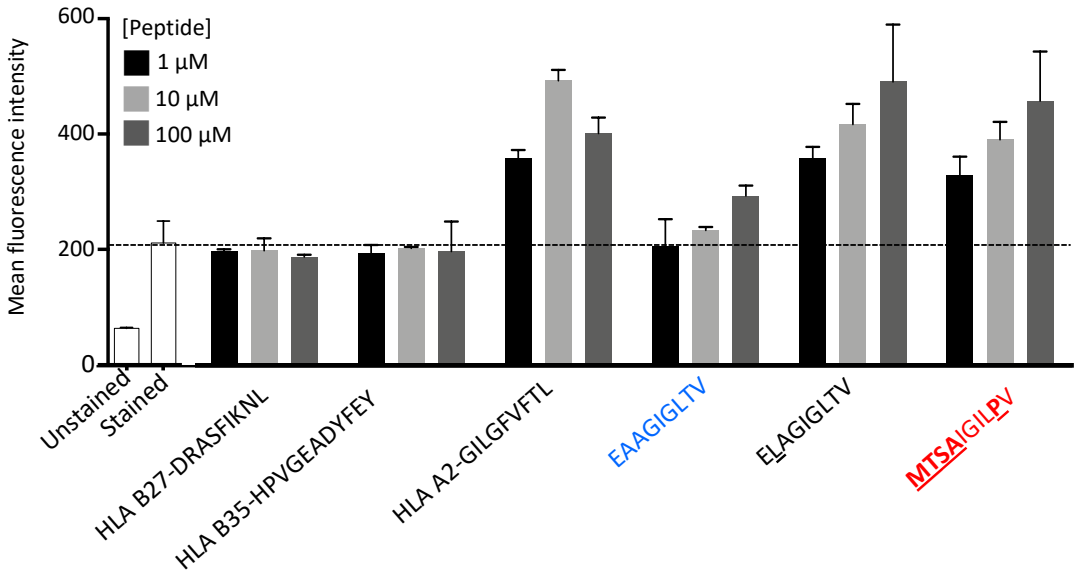


Figure 3.11: Super-agonist peptide MTSAIGILPV exhibits improved binding to HLA A2 compared to the EAAGIGILTV wild-type peptide sequence.

*2 x10^4 – 1 x10^6 T2 APCs were plated overnight at RT in serum-free media including peptides of interest (EAAGIGILTV, **MTSA**IGIL**P**V, E**L**AGIGILTV) at concentrations 1, 10 and 100 µM. HLA A2 binding peptide (GILGFVFTL) and non-HLA A2 binding peptides (DRASFIKNL and HPVGEADYFEY) were included as controls. The following day, cells were stained with HLA A2 FITC antibody and LIVE/DEAD VIVID and acquired by flow cytometry. Assay was carried out in duplicate. Results are displayed as the mean fluorescence intensity (MFI) of HLA A2 FITC. Standard deviation is shown. Complementary flow cytometry histograms are displayed in **Appendix figure 5.***

3.2.10. Clonotypic analysis of EAAGIGILTV and MTSAIGILPV primed CD8 T-cells

As **MTSA**IGIL**P**V-primed CD8 T-cells appeared to be more effective than those primed by the EAAGIGILTV natural sequence while exhibiting a similar phenotype, I next hypothesised that T-cell primed with the super-agonist ligand might bear different TCRs that enabled them to exhibit improved recognition of melanoma cells. Previous infection-based studies have demonstrated that different T-cell clonotypes that recognise the same peptide need not be equally effective (Alexander-Miller, Leggatt and Berzofsky, 1996). Other studies have also suggested that enhancing the quality of the TCRs at the clonotypic level might be more

important than increasing the overall magnitude of a T-cell response (Seder, Darrah and Roederer, 2008; Bangham, 2009a; Ekeruche-Makinde *et al.*, 2012).

CD8 T-cells isolated from the PBMC of healthy donor 9, primed with EAAGIGILTV and **MTSA**IGIL**P**V peptides were sorted based on EAAGIGILTV-tetramer positivity after 28 days. The EAAGIGILTV-tetramer positive cells were then subjected to TCR clonotyping. Priming healthy CD8 T-cells primed with **MTSA**IGIL**P**V elicited almost completely different TCRs compared to priming with EAAGIGILTV (**Figure 3.12**). Only two TRAV CDR3, and one TRBV CDR3 were shared between EAAGIGILTV and **MTSA**IGIL**P**V primed populations (**Figure 3.12A**), and all of these appeared at a low frequency. There was a strong bias towards TRAV 12-2 gene expression in the TCR-α chain in both peptides primed populations. This TRAV12-2 bias of the EAAGIGILTV-specific responses was expected and has been reported before (Trautmann *et al.*, 2002; Dietrich *et al.*, 2003). There is little overlap in the TRBV genes with the EAAGIGILTV-primed population showing a strong bias towards TRBV 20-1 and the **MTSA**IGIL**P**V-primed population having a large TRBV 7-4, and a small TRBV 20-1 population in their TCR-β chains (**Figure 3.12B**).

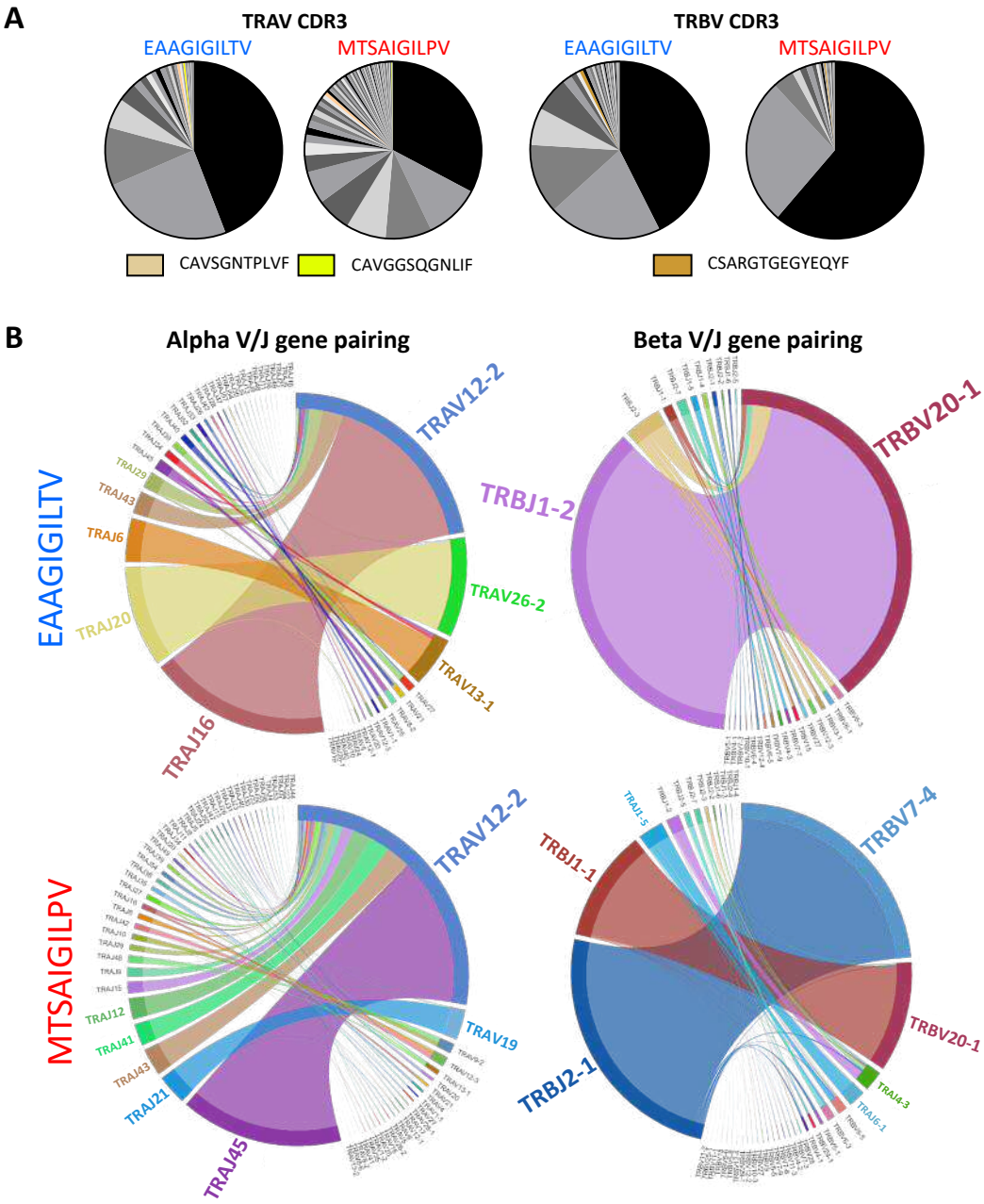


Figure 3.12: Priming CD8 T-cells with EAAGIGILTV and MTSAIGILPV elicits different T-cell clonotypes

*(**A**) CDR3 analysis of alpha (left) and beta TCR chains (right) from donor 9 CD8 T-cells bulk cell sorted on EAAGIGILTV-tetramer positivity, where each segment of the pie represents a distinct clonotype. Shared CDR3 sequences between the two priming conditions (**MTSA**IGIL**P**V or EAAGIGILTV peptide) are highlighted. (**B**) TCR variable (V) and joining (J) gene pairing analysis of alpha (left) and beta TCR genes (right), from donor 9 CD8 T-cells primed with **MTSA**IGIL**P**V or EAAGIGILTV and single cell sorted on EAAGIGILTV tetramer positivity. Dominant gene pairings highlighted in bold. Thanks to M. Attaf for carrying out the clonotyping. Clonotyping data was visualised using Circos plotting software* (Krzywinski *et al.*, 2009).

3.2.11. EAAGIGILTV-specific CD8 T-cells bind to MTSAIGILPV-tetramer with greater fluorescent intensity

I next sought to examine how TCRs bound to EAAGIGILTV and **MTSA**IGIL**P**V peptide using pMHC tetramers with each specificity. Previous studies have demonstrated that staining with pMHC multimers is dependent on the TCR-pMHC affinity (Laugel *et al.*, 2007; Tungatt *et al.*, 2015; Rius *et al.*, 2018). TCRs with low affinity for their cognate antigen often fail to produce a robust immune response (Schmid *et al.*, 2010). I first examined staining of EAAGIGILTV-specific T-cell clones with HLA A2-EAAGIGILTV and HLA A2-**MTSA**IGIL**P**V tetramers. Melan-A-specific CD8 T-cell clones; ST8.24, MEL13, MANUELA, CACTUS and EDDY, all stained with HLA A2-**MTSA**IGIL**P**V tetramers with a higher intensity than HLA A2-EAAGIGILTV tetramer in parallel assays (**Figure 3.13A**). Accordingly, **MTSA**IGIL**P**V was a stronger agonist for each clone than EAAGIGILTV in T-cell activation assays (**Figure 3.13A**).

I next examined staining of polyclonal CD8 T-cell lines from 2 healthy donors primed with **MTSA**IGIL**P**V. While each tetramer stained a similarly sized population of CD8 T-cells, the intensity of staining was brighter with the HLA A2-**MTSA**IGIL**P**V-tetramer (**Figure 3.13B**). Overall, the MFI was significantly greater when staining with the **MTSA**IGIL**P**V tetramer in all examples (clones and polyclonal donors) (**Figure 3.13C**). Collectively, these results indicate that the **MTSA**IGIL**P**V peptide has a higher affinity for a wide range of EAAGIGILTV-specific T-cell clonotypes explaining why it might act as a general super-agonist peptide for T-cells with this specificity.

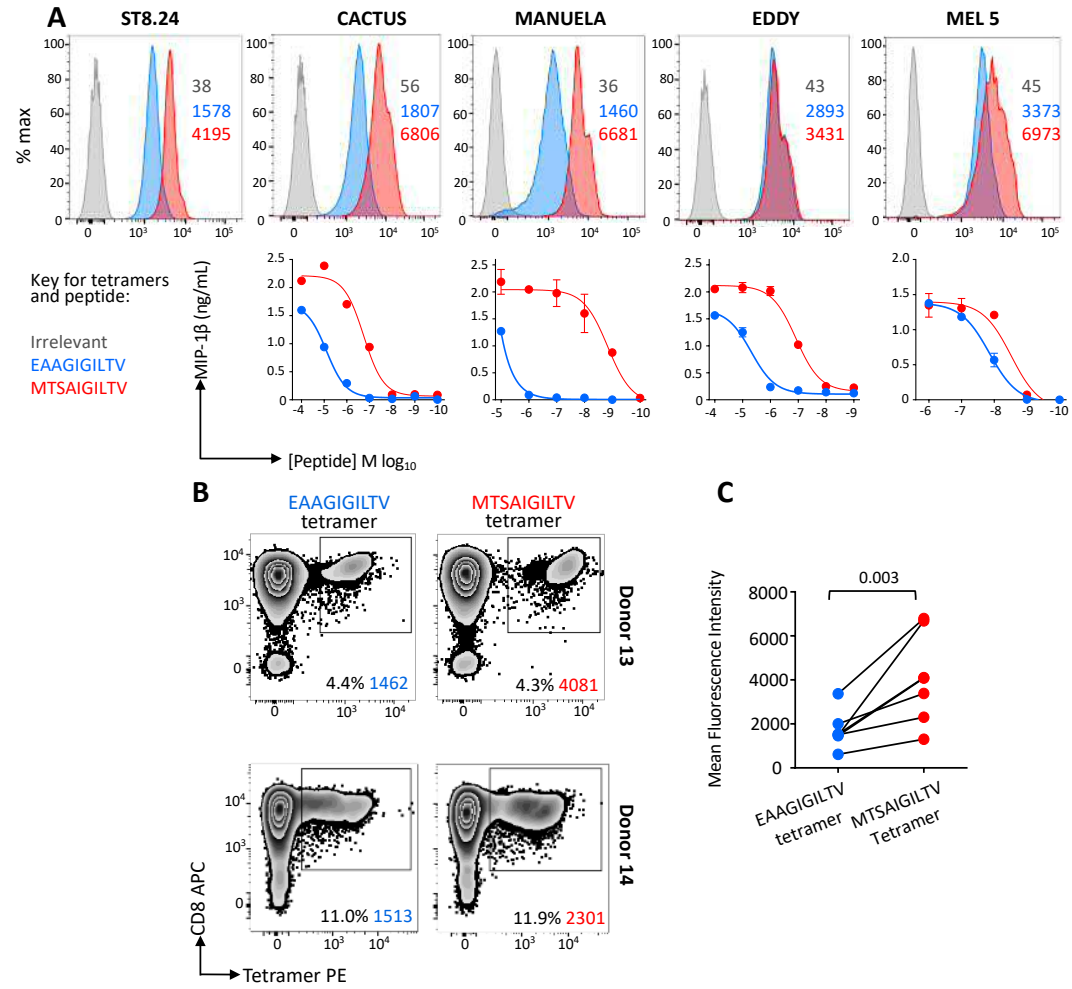


Figure 3.13: EAAGIGILTV-specific T-cells bind HLA A2-MTSAIGILPV tetramers with increased florescent intensity.

*(**A**) Five melan-A restricted CD8 T-cell clones were stained with ALWGPDPAAA (an irrelevant peptide derived from preproinsulin), EAAGIGILTV or **MTSA**IGIL**P**V tetramers using an optimised protocol (PKI + tetramer + 1° PE antibody). The mean fluorescence intensity (MFI) of PE-tetramer is indicated. Reactivity of each CD8 T-cell clone to EAAGIGILTV and **MTSA**IGIL**P**V peptides is shown in the corresponding line graphs, assessed by MIP-1ß ELISA after overnight activation. (**B**) CD8 T-cells isolated from healthy donors 13 and 14 were primed for 28 days with EAAGIGILTV peptide and subsequently stained with EAAGIGILTV or **MTSA**IGIL**P**V tetramers using an enhanced optimised staining protocol (PKI + tetramer + 1° anti-PE antibody + 2° anti-PE antibody). Percentage of tetramer positive cells is shown, alongside the MFI of PE-tetramer. (**C**) Accumulated MFI data from five CD8 T-cell clones and 2 polyclonal CD8 T-cell populations stained with EAAGIGILTV and **MTSA**IGIL**P**V tetramer. The difference in MFI between EAAGIGILTV and **MTSA**IGIL**P**V tetramer staining is statistically significant (P=0.003, paired one-tailed test).*

3.2.12. MTSAIGILPV is a structural mimic of EAAGIGILTV when bound to HLA A2

In order to study the binding of **MTSA**IGIL**P**V to HLA A2 in further detail the structure of **MTSA**IGIL**P**V bound to HLA A2 was resolved (**Table 3.3** and **Figure 3.14**). Structural analysis of peptide-MHC complexes can reveal intricate details about how a peptide binds to a given MHC and I was hoping that resolving the structure would elude to why **MTSA**IGIL**P**V displayed improved binding to HLA A2 and EAAGIGILTV-specific TCRs.

Upon first look, it was not clear why **MTSA**IGIL**P**V displayed improved binding to HLA A2 compared to EAAGIGILTV since at position 2 **MTSA**IGIL**P**V does not possess the leucine (L), methionine (M) or isoleucine (I) classically associated with improved HLA A2 binding. Structural analysis revealed that the number of contacts each peptide made with HLA A2 molecule was similar (134 EAAGIGILTV and 136 **MTSA**IGIL**P**V), but that the threonine (T) of **MTSA**IGIL**P**V protruded deeper into the B pocket of the HLA compared to the alanine (A) of EAAGIGILTV (**Figure 3.14**). This binding motif may allow for more stable binding of **MTSA**IGIL**P**V to HLA A2, equating for the more immunogenic properties of this peptide.

Even with **MTSA**IGIL**P**V and EAAGIGILTV differing by 5/10 amino acids, the structural analysis revealed that both peptides possess similar patterns of buried and exposed side chains, with residues 1 and 8 protruding away from the HLA providing TCR contact sites and residues 4,5 and 6 forming the outward facing central bulge. Despite the large variation in sequence, **MTSA**IGIL**P**V and EAAGIGILTV are close structural mimics when bound to HLA A2 (root mean square deviation = 0.38 Å), with both peptides having virtually identical backbone conformation. (**Figure 3.14**). This structurally mimicry provides a molecular basis as to why T-cells primed with **MTSA**IGIL**P**V can recognise the EAAGIGILTV peptide.

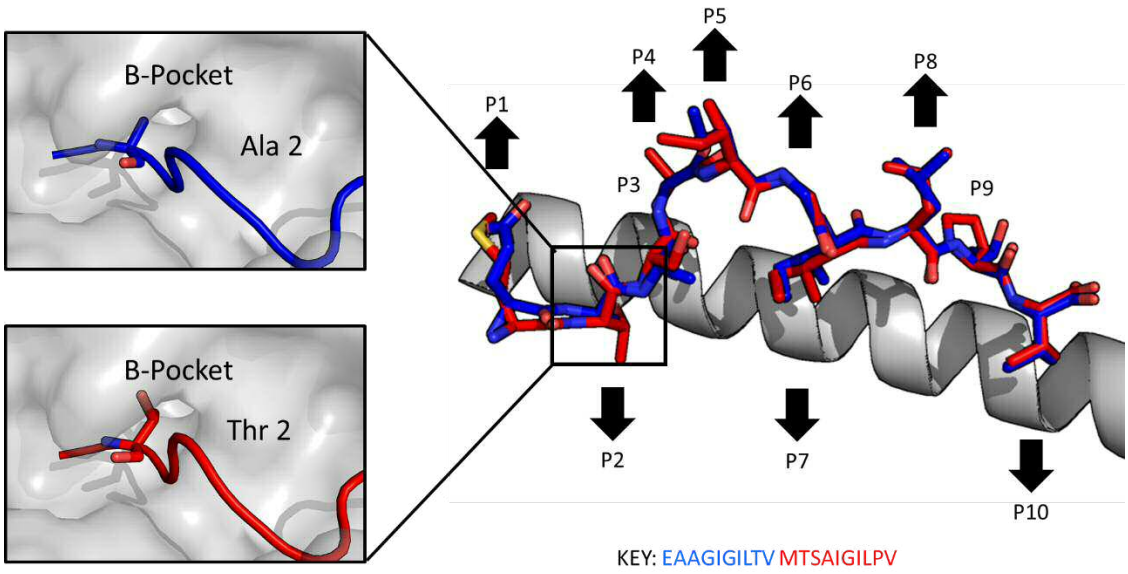


Figure 3.14: MTSAIGILPV is a structural mimic of EAAGIGILTV when bound to HLA A2.

*Structural analysis of the HLA A2-**MTSA**IGIL**P**V complex compared to HLA A2-EAAGIGILTV. The HLA α1 helix is shown as grey cartoon with the **MTSA**IGIL**P**V (red sticks) and EAAGIGILTV (blue sticks) superimposed. Black arrows demonstrate the upward facing solvent exposed and downward facing buried residues in each peptide. Boxes to the left show the interaction between the HLA A2 B-pocket (grey surface) and EAAGIGILTV (blue cartoon and sticks) and **MTSA**IGIL**P**V (red cartoon and sticks). Structural data provided by Aaron Wall.*

PDB code	*6G3J*
Data collection	
DLS Beamline	I24
Space group	C 1 2 1
Wavelength (Å)	0.96859
Cell Dimensions	
a, b, c *(Å)*	202.94, 50.28, 119.07
A, ß, γ (°)	90.0, 122.86, 90.0
Resolution (Å)	2.45-41.05
Outer Shell (Å)	2.51-2.45
R_{merge} *(%)*	10.4 (115.5)
R_{meas} *(%)*	12.3 (135.4)
CC1/2	0.993 (0.594)
I /σ I	9.1 (1.0)
Completeness *(%)*	98.7 (99.3)
Multiplicity	3.5 (3.6)
Unique reflections	37, 034 (2,764)
Wilson B Factor (Å²)	61.8
Refinement	
R-work reflections	35,216
R-free reflections	1,818
R_{works}/R_{free}	22.3/27.0
R.m.s. deviations	
Bond lengths (Å)	0.018
Bond angles (°)	1.9
Coordinate Error	0.331
Mean B value (Å²)	84.8
Ramachandran Statistics	
Favoured/Allowed/Outliers	725/27/5
(%)	95.8/3.6/0.7

Table 3.3: Refinement statistics

3.2.13. Increased killing of autologous tumour by MTSAIGILPV -primed CD8 T-cells in a metastatic melanoma patient

An important assessment of the clinical relevance of **MTSA**IGIL**P**V as a super-agonist peptide was to investigate the potency of T-cells primed with this peptide from samples taken from metastatic melanoma patients. Previously generated altered peptide ligand E**L**AGIGILTV failed to cause any measurable tumour regression in a clinical setting (Jäger *et al.*, 2002; Bins *et al.*, 2007; Filipazzi *et al.*, 2012) and **F**A**T**GIGI**I**TV was never tested on patient samples (Ekeruche-Makinde *et al.*, 2012).

PBMC, taken before TIL therapy, and autologous melanoma tumour were obtained from metastatic melanoma patients MM909.37 (Andersen *et al.*, 2016) and MM1413.12 (R Andersen *et al.*, 2018), kindly provided by the CCIT, Copenhagen. Patient MM909.37 received TIL therapy but succumbed to disease whilst patient MM1413.12 experienced a partial response to TIL therapy and subsequently had the remainder of their tumour surgically resected; this patient remains disease-free. CD8 T-cells from both patients were isolated and primed with EAAGIGILTV and **MTSA**IGIL**P**V for 28 days, as previously described. The magnitude of EAAGIGILTV-specific CD8 T-cells in the polyclonal populations from each patient was measured using tetramer staining. In both patients, the **MTSA**IGIL**P**V population had a greater frequency of EAAGIGILTV-tetramer specific CD8 T-cells than the EAAGIGILTV primed populations in accordance with results from healthy donor samples (MM909.37 0.06% and 0.8% and MM1413.12 0.029% and 2.62% respectfully) (**Figure 3.15A**). CD8 T-cells from patient MM909.37 primed with **MTSA**IGIL**P**V displayed enhanced killing of autologous tumour, compared to EAAGIGILTV primed cells after 4 and 8 hours of co-incubation (**Figure 3.15B**). Unfortunately, there was not enough sample remaining from patient MM1413.12 to carry out any further analysis.

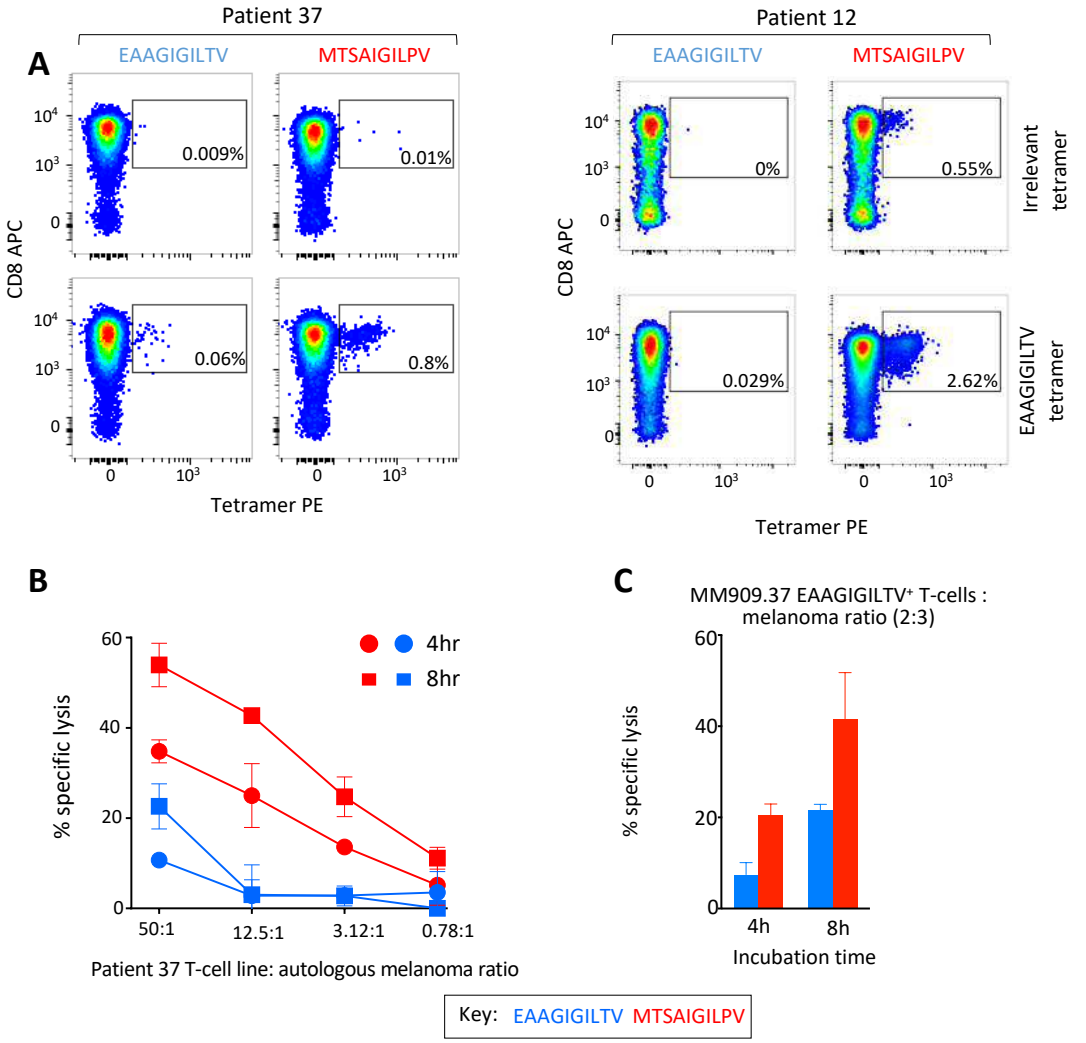


Figure 3.15: MTSAIGILPV primed EAAGIGILTV-tetramer positive cells from patient blood that exhibited improved killing of autologous melanoma cells.

*CD8 T-cells were isolated from the PBMC of two HLA A2+ metastatic melanoma patients and primed for 28 days with EAAGIGILTV and **MTSA**IGIL**P**V peptides. (**A**) On day 28 cells were stained with EAAGIGILTV and ALWGPDPAAA (an irrelevant peptide derived from preproinsulin)-tetramers using an optimised protocol, the percentage of tetramer positive cells is shown. (**B**) CD8 T-cells from patient 37 were co-incubated with chromium-labelled autologous tumour, at a range of T-cell: tumour ratios, and chromium release measured after 4 and 8 hours. Killing of autologous tumour by the 2 populations of CD8 T-cells is depicted at percentage specific lysis relative to maximum chromium release. Assay was set up in duplicate and results expressed as the average with the standard deviation from the mean shown. (**C**) Cytotoxicity assay as in **B**, but with the cell numbers adjusted according to EAAGIGILTV tetramer positivity shown in **A**, to give two EAAGIGILTV tetramer+ cells per three autologous melanoma cells, for both the EAAGIGILTV and **MTSA**IGIL**P**V primed T-cell lines (thus a different number of total cells was used from each primed line).*

3.3. Discussion

A hallmark of cancer is the dysregulation of protein expression in transformed cells. Changes in the cancer proteome include; overexpression of self-proteins, expression of neoantigens derived from mutations or expression of cancer-testis antigens. CD8 T-cells can target these changes in the proteome via the MHC class I peptide presentation pathway and there has been recent wide interest in harnessing these cells for cancer immunotherapy. Unlike neoantigens, which tend to be patient-specific, over-expressed self-proteins and cancer-testis antigens are shared by the cancers of multiple individuals and therefore make good candidates for development of a broad-spectrum vaccine for individuals that share a common HLA type.

This chapter focused on the over-expressed cancer antigen, melan-A which is upregulated in the majority of melanoma cancer cells (Valmori, *et al.*, 1998). Previous work aimed at exploiting this antigen in peptide vaccination has been largely unsuccessful as the natural peptide sequence recognised by CD8 T-cells, EAAGIGILTV, is poorly immunogenic (Yutaka Kawakami *et al.*, 1994). An alternative heteroclitic peptide E**L**AGIGILTV was designed to have improved binding to HLA A2, due to the substitution of alanine (A) at position 2 of the sequence with a known favoured binding residue of HLA A2, leucine (L) (Valmori, *et al.* 1998). E**L**AGIGILTV was also largely unsuccessful in clinical trials, as whilst it induced a greater number of EAAGIGILTV-specific CD8 T-cells these did not mediate measurable tumour regression (Bins *et al.*, 2007). E**L**AGIGILTV induces different TCRs compared to the natural sequence (Wieckowski *et al.*, 2009), with inferior functional capacity (Speiser *et al.*, 2008). Therefore, whilst E**L**AGIGILTV increased the magnitude of the CD8 T-cell responses these responses exhibited inferior recognition of melanoma.

Combinatorial peptide library (CPL) screening using CD8 T-cells clones has been identified as a method of identifying novel T-cell antigens (Linnemann *et al.*, 2001; Pinilla *et al.*, 2001; Zhao *et al.*, 2001; Wooldridge *et al.*, 2012a). CPL screening harnesses the cross-reactive nature of CD8 T-cells by exposing a given T-cell clone to all possible combinations of amino acids in a peptide of a given length, thus allowing an unbiased and non-competitive approach to identifying novel agonist peptides. CPL screens have previously been used to design a peptide for cancer vaccination (Linnemann *et al.*, 2001) whereby a HLA B8 restricted T-cell clone, reactive to cutaneous T-cell lymphoma (CTCL), was used to design a novel peptide which induced T-cells capable of lysing CTCL tumour *in vitro* (Linnemann *et al.*, 2001). In the resulting clinical trial, tumour regression was seen in two vaccinated CTCL patients (Tumenjargal *et al.*, 2003). Furthermore, it is possible to screen CD8 T-cell clones against CPL

screens comprised of D-amino acids. Using an influenza restricted HLA A2 T-cell clone in a D-amino acid CPL screen, the Sewell Lab identified a novel D-amino acid peptide capable of immunizing mice against a lethal challenge of influenza (Miles *et al.*, 2018). In this chapter I sought to use CPL screening to improve upon previous attempts to design an immunogenic agonist peptide for use in an anti-melanoma peptide vaccine.

3.3.1. Designing super-agonist peptides using dominant CD8 T-cell clonotypes

Tumour infiltrating lymphocyte (TIL) therapy has demonstrated remarkable results in metastatic melanoma patients in recent clinical trials (Andersen *et al.*, 2016; Andersen *et al.*, 2018), with as many as 50% of stage IV patients showing objective responses to treatment and proportion of these remaining disease free over 7 years after treatment. It has been demonstrated that the main drivers of this response are CD8 T-cells (Besser *et al.*, 2009; Dudley *et al.*, 2010b; Radvanyi *et al.*, 2012). With this evidence in mind I sought to use one such dominant and persistence melanoma-specific CD8 T-cell clonotype to design super-agonist peptides for an anti-cancer peptide vaccine.

T-cell clonotype, ST8.24, whose CDR3 sequence is found in the TIL infusion product given to metastatic melanoma patient MM909.24 and in the PBMC post-cure exhibited strong recognition of EAAGIGILTV and E**L**AGIGILTV peptides (**Figure 3.2**), highlighting it as a good candidate clonotype to try and induce via peptide vaccination. Data produced by a CPL screen of ST8.24 (**Figure 3.3**) identified ten candidate super agonist peptides (**Table 3.1**). Of the ten, two candidate agonist peptides, **ITS**GIG**V**L**P**V and **MTSA**IGIL**P**V, were identified as being strong activators of melan-A-specific T-cell clones. The agonist peptide **ITS**GIG**V**L**P**V was favoured when tested against a panel of 4 CD8 T-cell clones (**Figure 3.4**).

Vaccination involves breaking T-cell tolerance to prime antigen-specific T-cells from polyclonal naïve T-cell populations. I next tested all ten candidate super-agonist peptides by priming polyclonal CD8 T-cell populations isolated from HLA A2+ healthy individuals *in vitro*. Initially, agonist peptide **MTSA**IGIL**P**V primed more EAAGIGILTV-tetramer positive CD8 T-cells that the other candidate agonist peptides in 5 healthy donors (**Figure 3.5**). I examined **MTSA**IGIL**P**V priming of EAAGIGILTV-specific CD8 T-cells over 14 and 28 days in seven additional healthy donor samples, and found that **MTSA**IGIL**P**V priming elicited more EAAGIGILTV-tetramer positive cells in all donors (**Figure 3.6**). In addition, EAAGIGILTV-specific CD8 T-cells primed with **MTSA**IGIL**P**V peptide exhibited enhanced proliferation compared to those primed with EAAGIGILTV peptide in parallel assays (**Figure 3.7**). At this

stage, **MTSA**IGIL**P**V priming had elicited more EAAGIGILTV-specific CD8 T-cells than priming with the natural sequence in 14/14 healthy donors tested. Priming polyclonal CD8 T-cells with **MTSA**IGIL**P**V peptide elicited more EAAGIGILTV-tetramer positive cells than priming with the **ITS**GIG**V**L**P**V peptide in parallel assay (**Figure 3.8**). I therefore selected **MTSA**IGIL**P**V as the most promising peptide super-agonist to take forward for further studies.

3.3.2. MTSAIGILPV exhibits improved binding to HLA A2 and is a close structural mimic of EAAGIGILTV

I next sought to identify any differences in MHC binding between **MTSA**IGIL**P**V and EAAGIGILTV peptides that would account for the increased immunogenicity of **MTSA**IGIL**P**V primed T-cells in all 14 donors and 2 patients tested. **MTSA**IGIL**P**V exhibited enhanced binding to HLA A2 compared to EAAGIGILTV (**Figure 3.11**), the structural data attributes this to the longer side chain of threonine at peptide residue 2 extending deeper into the HLA A2 B pocket (**Figure 3.14**). Moreover, the structural data revealed that despite differing by 5/10 amino acids **MTSA**IGIL**P**V is a close structural mimic of EAAGIGILTV when bound to HLA A2, which may account for the cross-reactive nature of these peptides. These data were complimented by clonotypic analysis of the TCRs of **MTSA**IGIL**P**V primed cells which exhibit a strong bias towards TRAV 12-2 gene expression. Clonotypic analysis fit with previous studies showing a strong bias towards TRAV 12-2 α-chain TCRs in EAAGIGILTV-specific T-cell populations (Trautmann *et al.*, 2002; Dietrich *et al.*, 2003).

3.3.3. EAAGIGILTV primed T-cells bind MTSAIGILPV with increased affinity

Since it was not possible at this stage to resolve a complex structure of HLA A2- **MTSA**IGIL**P**V and a TCR, pMHC tetramer staining was used to gain some insight into any differences in affinity of TCRs to **MTSA**IGIL**P**V and EAAGIGILTV peptides bound to HLA A2. Five CD8 T-cell clones reactive to EAAGIGILTV and **MTSA**IGIL**P**V peptides and two polyclonal populations primed with EAAGIGILTV bound HLA A2-**MTSA**IGIL**P**V tetramer with greater intensity compared to HLA A2-EAAGIGILTV tetramer (**Figure 3.13**). These data infer that compared to HLA A2-EAAGIGILTV, TCRs bind to HLA A2- **MTSA**IGIL**P**V with a greater affinity, suggesting that **MTSA**IGIL**P**V affords a better antigenic stimulus and providing a potential explanation for the increased proliferation and improved functional capacity of T-cells primed with **MTSA**IGIL**P**V peptide. The affinity of **MTSA**IGIL**P**V-primed T-cells could also be assessed using surface plasmon resonance (SPR) analysis, this method cannot elude the avidity of the overall interaction but would give a real-time measure of TCR pMHC affinity.

3.3.4. **MTSA**IGIL**P**V primed T-cells exhibit improved killing of melanoma cells

I also examined the ability of **MTSA**IGIL**P**V primed CD8 T-cells to kill melanoma cells compared to T-cells primed with the natural sequence. **MTSA**IGIL**P**V primed T-cells from healthy donors 11 and 14 and metastatic melanoma patients MM909.37 and MM1413.12 exhibited enhanced killing of melanoma cell lines compared to EAAGIGILTV primed T-cells (**Figure 3.9** and **Figure 3.15**), demonstrating that priming with the agonist peptide **MTSA**IGIL**P**V can elicit better quality T-cells compared to priming with the natural peptide sequence, EAAGIGILTV.

3.3.5. Summary

In this chapter, CD8 T-cell clone ST8.24 derived from a metastatic melanoma patient who successfully cleared their tumour after undergoing TIL therapy was observed in patient blood long after successful clearance of tumour. ST8.24 was selected as a good example of a persistent T-cell clonotype. A CPL screen of ST8.24 was used to generate ten candidate super-agonist peptides which were then tested for their ability to elicit melan-A specific CD8 T-cell responses. A peptide of sequence **MTSA**IGIL**P**V elicited more EAAGIGILTV-specific CD8 T-cells than EAAGIGILTV in all donors tested, including two melanoma patients. Furthermore, the cells primed by **MTSA**IGIL**P**V were superior at killing melanoma tumour compared to EAAGIGILTV-primed cells. Overall, this work highlights the potential of CPL screens for designing optimal peptides for use in vaccination treatments for metastatic melanoma. The success of this approach in terms of using an altered peptide ligand to prime T-cells in greater *quantity* than those primed by the natural antigen that exhibited better overall *quality* in terms of melanoma killing suggest that this encouraging approach could be applied to different antigens in different cancers.

4. MTSAIGILPV peptide primes T-cells that recognise multiple cancer epitopes

4.1. Background

4.1.1. T-cell cross-reactivity

The clonal selection theory dictates that T-cells recognise one peptide antigen presented by an MHC molecule (Jerne, 1971). VDJ rearrangement, nucleotide insertion/deletion in the CDR3 loops and αβ chain pairing can theoretically produce 10^{15} and 10^{18} unique αβ TCRs in mouse and human respectively (Davis and Bjorkman, 1988; Sewell, 2012) sufficient for 1:1 receptor:peptide coverage. However, Mason first pointed out the numerical absurdity of the one receptor for one antigen paradox, given that T-cells appear capable of responding to ~10^{17} different peptides when there are less than 10^{12} of them in a human body (and considerably less in a mouse) (Mason, 1998). Based on theoretical considerations, Mason proposed that a very high level of cross-reactivity is an essential feature of the TCR and that each receptor could recognise millions of different individual peptides in the context of the restricting MHC molecule (Mason, 1998). My own laboratory went onto prove that an insulin-specific T-cell isolated from a patient with type 1 diabetes could recognise over a million different peptides (Wooldridge *et al.*, 2012). The fact that the strongest agonist sequence in this study was different from the insulin-derived sequence, that allowed this T-cell to kill human pancreatic β-cells, at seven of ten amino acid positions hinted at the extent of possible T-cell cross-reactivity.

The number of different peptides, of a length that could be recognised by T-cells, that can be generated from the 20 proteogenic amino acids is vast (**Table 4.1**). Furthermore, many studies have shown that T-cells can recognise peptides with post-translational modifications such as; citrullination (Ireland, Herzog and Unanue, 2006; Brentville *et al.*, 2016), disulphide bridges (Mannering *et al.*, 2005), glycosylation (Haurum *et al.*, 1994; Van den Steen *et al.*, 2004), deamidation (McAdam *et al.*, 2001) and phosphorylation (van Stipdonk *et al.*, 1998) and peptides composed of non-natural amino acids (Benkirane *et al.*, 1993; Miles *et al.*, 2018). These peptides further widen the scope of TCR recognition and must also be taken into account when numerating the pool of potential TCR antigens.

Peptide length (amino acids)	Predicted peptides	3% MHC binders	1% MHC binders
8	2.56×10^{10}	7.8×10^{8}	2.6×10^{8}
9	5.12×10^{11}	1.5×10^{10}	5.1×10^{9}
10	1.24×10^{13}	3.6×10^{11}	1.2×10^{11}
11	2.048×10^{14}	6×10^{12}	2×10^{12}
12	4.096×10^{15}	1.2×10^{14}	4.1×10^{13}
13	8.192×10^{16}	2.4×10^{15}	8.2×10^{14}
14	1.638×10^{18}	4.8×10^{16}	1.6×10^{16}

Table 4.1: Potential number of peptide epitopes for αβ T-cells

An early study by Wucherpfennig et al, demonstrated that CD4 T-cells specific for myelin basic protein (MBP) were able to tolerate substitutions in their cognate antigen and that CD4 T-cells derived from multiple sclerosis patients could recognise epitopes of bacterial and viral origin (Wucherpfennig and Strominger, 1995). Subsequently, many examples emerged showing CD8 T-cells capable of recognising peptides distinct from their cognate antigen (Hunt *et al.*, 1992; Fahrer *et al.*, 1995; Ignatowicz *et al.*, 1997; Acierno *et al.*, 2003; Nilges *et al.*, 2003; Clute, 2005; Vujanovic *et al.*, 2014). In some cases, the alternative peptides recognised by the T-cell induced greater T-cell activation than the wild type 'index' antigen (Valmori *et al.*, 1998; Ekeruche-Makinde *et al.*, 2012; Cole *et al.*, 2016).

In **Chapter 3**, I took advantage of T-cell cross-reactivity to generate the **MTSA**IGIL**P**V altered peptide ligand that was capable of inducing greater numbers of melan-A-specific T-cells from 16/16 different HLA A2+ donors. **MTSA**IGIL**P**V-induced T-cells were shown to express different TCRs than those induced by the wild type peptide. **MTSA**IGIL**P**V-induced T-cell clonotypes appeared to be more effective at killing HLA A2+ melanoma cells than those primed by the natural EAAGIGILTV melan-A peptide. In this chapter, I set out to try and answer the obvious question of why are **MTSA**IGIL**P**V-primed T-cells more effective than those primed by the natural sequence?

4.1.2. Aims

In **chapter 3**, priming with the super-agonist peptide **MTSA**IGIL**P**V was shown to elicit more melan-A restricted CD8 T-cells from 14 healthy HLA A2+ individuals tested, as well as two metastatic melanoma patient samples. CD8 T-cells primed with **MTSA**IGIL**P**V exhibited improved capacity to kill tumour cells in an HLA A2 restricted manner. **MTSA**IGIL**P**V exhibit superior binding to HLA A2 than the WT peptide EAAGIGILTV which was reasoned to be one plausible explanation as to why **MTSA**IGIL**P**V elicited more EAAGIGILTV-specific T-cells. However, this did not explain the improved functional capacity of **MTSA**IGIL**P**V-primed CD8 T-cells. Work by previous students in my laboratory showed that the ST8.24 T-cell clonotype that was used in **Chapter 3** was present within the population of T-cells within patient MM909.24 TIL that responded to multiple HLA A2+ cancer types (Bianchi, 2016; Theaker, 2018; Rius Rafael, 2019). Most of these cancer types did not express melan-A suggesting that the ST8.24 T-cell might recognise a further cancer-specific epitope. Sarah Theaker's work focussed on breast cancer and found that ST8.24 could respond to residues 22-20 from putative breast cancer antigen bone marrow stromal antigen 2 (BST2; sequence LLLGIGILVL). Cristina Rius took this work to the next stage using a clonotype isolated from MM909.24 TIL called CR24 that was shown to recognise residues 367-376 of insulin-like growth factor 2 RNA binding protein (IMP2) in addition to melan-A. Sequencing of the CR24 TCR showed it was identical to that isolated from ST8.24 (i.e. CR24 and ST8.24 were the same T-cell clone). Thus, the ST8.24/CR24 TCR could recognise three different tumour-derived peptides in the context of HLA A2: melan-$A_{26\text{-}35}$ (EAAGIGILTV), $BST2_{22\text{-}31}$ (LLLGIGILVL) and $IMP2_{367\text{-}376}$ (NLSALGIFST). This new type of T-cell recognition was termed "multipronged' to reflect that such T-cells have capacity to attack cancer via different peptide epitopes. Dr Rius also showed that the CR24 (ST8.24) T-cell clone could still recognise the patient MM909.24 tumour when the melan-A gene was knocked out using CRISPR-Cas9 (Rius Rafael, 2019). Importantly, this result indicates that some T-cells really can recognise tumour cells via multiple different peptide epitopes.

The above findings, made during the course of my own studies, led us to hypothesise that priming with the super-agonist **MTSA**IGIL**P**V might elicit cross-reactive T-cells, capable of seeing epitopes from both melan-A and/or BST2 and/or IMP2. Such multipronged recognition might provide a rationale as to why **MTSA**IGIL**P**V-primed CD8 T-cells exhibit improved functionality. My aim was to determine whether **MTSA**IGIL**P**V-primed T-cells might recognise melanoma cells in different ways, possibly including recognition via additional epitopes, compared to T-cells primed with the EAAGIGILTV natural melan-A sequence.

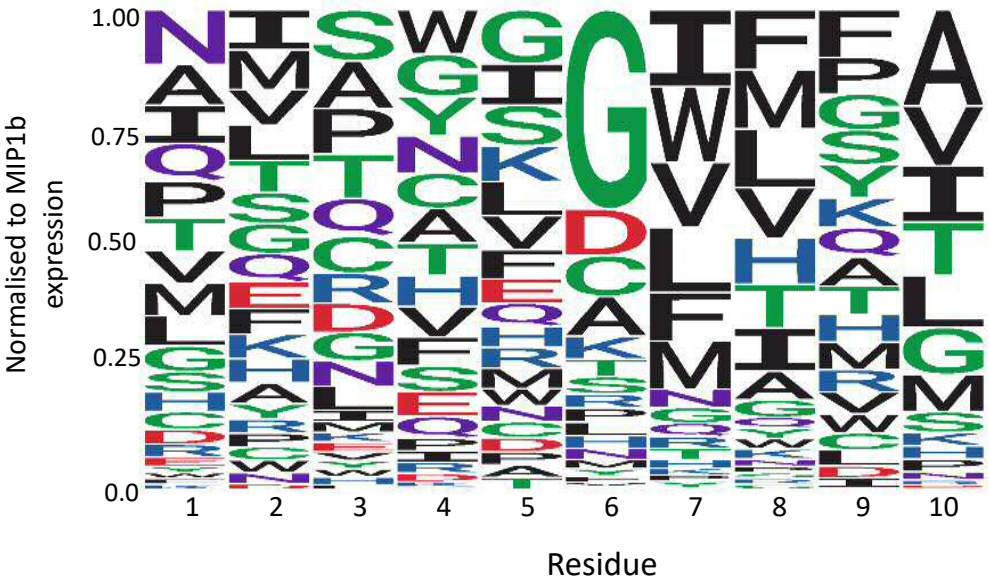
4.2. Results

4.2.1. MTSAIGILPV-T-cell clone ST8.24 recognises multiple TAA epitopes

TCR clonotype ST8.24 was isolated from the TIL infusion product given to patient MM909.24, the TCR sequence was also found in the PBMC 6-months post-cure. The cognate peptide for TCR clonotype ST8.24 is the melan-A-derived peptide EAAGIGILTV. The CPL screen carried out in **Chapter 3** highlighted the ability of ST8.24 to recognise multiple peptides distinct from its cognate antigen. These data fitted with that produced by other students in my laboratory while I was undertaking my studies with the **MTSA**IGIL**P**V peptide. I therefore decided to further interrogate the data generated by the combinatorial peptide library screen carried out in **Chapter 3**. The CPL data were entered into a bespoke database composed of known tumour associated proteins (Tumour Antigen Database, Dr Garry Dolton and Dr Barbara Szomolay, publication pending). A similar approach for screening autoimmune T-cells was recently published by my laboratory (Whalley *et al.*, 2020).

The TAA database produced a list of potential epitope 'hits' which were ranked based on the likelihood that they would activate TCR clonotype ST8.24 (**Figure 4.1**). The results from the database highlighted several potential ST8.24 epitopes derived from known tumour associated proteins (**Figure 4.1B**). Although all of the proteins identified by the screen have been previously associated with cancer, some of the epitopes predicted to activate ST8.24 had not been previously described in the literature.

A



B

Rank	Peptide	Protein	Abbrev.
-22.065	ELAGIGILTV	Melanoma antigen recognised by T-cells 1 (analogue)	Melan-A
-22.488	NLAAVGLFPA	Insulin-like growth factor 2 mRNA-binding protein 1	IMP2
-22.659	EAAGIGILTV	Melanoma antigen recognised by T-cells 1	Melan-A
-22.969	LLLGIGILVL	Bone marrow stromal antigen 2	BST2
-23.128	NLSALGIFST	Insulin-like growth factor 2 mRNA-binding protein 1	IF2B2
-23.301	VYAALGILQG	Canalicular multispecific organic anion transporter 2	MRP3
-23.373	LILNIAIFFV	Dermatan-sulfate epimerase	DSE
-23.373	ATSAMGTISI	Mucin-16	MUC16
-23.435	ISAVVGILLV	Tyrosine kinase-type cell surface receptor HER2	HER2
-23.477	TSSAIPIMTV	Mucin-16	MUC16

Figure 4.1: Peptide hits from a combinatorial peptide library (CPL) of ST8.24

***A**) CD8 T-cell clone ST8.24 isolated from melanoma patient MM909.24 who is 7+ years cancer-free after undergoing TIL therapy, showed degenerate recognition of 10 amino acid peptides when subjected to a combinatorial peptide library scan. Sequence Logo plot generated in R by Tom Whalley. **B**) Top ten predicted tumour associated antigens (TAA) peptide sequences identified through CPL screening of ST8.24. Ranking is based on the likelihood of ST8.24 antigen recognition. List of TAA generated with help from Dr Barbara Szomolay and a TAA database (publication pending). CPL screen was carried out by Dr Valentina Bianchi* (Bianchi, 2016).

In vitro validation of the top peptide 'hits' from ST8.24 CPL was required to validate the *in silico* result (**Figure 4.2**). The reactivity of ST8.24 to the identified epitopes was initially assessed using peptide titrations through a MIP-1β ELISA, with ST8.24 recognising three of the top five hits; peptides EAAGIGILTV, LLLGIGILVL and NLSALGIFST. Peptide LLLGIGILVL titrated as well as the cognate antigen EAAGIGILTV (**Figure 4.2A**). Further validation of the TAA epitopes was carried out using peptide-MHC tetramer staining with tetramers for EAAGIGILTV, LLLGIGILVL and NLSALGIFST (**Figure 4.2B**). NLSALGIFST bound with an increased MFI compared to the cognate antigen EAAGIGILTV (**Figure 4.2B**). The discovery that ST8.24 recognises three TAA epitopes gave rise to the hypothesis that it might be able to see all of these epitopes on the surface of the same tumour cell. We termed such recognition as being multipronged to reflect that cancer cells were being targeted through more than one antigen by a single TCR. Multipronged T-cell recognition would mean that cancer cells might not be able to escape by deleting expression of an individual TAA. As the TCR clonotype ST8.24 was found in the TIL infusion product given to patient MM909.24 (TIL 24), and the presence of such multipronged clonotypes may help to explain the remarkable success of patient MM909.24's TIL therapy treatment. I set out to extend the work done by Dr Rius by assessing whether TIL 24 contained CD8 T-cells specific for EAAGIGILTV, LLLGIGILVL and NLSALGIFST epitopes using peptide-MHC tetramers.

4.2.2. TIL 24 infusion product contains T-cells specific for melan-A, BST2 and IMP2

Peptide-MHC tetramer staining of TIL 24, highlighted distinct populations of CD8 T-cells specific for the three tumour associated antigens, 7.05% EAAGIGILTV, 0.26% LLLGIGILVL and 0.65% NLSALGIFST. (**Figure 4.2C**). Given the vast numbers of cells in the TIL infusion product given to patient MM909.24 (1 $x10^{11}$), these small percentages actually infer that ~2.86 $x10^{8}$ LLLGIGILVL and 7.5 $x10^{8}$ NLSALGIFST-specific T-cells were given to patient MM909.24 before they went onto achieve complete remission (i.e. the patient received over a billion T-cells that could engage BST2 and IMP2 epitopes). Tetramer staining also suggested that the vast majority of melan-A-specific T-cells in MM909.24 TIL did not co-recognise BST2 or IMP2.

The new epitopes identified for ST8.24 are derived from the proteins insulin-like growth factor 2 mRNA-binding protein 1 (IMP2, also known as IF2B2) (NLSALGIFST) and bone marrow stromal antigen 2 (BST2) (LLLGIGILVL). IMP2, is a member of the highly-conserved IMP family RNA binding proteins which mediate post-transcriptional regulation of RNA.

Unlike the other IMP family members, IMP1 and IMP3, IMP2 is reportedly expressed in adult tissues, all be it at a low levels, and is thought to regulate mitochondrial activity, specifically oxidative phosphorylation (Bell *et al.*, 2013). Overexpression of IMP2 has been linked to poor prognosis in a number of cancer types, such as glioblastoma (Janiszewska *et al.*, 2012), pancreatic cancer (Dahlem *et al.*, 2019), breast cancer (Kim, Ha Thi and Hong, 2018) and oesophageal cancer (Barghash *et al.*, 2016). This correlation could be due to the influence of IMP2 on cellular metabolism (Christiansen *et al.*, 2009; Wang *et al.*, 2019) and tumour metastasis (Mu *et al.*, 2015). BST2, also known as HM1.24 or CD137, is commonly overexpressed in multiple cancers including breast cancer (Mahauad-Fernandez *et al.*, 2018), myeloma (Hundemer *et al.*, 2006) and lung cancer (Wang *et al.*, 2009). The 9-mer variation of this epitope, LLLGIGILV, has previously been identified as an HLA A2 restricted epitope (Hundemer *et al.*, 2006). NLSALGIFST and the 10-mer LLLGIGILVL were first identified and validated as tumour associated epitopes by my laboratory (Bianchi, 2016; Theaker, 2018; Rius Rafael, 2019).

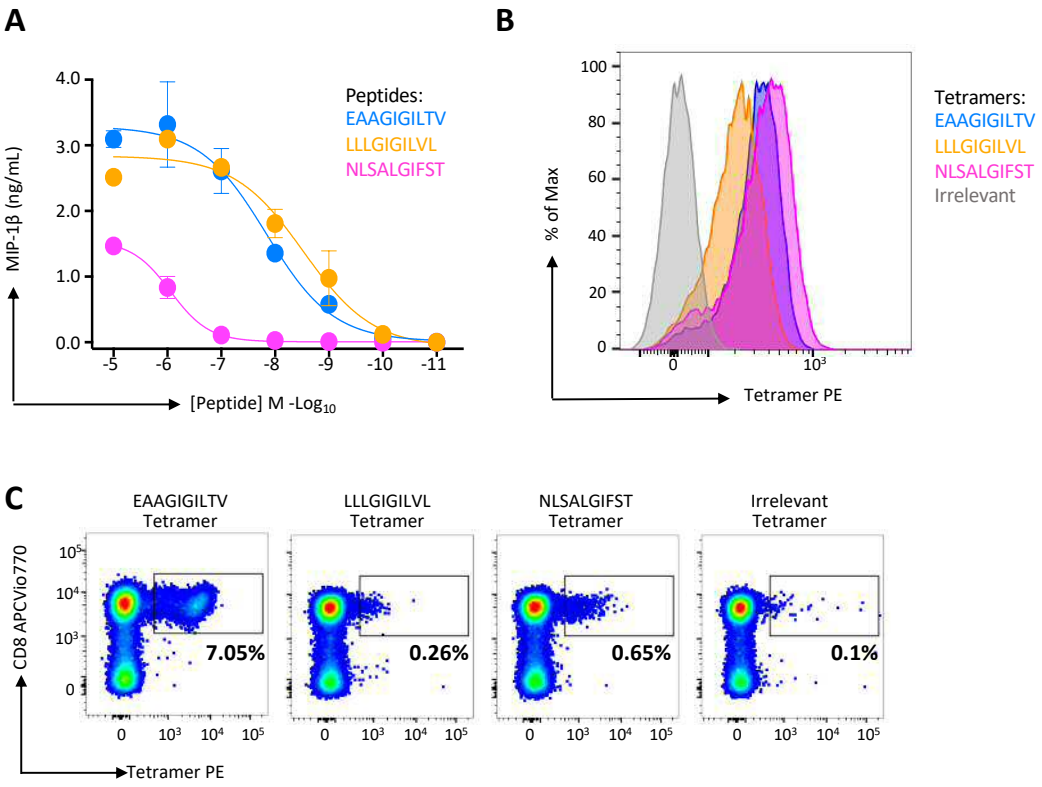


Figure 4.2: Validation of potential ST8.24 tumour associated epitopes

***A**) EAAGIGILTV, LLLGIGILVL and NLSALGIFST peptide activation of T-cell clone ST8.24 at concentrations 10^{-5} to 10^{-11} M assessed by MIP-1β ELISA. Data is shown as the average of two replicates, standard error of the mean is shown **B**) pMHC-tetramer staining of ST8.24 with cognate antigen EAAGIGILTV (melan-A) and additional epitopes LLLGIGILVL (BST2) and NLSALGIFST (IMP2), plus an irrelevant peptide ALWGPDPAAA (pre-pro insulin) using an optimised protocol (PKI + tetramer + anti-PE 1◦ antibody). Mean fluorescent index of PE-tetramer is shown. ST8.24 was stained with LIVE/DEAD vivid pacific blue to allow exclusion of dead cells and CD8 APC. **C**) p-MHC tetramer staining of tumour infiltrating lymphocyte (TIL) infusion product from metastatic melanoma patient MM909.24 using tetramers for EAAGIGILTV, LLLGIGILVL and NLSALGIFST. Percentage of CD8+ Tetramer+ cells shown. Staining was carried out using an optimised 'tricks' protocol (PKI + tetramer + 1◦ antibody) and dead cells excluded using LIVE/DEAD VIVID pacific blue.*

4.2.3. Tumour associated antigens BST2 and IMP2 are expressed in multiple cancer types

In order to assess the potential clinical relevance of LLLGIGILVL and NLSALGIFST-derived epitopes, I compiled expression data of BST2 and IMP2 in primary melanoma tumours and a collection of tumour cell lines from distinct anatomical locations. Abundant expression of TAAs BST2 and IMP2 in multiple tumours would help to confirm the importance of eliciting multipronged T-cells for effective tumour regression. The RNAseq expression data is normalised for sequencing depth and gene length using two well established metrics; reads

per kilobase million (RPKM) and fragments per kilobase million (FPKM). The expression of insulin in each tumour is included in the analysis and is considered to be baseline as it was not expressed by any tumour cell line.

Given the ability of T-cell clone ST8.24, isolated from a metastatic melanoma patient, to react to multiple tumour epitopes derived from tumour associated antigens melan-A, BST2 and IMP2, I initially wanted to ascertain the prevalence of TAAs melan-A, BST2 and IMP2, in primary melanoma tumour samples. RNAseq data was extracted from Lauss *et al*. 2017, which contains a cohort of tumours isolated from metastatic melanoma patients enrolled in TIL therapy trials, at the CCIT, Copenhagen (Lauss *et al.*, 2017). The TAA melan-A was highly expressed in the majority of melanoma tumours, apart from in three patients, 11, 22 and 36, which had low melan-A expression. A high level of melan-A expression was expected due to the known association of melan-A and melanoma (Valmori, *et al.*, 1998) (**Figure 4.3A**). All of the melanoma patient tumours expressed BST2 and all but three patient tumours expressed IMP2. All the melanoma tumours expressed at least two of the three antigens; melan-A, BST2 or IMP2.

In addition to melanoma samples, I wanted to determine the expression levels of melan-A, BST2 and IMP2 in cancer cell lines from diverse anatomical locations such as liver, lung, bone, brain, pancreas, cervix, prostate, breast, kidney, colon, and the blood (**Figure 4.3B**). Expression data was compiled using the TRON Cell Line Portal (Scholtalbers *et al.*, 2015). BST2 and IMP2 were highly expressed in the majority of tumour cell lines analysed, with only breast cancer cell line T47D exhibiting low IMP2 expression. Eleven cell lines analysed had low expression of BST2; MIA-PACA-2, HPAC, SAOS, U2OS, 143B, ACHN, LS147T, DLD-1, PC3, 549 and CHP-212. Melan-A was expressed at very low levels compared to the melanoma samples displayed in part A. Importantly, every cell line assessed expressed at least one of the TAAs BST2 or IMP2, with 21/32 cell lines expressing both antigens.

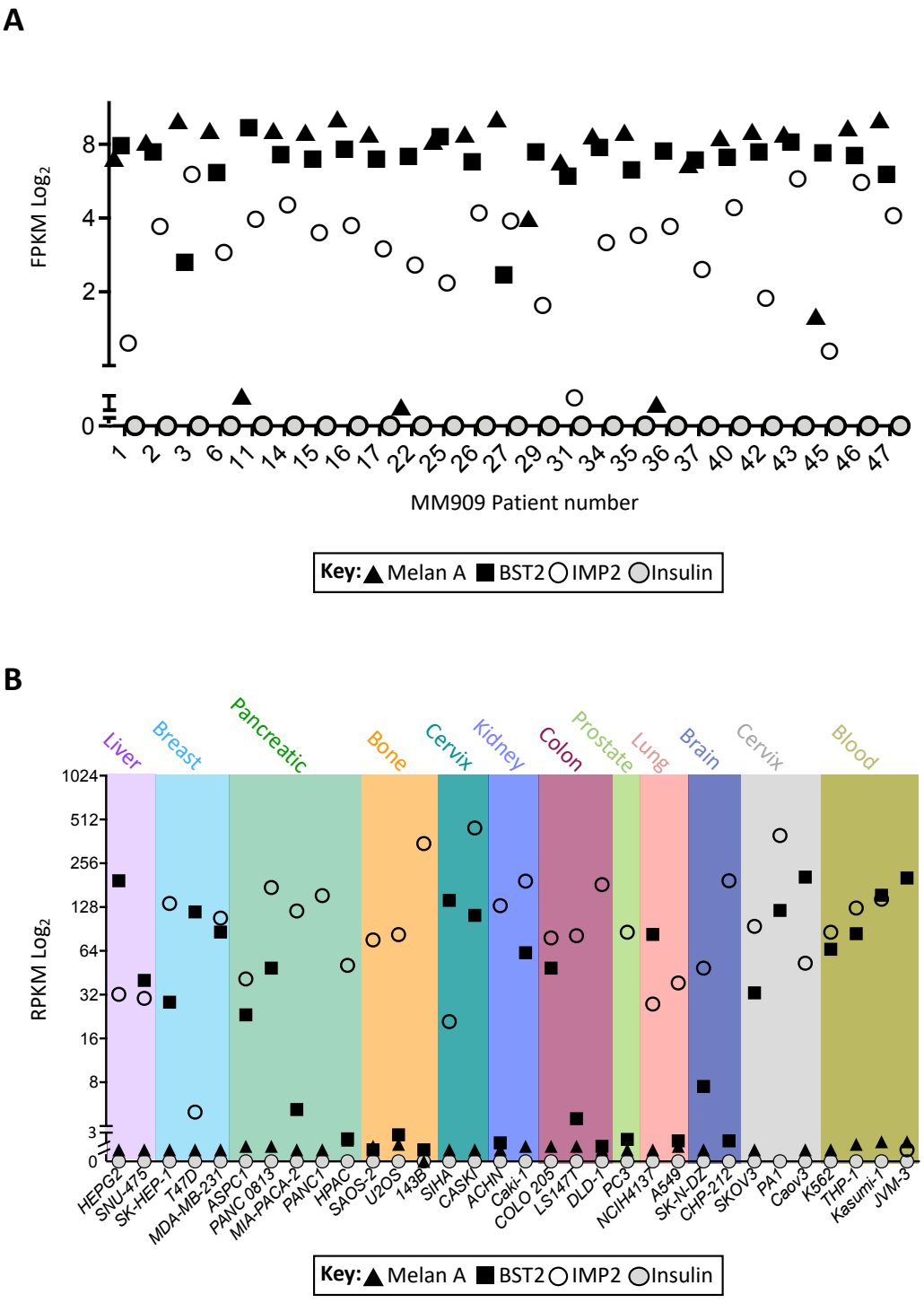


Figure 4.3: Ubiquitous expression of tumour associated antigens BST2 and IMP2 in cancer cell lines

***A**) Expression data of TAA melan-A, BST2 and IMP2, in tumour samples from 25 metastatic melanoma patients enrolled in TIL therapy trials at the CCIT, Copenhagen. RNAseq data was extracted from (Lauss et al., 2017). Expression of TAA is compared to insulin as a baseline control. Results are expressed as fragments per kilobase million (FPKM) Log_2. **B**) Expression data of TAA melan-A, BST2 and IMP2 in cancer cell lines from a variety of malignant diseases. Expression data was extracted from the TRON Cell Line Portal (Scholtalbers et al., 2015). Results are expressed as reads per kilobase million (RPKM) Log_2.*

4.2.4. TCR clonotype ST8.24 is reactive to multiple tumour types

TCR clonotype ST8.24 recognises three tumour-associated antigen epitopes (**Figure 4.2**), which are commonly expressed on many tumour cell lines (**Figure 4.3**). I therefore reasoned that ST8.24 could recognise these epitopes on tumours distinct from its autologous tumour, MM909.24. Initially, reactivity of ST8.24 to different HLA A2 positive and HLA A2 negative melanoma cell lines was assessed after 4 h of co-incubation using a TAPI assay (**Figure 4.4A**). ST8.24 showed strong reactivity to HLA A2+ melanoma cell lines MEL-624 and MEL-526 as well as its autologous tumour, MM909.24, conversely ST8.24 didn't react to HLA A2- melanoma cell lines FM-2 and FM-56. Reactivity of ST8.24 to a variety of tumour cell lines from different anatomical locations was then assessed after an overnight co-incubation by a MIP-1β ELISA (**Figure 4.4B**). ST8.24 demonstrated broad reactivity to the tumour cell lines tested, these included; MDA-MB-231 (breast), SAOS (bone), SIHA (cervix), PC3 (prostate), ACHN and RCC 17 (kidney). Given that TCR clonotype ST8.24, which was isolated from the TIL infusion product of melanoma patient MM909.24, is cross-reactive, it was reasoned that TIL 24 might also exhibit reactivity to tumour cell lines distinct from its autologous tumour (**Figure 4.4C**). Reactivity of TIL 24 to HLA A2+ tumour cell lines; colo 205 (colon), LnCAP (prostate), MCF-7 (breast), MDA-MB-231 (breast), MS751 (cervix) and SAOS (bone) was assessed as CD107a and TNF production following a 5 h activation assay (**Figure 4.4**). TIL 24 demonstrated broad reactivity to the tumour cell lines assessed.

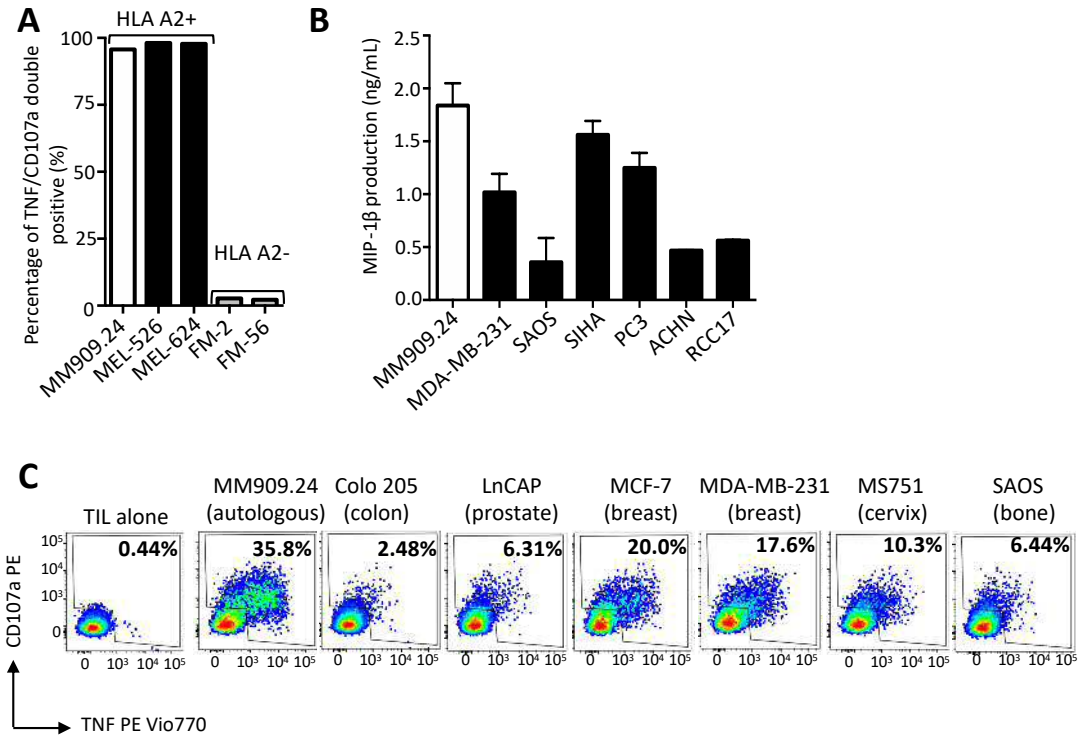


Figure 4.4: CD8 T-cell clonotype ST8.24 reacts to multiple tumour types

***A**) Reactivity of TCR clonotype ST8.24 to HLA A2+ (MEL-526 and MEL-624) and HLA A2- (FM-2 and FM-56) melanoma cell lines including its autologous tumour (MM909.24) assessed by a TAPI-O assay. 3 x10^4 T-cells were co-incubated with 1 x10^5 tumour cells alongside TAPI, CD107a FITC and TNF Pe vio770, for 4 h at 37 ℃. TNF and CD107a release was measured by flow cytometry. CD8 APC Vio770 and VIVID PB were used to allow identification of live, CD8 + T-cells. Results are expressed as the percentage of CD107a/TNF double positive CD8 T-cells. **B**) The reactivity of ST8.24 to tumour cell lines from different anatomical locations was measured by MIP1-β ELISA. 3 x10^4 T-cells were co-incubated with 1.5 x10^4 tumour cells overnight at 37 ℃. The following day the supernatants were harvested and MIP1-β production assessed. Results are displayed as the MIP-1β reactivity of ST8.24 to HLA A2+ tumours, with the MIP-1β production to HLA A2- tumours subtracted. Experiment was carried out in duplicate, standard error of the mean is shown. **C**) Reactivity of TIL 24 to its autologous tumour MM909.24 and a panel of HLA A2+ cancer cell lines from different anatomical locations was assessed by TAPI assay following a 5 h incubation. Results are expressed as the percentage of CD8+ CD107a+ TNF+ cells. Cells were stained with LIVE/DEAD VIVID pacific blue to allow the exclusion of dead cells from the analysis. Data generated by Dr Valentina Bianchi.*

4.2.5. Multipronged T-cells can prevent melanoma tumour escape

I next wanted to confirm that multipronged TCR clonotype ST8.24 was recognising tumour cells by recognising multiple antigens. To do this, a melan-A KO of ST8.24 autologous tumour MM909.24 was generated by CRISPR/Cas9. Reactivity of ST8.24 to the melan-A negative MM909.24 tumour cell line was assessed by a TAPI-O assay after 4 h incubation (**Figure 4.5A**). ST8.24 was still reactive to the melan-A negative MM909.24, although reactivity was reduced compared to the WT MM909.24. This result infers that ST8.24 recognised more than one tumour associated antigen on the surface of MM909.24. To explore this 'additive' effect further, I compared the reactivity of ST8.24 to the TAA epitopes EAAGIGILTV, LLLGIGILVL and NLSALGIFST alone and in combination (**Figure 4.5B**). At the peptide concentration 10^{-6} M the peptides EAAGIGILTV, LLLGIGILVL and NLSALGIFST activated ST8.24 following overnight stimulus, measured by a MIP1-β ELISA (**Figure 4.5B**). At the concentration of 10^{-8}, the peptides EAAGIGILTV, LLLGIGILVL and NLSALGIFST could not activate ST8.24 above baseline (**Figure 4.5B**). However, in combination with each peptide at the concentration of 10^{-8}, the TAA epitopes EAAGIGILTV, LLLGIGILVL and NLSALGIFST restored activation of ST8.24 to the levels seen at 10^{-6} M (**Figure 4.5B**). The result infers an additive effect of the TAA epitopes at the peptide concentration 10^{-8} M, leading to activation of ST8.24 at a sub-optimal peptide concentration. This suggests that tumours expressing lower frequencies of tumour-associated antigens could induce effective activation of multipronged T-cells.

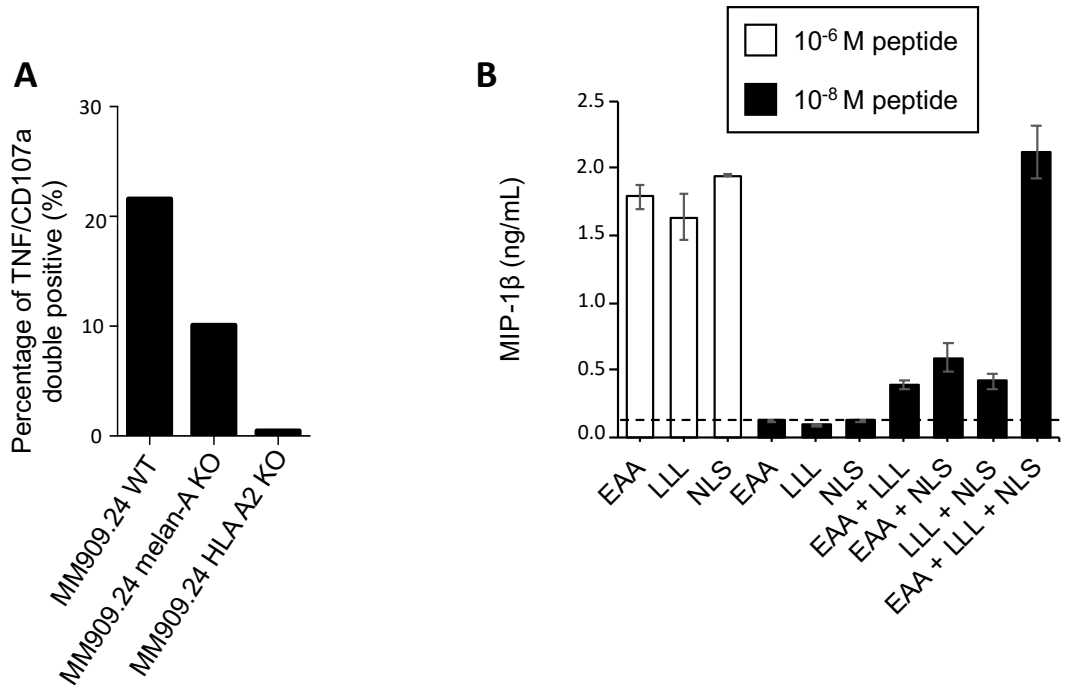


Figure 4.5: Additive effect of multipronged TCR ST8.24

***A**) HLA A2 and melan-A KO of melanoma tumour cell line MM909.24 were generated using CRISPR technology. Melan-A and HLA A2 negative clones were generated using single cell dilution and confirmed to be negative by flow cytometry. The reactivity of multipronged TCR clonotype ST8.24 to melan-A and HLA A2 negative MM909.24 tumour was assessed by TAPI assay following a 4 h incubation. 5 x10^4 T-cells were cultured with 1 x10^5 tumour at 37 °C. Reactivity is displayed as the percentage of CD8+ CD107a+ TNF+ cells with the percentage of CD107a and TNF produced by the T-cells alone subtracted. T-cells were also stained with LIVE/DEAD VIVID pacific blue to allow the exclusion of dead cells. **B**) Reactivity of TCR clonotype ST8.24 to TAA epitopes EAAGIGILTV, LLLGIGILVL and NLSALGIFST was assessed using a MIP-1β ELISA following overnight activation. 6 x10^4 T2 APC were incubated with 3 x10^4 T-cells and peptides at concentrations 10^{-6} M and 10^{-8} M. Control wells of T-cells + T2s with no peptide were included and the MIP-1β produced subtracted from the presented values. Results are expressed as the average of two replicates and the standard deviation is shown. Data generated by Dr Cristina Rius during the course of my studies.*

T-cell clones AL.91, AL.84 and AL.86 were isolated from the PBMC of a healthy HLA A2+ individual based on reactivity to the TAA epitopes EAAGIGILTV and NLSALGIFST. AL.91 is reactive to EAAGIGILTV, AL.84 to NLSALGIFST and AL.86 to both peptide antigens (**Figure 4.6A**). In the context of this study AL.91 and AL.84 recognise just one tumour-associated epitope (i.e. they were considered to be single pronged) whilst AL.86 is multipronged, given its capacity to recognise two TAA peptide epitopes. To further confirm the improved recognition of multiple peptide antigens by multipronged T-cells it would be interesting to assess the avidity of the T-cell clones AL.91, AL.84 and AL.86 to EAAGIGLTV and NLSALGIFST

peptides using a peptide titration. To further explore the potential improved tumour-reactivity of multipronged CD8 T-cells, recognition of three melanoma cell lines, MEL562, MEL 624 and MM909.24 by AL.91, AL.84 and AL.86 was assessed by TAPI-O assay following 4 h incubation (**Figure 4.6B**). Multipronged T-cell clone AL.86, demonstrated greater reactivity to all three melanoma cell lines compared to the single pronged T-cell clones AL.91 and AL.84 (**Figure 4.6B**). T-cell clones AL.91, AL.84 and AL.86 all demonstrated similar reactivity to a non-specific stimulus, CD28/CD3 which acted as a positive control.

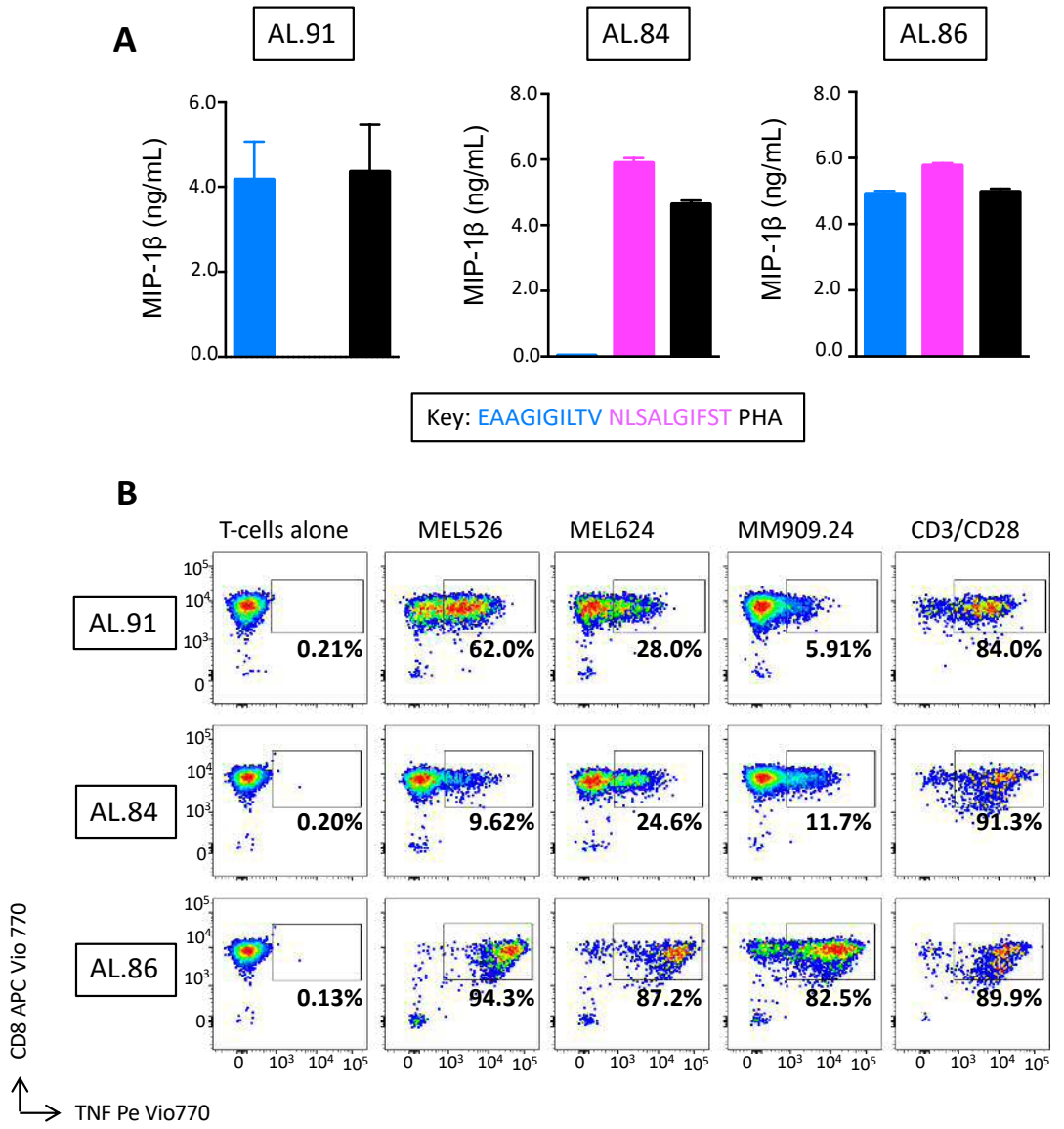


Figure 4.6: Multipronged T-cells exhibit improved recognition of melanoma

TCR clonotypes AL.91, AL.84 and AL.86 were isolated from a healthy HLA A2+ individual based on reactivity to the TAA epitopes EAAGIGILTV and NLSALGIFST. ***A****) Reactivity of* AL.91, AL.84 and AL.86 *to the peptide epitopes EAAGIGILTV and NLSALGIFST was assessed by MIP-1β ELISA at the peptide concentration* 10^{-6}*. Results are shown as the average of two replicates with the error expressed as standard deviation.* ***B****) Reactivity of T-cell clones* AL.91, AL.84 and AL.86 *to melanoma tumour cell lines MEL624, MEL526 and MM909.24 was assessed by TAPI-O assay. Percentage of CD8+ TNF+ T-cells is shown. 5 x*10^4 *T-cells were cultured with 1 x*10^5 *tumour cells for 4 h at 37 °C. Cells were stained with CD8 APC Vio770 and LIVE/DEAD VIVID to allow the identification of live, CD8+ cells. Control wells of T-cells alone and T-cells + CD3/CD28 were included as negative and positive controls.*

4.2.6. Analysis of the peptide specificity and tumour reactivity of therapeutically unsuccessful TCR, DMF4

Redirecting a patients T-cells to attack their tumour, using an HLA class-I restricted cytotoxic TCR is a promising form of cancer immunotherapy. TCR therapy involves the transduction of a tumour-specific TCR into autologous cancer patient T-cells; these T-cells are then transfused back into the patient (so called TCR-T therapy). The EAAGIGILTV-reactive DMF4 TCR, which was isolated from a metastatic melanoma patient who underwent TIL therapy was used as a TCR-T therapy (Morgan *et al.*, 2006). The DMF4 TCR was used to treat 17 metastatic melanoma patients by adoptive transfer. Despite positive *in vitro* results, the DMF4 TCR transduced T-cells mediated partial tumour regression in only two patients (Morgan *et al.*, 2006). It was reasoned that lower peptide sensitivity of DMF4 transduced T-cells for EAAGIGILTV could provide an explanation for the lack of clinical efficacy of the DMF4 TCR (Laura A. Johnson *et al.*, 2009). I wanted to compare the DMF4 TCR to a TCR like ST8.24 that could recognise multiple cancer-derived epitopes.

The MEL5 TCR (Cole *et al.*, 2009b) was derived from a T-cell clone judged to be the most potent of 40 different EAAGIGILTV-reactive clones by my supervisor (Professor Andrew Sewell, personal communication). The MEL5 T-cell has grown very well in culture for over 10 years. Our discovery that the ST8.24 T-cell could simultaneously recognise BST2-and IMP2-derived epitopes in addition to EAAGIGILTV led to my colleague testing whether the potency of the MEL5 T-cell clone was the result of its recognising other epitopes. As working lentiviral transduction systems were available for both DMF4 and MEL5 TCRs as a result of a previous study (Madura *et al.*, 2019), I could compare these two TCRs in an identical system.

Transduction efficiency of the DMF4 and MEL5 TCRs into CD8 T-cells isolated from a healthy, HLA A2 negative donor was assessed by flow cytometry by the expression of transduction marker rCD2. DMF4 and MEL5 TCR transduced CD8 T-cells, matched for rCD2 expression (**Figure 4.7A**), exhibited similar levels of CD3 staining (**Figure 4.7B**). TCR expression could also have been confirmed using tetramers or clonotypic monoclonal antibody staining.

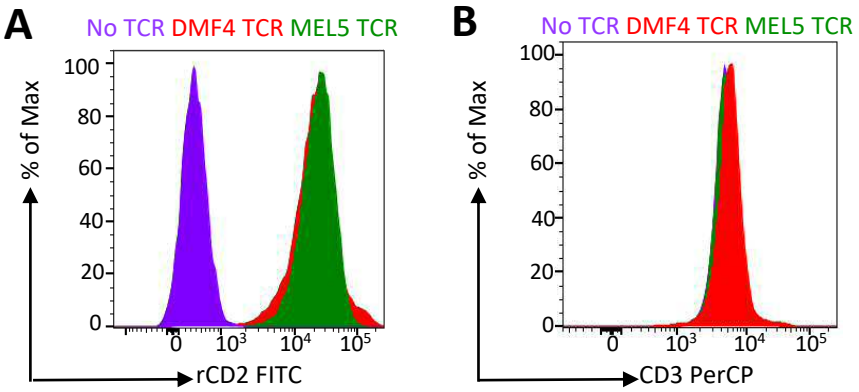


Figure 4.7: rCD2 and CD3 expression of DMF4 and MEL5 TCR transduced CD8 T-cells

CD8 T-cells isolated from a healthy, HLA A2 negative donor were transduced with the MEL5 or DMF4 TCRs by lentiviral transduction. The success and purity of TCR transduction was assess using the surface-expressed marker, rCD2. ***A****) Expression of the transduction marker rCD2 in CD8 T-cells transduced with the MEL5 TCR or the DMF4 TCR compared to non-transduced cells. Cells were also stained with LIVE/DEAD VIVID pacific blue, CD3 PerCP and CD8 APC Vio770 to allow the identification of live, CD3+, CD8+ T-cells.* ***B****) CD3 expression in CD8 T-cells transduced with the DMF4 and MEL5 TCRs compared to non-transduced T-cells. Cells were stained with VIVID pacific blue and CD8 APC Vio770 to allow the exclusion of dead cells. The pELNs plasmid map for TCR transduction can be found in* ***Appendix Figure 6****.*

Reactivity of DMF4 and MEL5 TCR transduced CD8 T-cells to melan-A-derived antigen EAAGIGILTV was assessed by MIP-1β ELISA following overnight activation (**Figure 4.8A**). Both DMF4 and MEL5 transduced CD8 T-cells showed similar reactivity to EAAGIGILTV peptide (**Figure 4.8A**). However, DMF4 TCR transduced T-cells did not display any reactivity to the peptide antigens LLLGIGILVL and NLSALGIFST above the level of untransduced CD8 T-cells at a peptide concentration of 10^{-7} M, whilst the MEL5 TCR demonstrated good reactivity to LLLGIGILVL and NLSALGIFST at the same peptide concentration (**Figure 4.8B**). I compared recognition of melanoma tumour cell lines by T-cells transduced with the DMF4 or MEL5 TCR (**Figure 4.8C and D**). Reactivity of transduced T-cells was assessed by a TAPI-O assay as TNF production following a 4.5 h incubation. T-cells transduced with the DMF4 TCRs demonstrated reduced activation to the three non-autologous melanoma cell lines, MEL624, MM909.24 and MEL526, compared to MEL5 transduced CD8 T-cells (**Figure 4.8C and D**). These results suggest that the recognition of BST2- and IMP2- derived epitopes in addition to the melan-A peptide by the MEL5 TCR could explain why this multipronged TCR is more effective at recognising HLA A2+ melanoma cell lines than the DMF4 TCR. I next hypothesised that this same effect might explain why T-cells primed with the **MTSA**IGIL**P**V peptide in **Chapter 3** were more effective than those primed with the wildtype EAAGIGILTV peptide.

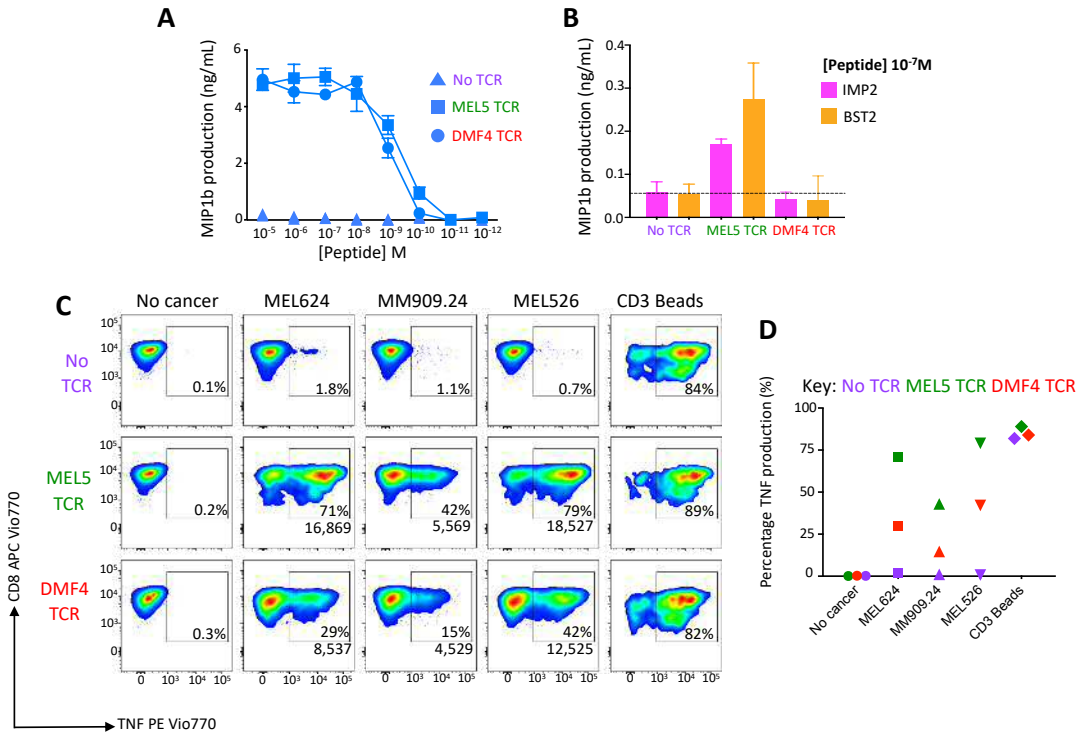


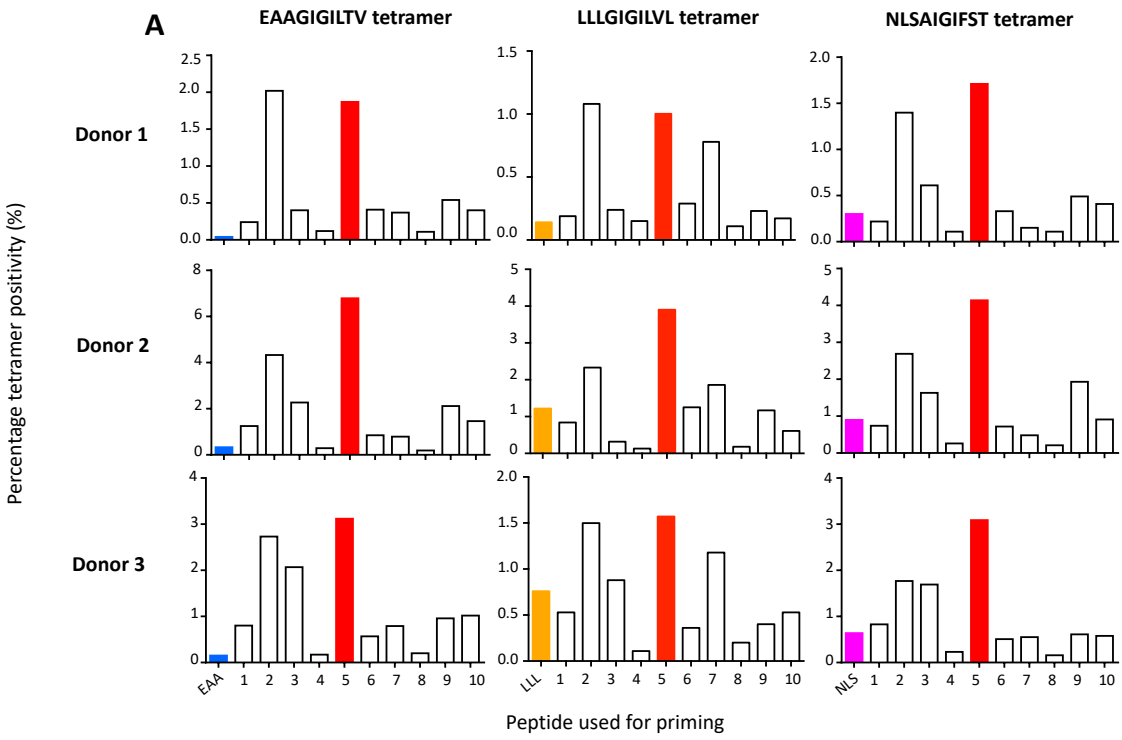
Figure 4.8: Analysis of the peptide specificity and tumour reactivity of therapeutically unsuccessful TCR, DMF4

TCR constructs of DMF4, which was used in a clinical trial for metastatic melanoma, and MEL5, a TCR isolated from a healthy individual, were transduced by lentivirus into healthy donor CD8 T-cells isolated from a HLA A2 negative individual. Untransduced CD8 T-cells from the same healthy donor were also used. rCD2 was included in each construct to act as a marker of successful transduction. ***A****) Reactivity of DMF4 and MEL5 transduced T-cells to EAAGIGILTV at concentration range 10^{-5} to 10^{-12} M was assessed by a MIP-1β ELISA following overnight activation. Data is shown as an average of two replicates and error is depicted as standard deviation.* ***B****) The reactivity of DMF4, MEL5 and untransduced CD8 T-cells to tumour associated antigens LLLGIGILVL and NLSALGIFST was assessed by ELISA, MIP-1β production is shown at the peptide concentration of 10^{-7} M. Data is depicted as an average of two replicates with the standard deviation shown.* ***C****) Reactivity of DMF4, MEL5 and non-transduced CD8 T-cells to three HLA A2+ melanoma cell lines, MEL624, MM909.24 and MEL526, was assessed after 4.5 h by a TAPI-O assay. Reactivity is expressed as the percentage of CD8+ cells producing TNF. T-cells were incubated alone and with CD3/CD28 beads as negative and positive control. Cells were stained for LIVE/DEAD VIVID pacific blue to allow the exclusion of dead cells.* ***D****) TNF production by DMF4, MEL5 and non-transduced CD8 T-cells after 4.5 h incubation with three melanoma tumours as in* ***C****.*

4.2.7. Peptide priming with super-agonist peptide MTSAIGILPV elicits CD8 T-cells specific for LLLGIGILVL and NLSALGIFST from HLA A2+ healthy donors

In **Chapter 3** I described the identification of a panel of super-agonist peptides. By screening these peptides using healthy polyclonal CD8 T-cell populations and a panel of T-cell clones, the optimal super-agonist peptide **MTSA**IGIL**P**V was identified. **MTSA**IGIL**P**V primed more

EAAGIGILTV-restricted CD8 T-cells than EAAGIGILTV in all healthy donors tested and two melanoma patient samples. Since the cross-reactive TIL derived CD8 T-cell clone ST8.24 was used to identify **MTSA**IGIL**P**V, I reasoned that **MTSA**IGIL**P**V may be able to elicit CD8 T-cells that could also recognise the novel TAA epitopes, LLLGIGILVL and NLSALGIFST. Polyclonal CD8 T-cells isolated from three HLA A2+ healthy donor PBMC, isolated from buffy coats supplied by the WBS, were primed with EAAGIGILTV, LLLGIGILVL, NLSALGIFST or one of the ten candidate super-agonist peptides for 14 days. The success of peptide priming was assessed using peptide-MHC tetramers for EAAGIGILTV, LLLGIGILVL and NLSALGIFST. Previously identified super-agonist peptide **MTSA**IGIL**P**V elicited more NLSALGIFST-tetramer positive cells in all three donors than the WT peptide and all other super-agonist peptides (**Figure 4.9A**, right hand column). In donors 2 and 3, **MTSA**IGIL**P**V elicited more LLLGIGILVL and EAAGIGILTV tetramer positive cells than the WT peptides and the other nine super-agonist candidates (**Figure 4.9A**, left hand and central columns). However, in the case of donor 1, whilst **MTSA**IGIL**P**V elicited more WT-tetramer positive cells than LLLGIGILVL and EAAGIGILTV WT peptides, peptide 2 (**ITSA**IGIL**P**V) elicited more tetramer positive cells overall (**Figure 4.9A**, left hand and central columns). The data in **Figure 4.9** confirms that the **MTSA**IGIL**P**V peptide induces more EAAGIGILTV-, LLLGIGILVL- or NLSALGIFST-specific T-cells than any of the natural peptide sequences and confirms that this super-agonist peptide has enhanced potential to prime poly-specific, multipronged T-cells in all donors tested.



B

Code	Peptide sequence	Code	Peptide sequence
EAA	EAAGIGILTV	5	MTSAIGILPV
LLL	LLLGIGILVL	6	LTSAIGVLPV
NLS	NLSAIGIFST	7	ITSGIGVLPV
1	ITSAIGVLPV	8	ITSAIGVLPI
2	ITSAIGILPV	9	QTSAIGILPV
3	MTSAIGVLPV	10	ITSAIGVLPV
4	QTSAIGVLPV		

Figure 4.9: Super-agonist peptide MTSAIGILPV primes more EAAGIGILTV, LLLGIGILVL, NLSALGIFST CD8 T-cells than the WT peptides in 3 HLA A2+ healthy donors.

*Polyclonal CD8 T-cells isolated from 3 different HLA A2+ healthy donors were primed for 14 days with EAAGIGILTV (blue), LLLGIGILVL (yellow), NLSAIGIFST (pink) and a panel of 10 potential super-agonist peptides including previously identified super-agonist peptide MTSAIGILPV (red). Polyclonal CD8 T-cells were then subjected to pMHC tetramer staining with WT tetramers; EAAGIGILTV, LLLGIGILVL and NLSALGIFST using an optimised protocol (PKI + tetramer + anti-PE 1◦ antibody). VIVID Live/Dead Pacific Blue was used to allow exclusion of dead cells and CD8 APC and CD3 PerCP to identify CD8 T-cells. Plots depict tetramer staining with one of the WT tetramers and include the corresponding WT peptide primed CD8 T-cells alongside the 10 candidate super-agonists. Associated flow cytometry plots can be found in **Appendix figures 6, 7 and 8**. **B**) Table of peptides used for priming in **A.** Agonists designed by CPL screening TCR clonotype ST8.24 are depicted in black. Validated epitopes identified from the ST8.24 CPL screen from TAA melan-A (blue), BST2 (orange) and IMP2 (pink) are also shown.*

4.3. Discussion

4.3.1. T-cell cross-reactivity

The potential peptide pool available to be presented by MHC class I molecules to CD8 T-cells is vast. This peptide pool is comprised of peptides from self, foreign and mutated proteins. Furthermore, proteins can be post-translationally modified, leading to the generation of an additional selection of peptides with potential to become T-cell ligands. It is not possible that the original dogma of one T-cell to one antigen, first proposed in the clonal selection theory (Jerne, 1971), is realistic. It is essential that CD8 T-cells possess the capacity to recognise more than one peptide-MHC molecule. This distinct ability is termed T-cell cross-reactivity and it is critical to enable T-cells to protect against all invading pathogens that breach the innate immune system.

Cross-reactive TCRs have been identified in several disease settings. Primary viral infections have been demonstrated to activate CD8 T-cells specific for an unrelated virus to which the patient has already been exposed. Such cross-reactive T-cells been linked to the rapid expansion of influenza A virus-specific CD8 T-cells which occurs during EBV-associated infectious mononucleosis (Clute, 2005). Memory CD8 T-cells cross-reactive to the EBV $BMLF1_{280-288}$ and influenza A virus $M1_{58-66}$ epitopes were identified by pMHC-tetramer staining in HLA A2 positive individuals. The EBV and M1 antigens are distinct and only possess 33% sequence homology (Clute, 2005; Clute *et al.*, 2010). The recognition of a peptide by a TCR may be dependent on conserved amino acids in the peptide sequence, termed a motif. For example, a preproinsulin specific CD8 TCR which was demonstrated to recognise over one million peptide epitopes (Wooldridge *et al.*, 2012) was found to recognise multiple peptides through a conserved hotspot 'lock and key' binding motif, allowing for variability in the remaining peptide residues (Cole *et al.*, 2016). Identifying cross-reactivity can provide therapeutic benefits, activating tumour associated antigen T-cells with bacterial epitopes, overcoming central tolerance and eliciting a more potent anti-tumour response. For example, peptide priming with an epitope, HF-$2_{216-229}$, derived from *mycoplasma penetrans*, a common microbe, elicits MAGE A6 reactive T-cells in HLA A2+ patients and healthy donors. The cross-reactive T-cells able to recognise the MAGE $A6_{172-187}$ epitope and could kill MAGE A6+ tumours *in vitro* (Vujanovic *et al.*, 2014).

It has been previously demonstrated that melan-A reactive CD8 T-cells from HLA A2 positive individuals are cross-reactive (Dutoit *et al.*, 2002). Melan-A restricted TCRs have been shown to have a bias towards the TRAV 12-2 gene which interacts with antigen presented on HLA

A2 through its germline encoded CDR1 region (Trautmann *et al.*, 2002; Dietrich *et al.*, 2003; Cole *et al.*, 2009). This is an unusual interaction as the dogma dictates that TCRs interact with peptide mainly through their hypervariable CDR3 regions. The unusual binding mode of melan-A-specific TCRs, including MEL5 (Cole *et al.*, 2009), might explain the high degree of cross-reactivity displayed by melan-A restricted TCRs.

This chapter focused on a TCR clonotype, ST8.24, isolated from the TIL infusion product given to a metastatic melanoma patient MM909.24 who underwent complete remission after TIL therapy (Rikke Andersen *et al.*, 2016). This TCR clonotype recognises the wild type antigen from melan-A, EAAGIGILTV, and was used to identify the super-agonist peptide **MTSA**IGIL**P**V in **Chapter 3.**

4.3.2. The ST8.24 T-cell recognises multiple melanoma-derived antigens

Interrogation of the combinatorial peptide library (CPL) screen of ST8.24 shown in **Chapter 3** using an online database of known tumour associated proteins (Tumour Antigen Database, Dr Garry Dolton and Dr Barbara Szomolay, publication pending) generated a ranked list of potential novel epitopes for ST8.24 (**Figure *4.1***). Whilst all the proteins identified by the search have already been associated with cancer, some of the peptide epitopes had not previously been described in the literature. Peptide titrations and peptide-MHC tetramer staining confirmed the reactivity of ST8.24 to two novel tumour-associated antigen epitopes, LLLGIGILVL (derived from BST2) and NLSALGIFST (derived from IMP2) (**Figure *4.2***). Both BST2 and IMP2 have previously been described in the literature as being upregulated in a range of cancers, however the epitopes LLLGIGILVL and NLSALGIFST were first described by the Sewell Laboratory (unpublished). The ability of ST8.24 to recognise multiple tumour associated antigen epitopes, on the same tumour cell, could be advantageous to the patient when attempting to clear their tumour as such multipronged recognition would prevent tumour escape by loss of antigen.

Dunn's theory of cancer immunoediting comprises what is known as 'the three E's'; elimination, equilibrium and escape (Dunn *et al.*, 2002). This theory implicates a role of the immune system in removing, maintaining and ultimately allowing the 'escape' of malignant cells. Initially, T-cells recognise and kill transformed cells due to the aberrant display of an unusual array of peptide antigens on the tumour surface. T-cells are also able to maintain a pre-cancerous mass in a state of equilibrium, however during this time sustained T-cell activity induces selection pressure on the tumour cells removing the immunogenic cells and

allowing the non-immunogenic cells to thrive. After a period of selection, tumour cells not expressing the original immunogenic antigens can escape immune control, leading to the development of a tumour mass. The ability of multipronged T-cells to recognise multiple distinct peptide epitopes on the surface of a malignant cell, could prevent the escape of cancerous cells from the control of the immune system and ultimately prevent the development of metastatic disease (**Figure 4.10**).

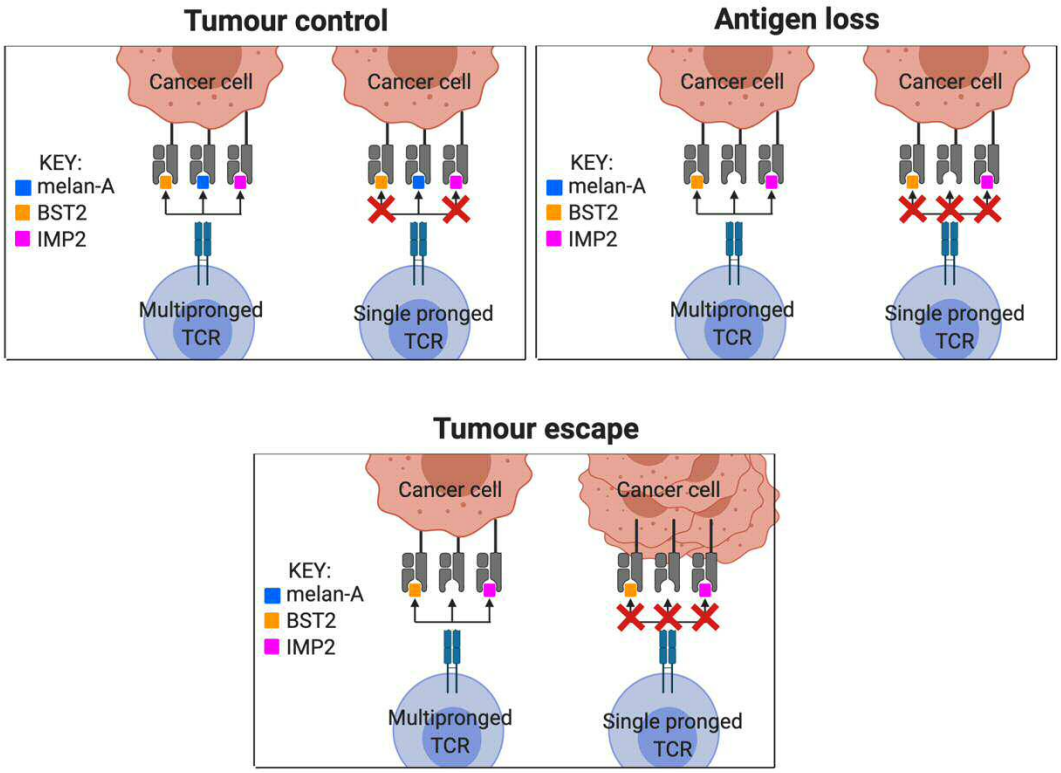


Figure 4.10: Multipronged TCRs can prevent tumour escape

Multipronged TCRs have the capacity to recognise different tumour associated antigen peptides on the surface of the same tumour cell, whilst single pronged TCRs can only recognise one peptide antigen. During the lifetime of a cancer cell mutations can occur leading to antigen loss, sometimes these mutations are driven by non-specific T-cell selection pressure. For a multipronged TCR, antigen loss does not affect the T-cells ability to recognise tumour, preventing tumour escape. However, for a single pronged TCR, antigen loss could lead to lack of tumour recognition, tumour escape and development of malignant disease.

4.3.3. Tumour associated antigen BST2 and IMP2 are widely expressed

We identified LLLGIGILVL (BST2) and NLSALGIFST (IMP2) as novel epitopes for TCR clonotype ST8.24. I next examined the expression levels of BST2 and IMP2 in a cohort of melanoma tumours and on cell lines from different cancers (**Figure 4.3**). This was done using published RNAseq data obtained from the CCIT, Copenhagen (Lauss *et al.*, 2017) and cell line data

obtained from TRON Cell Line Portal (Scholtalbers *et al.*, 2015). All melanoma tumours assess expressed at least two of the TAA melan-A, BST2 and IMP2. Three melanoma tumours, from patients 11, 22 and 36, did not express TAA melan-A which is commonly overexpressed in melanoma (**Figure 4.3A**). The lack of melan-A antigen expression might have been the result of selection pressures applied by mono-specific melan-A-reactive T-cells. The melan-A negative tumour cells expressed the TAAs BST2 and IMP2, therefore multipronged T-cells, capable of recognising BST2 or IMP2 would still be able to kill melan-A negative tumours, whilst single pronged melan-A-reactive T-cells would no longer be able to kill these tumours leading to enhanced tumour growth.

The TAAs BST2 and IMP2 were also found to be expressed in several non-melanoma tumour cells lines (**Figure 4.3B**). Every tumour cell line assessed expressed BST2 or IMP2, with many cell lines expressing both antigens. Ubiquitous expression of BST2 and IMP2 highlights these antigens as pan-cancer vaccine targets.

4.3.4. TCR clonotype ST8.24 recognises multiple tumour cell lines

As demonstrated in **Figure 4.2**, TCR clonotype ST8.24 can recognise the TAA epitopes EAAGIGILTV, LLLGIGILVL and NLSALGIFST. The proteins from which these peptides derive; melan-A, BST2 and IMP2 are widely expressed in many tumour cell lines (**Figure 4.3**). It was then hypothesised that ST8.24 would be able to recognise these TAA on other melanomas and other tumour cell lines from different sources. ST8.24 recognised multiple different cancer cell lines in an HLA A2 restricted manner (**Figure 4.4**). This result further strengthened the hypothesis that ST8.24 is multipronged and can recognise multiple TAA epitopes via HLA A2. Eliciting multipronged TCRs through cancer immunotherapy, could be beneficial for patients with non-melanoma tumours. Furthermore, MHC-class I-restricted TCRs can be used as TCR-T therapies. Multipronged TCR clonotypes, such as ST8.24, are attractive for this purpose given their capacity to recognise multiple TAAs and kill a wide range of tumour cell types.

4.3.5. Multipronged CD8 T-cells can prevent melanoma tumour escape from the immune response

The escape of tumour cells from the control of the immune system can lead to rapid development of metastatic disease. TIL-derived T-cell clone ST8.24 can recognise the autologous MM909.24 tumour via more than one TAA, as demonstrated by melan-A

negative MM909.24 tumour cell line (**Figure 4.6**). Furthermore, an additive effect of the TAA epitopes, EAAGIGILTV, LLLGIGILVL and NLSALGIFST allows ST8.24 to recognise peptides at a sub-optimal concentration (**Figure 4.6**). Tumour cells can downregulate pMHC at their surface, leading to low frequencies of peptide antigen being displayed to CD8 T-cells. The additive effect demonstrated here, could allow multipronged TCRs to recognise tumour cells with low frequencies of antigen, preventing tumour escape *in vivo*. These experiments also demonstrate how the recognition of multiple TAA by a single TCR can enhance the sensitivity of cancer cell killing.

The ability of multipronged TCRs to recognise tumours more efficiently than single pronged TCRs was further demonstrated by T-cell clonotypes AL.91, AL.84 and AL.86. TCR clone AL.86 is multipronged, demonstrated by its increased capacity to recognise three melanoma cell lines MEL624, MEL 526 and MM909.24 and recognise TAA peptides EAAGIGILTV and NLSALGIFST (**Figure 4.6**). TCR clonotypes AL.91 and AL.84 are single pronged and were not as reactive to MEL526, MEL624 or MM909.24 as AL.86 following a 4 h activation assay (**Figure 4.6**).

4.3.6. The DMF4 TCR does not recognise BST2 or IMP2

Multipronged TCR clonotypes are attractive TCRs for use in adoptive cell transfer, given their ability to recognise multiple TAAs. Melan-A-specific (Morgan *et al.*, 2006; Johnson *et al.*, 2009) and MAGE-A3- specific (Gerald P. Linette *et al.*, 2013; Richard A Morgan *et al.*, 2013) TCRs have been used to treat metastatic melanoma patients as a TCR-T therapy with overall disappointing clinical outcomes. I assessed the epitope specifies of the DMF4 TCR which demonstrated poor clinical outcomes in metastatic melanoma patients (Morgan *et al.*, 2006). In order to improve upon clinical outcomes, the same research team identified the TCR DMF5, which has a higher affinity for melan-A derived peptide EAAGIGILTV. This TCR was more effective, and treatment resulted in an increase in objective clinical responses however many patients experienced severe side-effects (Johnson *et al.*, 2009). The DMF4 TCR recognises the melan-A antigen EAAGIGILTV, however it does not recognise the TAA epitopes identified in this chapter; LLLGIGILVL or NLSALGIFST (**Figure 4.8A and B**). The MEL5 TCR clonotype, which was isolated from a healthy HLA A2+ individual, recognises TAA epitopes; EAAGIGILTV, LLLGIGILVL and NLSALGIFST and is multipronged. When compared to T-cells transduced with the MEL5 TCR, DMF4 TCR transduced T-cells produced less TNF upon encountering MEL 624, MEL 526 and MM909.24 melanoma tumour cell lines (**Figure 4.8C and D**). These data infer that DMF4 transduced T-cells are less effective at killing HLA A2+

melanoma cell lines when compared to MEL5 TCR transduced T-cells. One such explanation for the improved functional capacity of MEL5 TCR transduced T-cells is their capacity to recognise multiple TAA epitopes from the TAAs; melan-A, BST2 and IMP2. TAAs BST2 and IMP2 are widely expressed by melanoma tumour cells, as demonstrated in **Figure 4.3**. The inability of DMF4 to recognise these epitopes could provide an explanation as to why the DMF4 TCR provided poor results in the clinic. The ST8.24 T-cell was derived from successful TIL therapy and shown to persist in patient blood after complete remission. My staining results in **Figure 4.2C** indicate that >90% of the melan-A specific T-cells within the TIL used to treat patient MM909.24 did not recognise BST2 or IMP2. It is interesting to speculate that part of the reason that patient MM909.24 cleared their cancer might have been because the dominant persistent T-cell clonotype ST8.24, that was expanded in patient blood, could also recognise the patient's tumour in the absence of melan-A (**Figure 4.5**).

Given that the DMF5 TCR has a higher affinity for EAAGIGILTV, compared to the DMF4 TCR, and was slightly more clinically successful, it would be of interest to this study to assess whether it recognises TAAs epitopes LLLGIGILVL and NLSALGIFST and whether the tumour reactivity of DMF5 TCR transduced T-cells is comparable to those transduced with the MEL5 TCR. This work was in progress at the time of writing but the DMF5 lentiviral construct was not made prior to our laboratory shutting down due to the COVID-19 pandemic.

In this chapter, I studied ST8.24, MEL5 and AL.86 T-cells that could all recognise both BST2 and IMP2 epitopes in addition to melan-A. These TCR clonotypes were isolated from a melanoma patient (ST8.24) and different healthy HLA A2+ individuals (AL.86 and MEL5). Therefore, it is likely that EAAGIGILTV-specific multipronged TCRs might be common in HLA A2+ individuals. Furthermore, the presence of multipronged TCRs in healthy individuals infers that they might be safe to target with immunotherapeutic strategies as these TCRs have passed thymic selection and do not cause autoimmunity.

4.3.7. **MTSA**IGIL**P**V peptide-induced CD8 T-cells that recognise multiple cancer antigens

The super-agonist peptide **MTSA**IGIL**P**V was identified in **Chapter 3** as a potential peptide vaccine candidate. Increased binding of **MTSA**IGIL**P**V to HLA A2 compared to the WT melan-A peptide EAAGIGILTV and a remarkable structural mimicry when bound to HLA A2 provided some explanation to the superior peptide priming demonstrated by **MTSA**IGIL**P**V. The identification that the TCR clonotype, ST8.24, used to design super-agonist peptide

MTSAIGIL**P**V can also respond to epitopes from BST2 and IMP2 provides a potential reason as to why **MTSA**IGIL**P**V-primed CD8 T-cells demonstrated superior killing of melanoma tumour cells. Peptide priming of HLA A2+ healthy donor CD8 T-cells with **MTSA**IGIL**P**V peptide and subsequent pMHC-tetramer staining with LLLGIGILVL and NLSALGIFST pMHC-tetramers demonstrated that **MTSA**IGIL**P**V priming can elicit LLLGIGILVL and NLSALGIFST tetramer positive cells (**Figure 4.9**). Furthermore, in all three healthy donors tested super-agonist peptide **MTSA**IGIL**P**V primed more LLLGIGILVL and NLSALGIFST-tetramer positive cells than the WT peptides themselves. This data indicates that priming with super-agonist peptide **MTSA**IGIL**P**V may preferentially elicit multipronged CD8 T-cells over those that recognise only individual antigens. **MTSA**IGIL**P**V peptide vaccine may therefore elicit multipronged T-cells and could prevent tumour escape.

The ubiquitous expression of BST2 and IMP2 in a wide array of different tumours, as demonstrated in **Figure 4.3**, led me to hypotheses that super-agonist peptide **MTSA**IGIL**P**V could elicit multipronged T-cells in different cancer setting, distinct from metastatic melanoma. Super-agonist peptide **MTSA**IGIL**P**V's ability to prime multipronged T-cells in samples from renal cell carcinoma (RCC), chronic lymphocytic leukaemia (CLL) and acute myeloid leukaemia (AML) was assessed in **Chapter 5.**

4.3.8. Summary

In this chapter, the **MTSA**IGIL**P**V and EAAGIGILTV-reactive CD8 T-cell clone ST8.24 was shown to be cross-reactive. The data from the CPL screen, shown in **Chapter 3**, was used in conjunction with an online database to generate a list of potential epitopes for ST8.24 derived from known TAA. Two of the predicted epitopes, LLLGIGILVL and NLSALGIFST derived from TAA BST2 and IMP2 respectively, have not been previously described. ST8.24 was shown to effectively recognise these new epitopes (**Figure 4.2**). This poly-specificity was termed "multipronged" in our laboratory to indicate that such T-cells targeted cancer via multiple TAA. TAA BST2 and IMP2 are frequently expressed in tumours from multiple different cancers, including melanoma. Peptide and tumour reactivity of T-cells transduced with another multipronged TCR MEL5 and a therapeutic TCR DMF4, demonstrated that multipronged TCRs are functionally superior. Furthermore, T-cell clones with the capacity to recognise more than one TAA epitope, EAAGIGILTV and NLSALGIFST, were also found to be better at recognising melanoma tumour lines compared to T-cells that could only recognise one of the epitopes. Finally, **MTSA**IGIL**P**V peptide priming of polyclonal CD8 T-cells from healthy donors elicited more LLLGIGILVL and NLSALGIFST-reactive CD8 T-cells than priming

with the WT peptides. Overall, this work highlights the superiority of multipronged CD8 T-cells and demonstrates the importance of harnessing them for cancer immunotherapy. This work also begins to demonstrate that super-agonist peptide **MTSA**IGIL**P**V could be a pan-cancer peptide vaccine candidate given its ability to prime CD8 T-cells specific for TAA epitopes, EAAGIGILTV, LLLGIGILVL and NLSALGIFST which are widely expressed on a range of tumour cell lines.

5. Super-agonist peptide MTSAIGILPV as a pan-cancer vaccine candidate

5.1. Background

In **Chapter 3**, I showed that the **MTSA**IGIL**P**V peptide could induce large numbers of melan-A-specific T-cells that have an improved ability to kill melanoma targets over those that were primed with the natural melan-A epitope EAAGIGILTV. The improved binding of **MTSA**IGIL**P**V to both HLA A2 and EAAGIGILTV-specific TCRs provided an explanation as to why the super-agonist peptide might induce a greater number of T-cells than the wildtype melan-A-derived sequence. These properties did not explain why **MTSA**IGIL**P**V-primed T-cells exhibited improved function in terms of killing HLA A2+ melanoma cells. In **Chapter 4**, I showed that **MTSA**IGIL**P**V-primed T-cells were far more likely to recognise two other, new cancer epitopes LLLGIGILVL derived from BST2 and NLSALGIFST derived from IMP2. This finding provided a reason for why **MTSA**IGIL**P**V-primed T-cells might exhibit improved cancer recognition over those primed with the natural EAAGIGILTV peptide. **MTSA**IGIL**P**V- primed T-cells were shown to be more likely to recognise multiple antigens than those primed with LLLGIGILVL or NLSALGIFST peptide in all three donors tested. I further demonstrated that BST2 and IMP2 were expressed by many types of cancer. Collectively, my findings in **Chapter 3** and **Chapter 4** suggest that the **MTSA**IGIL**P**V peptide might make a good candidate vaccine for many cancer types.

I was keen to extend my finding with the **MTSA**IGIL**P**V peptide in an *ex vivo* situation by using it to prime T-cells that killed autologous non-melanoma cancers. For this work I used PBMC samples from the CCIT from renal cell carcinoma patients (Andersen *et al.*, 2015; Rikke Andersen *et al.*, 2018). I also decided to examine blood cancers as both the cancer and the autologous CD8 T-cells could be obtained from a single, minimally invasive blood donation.

5.1.1. Renal cell carcinoma

Renal cell carcinoma is the 9th most diagnosed cancer in the world, with cases predominantly occurring in developed nations (Jonasch, Gao and Rathmell, 2014). RCC patients often present as metastatic due to the asymptomatic nature of early stage disease. Stage IV RCC is often treatment resistant and predicted long term survival of patients is poor, with <10% of patients surviving beyond two years (van den Berg, 2013). For patients who present with metastasis or where surgical intervention is not possible standard cancer therapies have

demonstrated little effect. Initially, RCC was found to be resistant to chemotherapy and hormone therapy (Harris, 1983; Yagoda and Bander, 1989). Furthermore, biologics such as IFNα and IL-2 provide minimal clinical success with only a small subset of patients responding (Fyfe *et al.*, 1995; Motzer *et al.*, 2002). Currently the most successful treatments are inhibitors of key cellular processes such as tyrosine kinases, mTOR inhibitors, and VEGF inhibitors which disrupt angiogenesis (Motzer *et al.*, 2007). Genetic, phenotypic and subtype heterogeneity within RCC provides challenges for treatment of the disease, specifically when attempting to delineate resistance to therapy and when designing personalised therapeutics (Fisher, Larkin and Swanton, 2012).

Recent immunotherapy trials have demonstrated a positive role for CD8 T-cells in RCC treatment. A dendritic cell (DC) vaccine consisting of autologous DCs transfected with autologous tumour RNA and CD40 ligand administered in conjunction with sunitinib, a protein kinase inhibitor, demonstrated clinical benefit in 62% of patients with a greater than five year survival in 24% of patients (Amin *et al.*, 2015). This result was attributed to CD8 T-cell infiltration as the number of effector/memory CD8+ T-cells seen after treatment correlated with overall survival (Amin *et al.*, 2015). Moreover, a 29% of metastatic RCC patients treated with nivolumab, an anti-PD1 monoclonal antibody, achieved objective responses to the treatment (McDermott *et al.*, 2015). Tumour infiltrating lymphocyte (TIL) therapy has shown remarkable success in patients with metastatic melanoma, with CD8 T-cells appearing to be the dominant effectors of cancer regression (Rosenberg, *et al.*, 1986; Rosenberg *et al.*, 1994; Andersen *et al.*, 2016). Recent attempts to generate TIL for therapy from RCC have been successful (Baldan *et al.*, 2015; Rikke Andersen *et al.*, 2018), with Baldan *et al.* successfully expanding TIL cultures in 90% of patients. Whilst these results are promising, there is still room for improvement highlighting the need for further research. My research laboratory had samples from RCC patients including autologous tumour line. This allowed me to extend my studies beyond melanoma to determine if it was possible to elicit tumour-reactive cells using super-agonist peptide **MTSA**IGIL**P**V in RCC patient samples.

5.1.2. Leukaemia

Leukaemia encompasses any cancer that originates in blood-forming tissue, most commonly bone marrow, and ultimately leads to an over-production of white blood cells. Leukaemia can be characterised by the type of immune cell it derives from, myeloid or lymphoid, and as acute or chronic, based on the progression of the disease. Generally speaking, chronic leukaemia develops slower, in more differentiated cell types whilst acute leukaemia occurs

rapidly in immature haematopoietic cells. There are four main classifications of leukaemia; acute myeloid leukaemia (AML), acute lymphoid leukaemia (ALL), chronic myeloid leukaemia (CML) and chronic lymphoid leukaemia (CLL). First line treatment of leukaemia is generally chemotherapy followed by allogenic hematopoietic cell transplant (HCT) when a HLA matched donor is available. HCT is widely considered the most effective pre-remission treatment as it removes any minimal residual disease (MRD) which has been attributed to relapse experienced by many patients (Hamilton and Copelan, 2012).

Leukaemia has become somewhat of a model cancer for T-cell immunotherapies due to the remarkable success of CAR T-cell therapy or treating some lymphoid malignancies in some patients. This success is reflected in the fact that two CAR T therapies have been licenced by the FDA in recent years (Neelapu *et al.*, 2017; Maude *et al.*, 2018). I focussed my own studies on CLL and AML to assess two leukaemia's of different cellular origin, one chronic and one acute. CLL and AML will be introduced below.

5.1.2.1. Chronic lymphocytic leukaemia (CLL)

Chronic lymphocytic leukaemia (CLL) is the most common adult leukaemia in the western world (Zenz *et al.*, 2010). It is characterised by the slow accumulation of a clonal population of B-lymphocytes in the peripheral blood, bone marrow, lymph nodes and other secondary lymphoid tissues. Early stage CLL is asymptomatic, inert and does not require active treatment, in fact a randomised clinical trial demonstrated that the treatment of indolent CLL has no clinical benefit to patients (Dighiero *et al.*, 1998). Patients undergo routine monitoring of disease and are only treated once their disease becomes active.

CLL tumour cells are dependent on their tumour microenvironment for proliferation and survival, requiring positive signals from CD40L and interleukin-4 (IL-4) to maintain homeostasis, proliferation and prevent apoptosis (Lagneaux *et al.*, 1998). CLL cells form pseudofollicles in secondary lymphoid tissues to maintain a conducive microenvironment for survival. CLL cells recruit other immune cells, such as T-cells, and bone marrow stromal cells, into their periphery to provide stimulation though CD40L expression and the release of cytokines such as IL-4. Specifically, studies have demonstrated the inherent ability of CLL tumour cells to secrete T-cell chemokines and chemoattractants such as CCL22 (Ghia *et al.*, 2002) and CCL3 and CCL4 (Burger *et al.*, 2009). CLL tumour cells appear to benefit from the presence of CD3+ T-cells and stromal cells in their microenvironment as contact with CD40L+

cells reduces expression of intracellular, pro-apoptotic proteins such as survivin (Granziero *et al.*, 2001), prolonging the life of CLL tumour cells.

5.1.2.2. Acute myeloid leukaemia (AML)

Acute myeloid leukaemia (AML) results from the accumulation of myeloid blasts in the bone marrow, which ultimately escape into the peripheral blood and infiltrate vital organs. AML is a highly heterogeneous disease, both within and between patients. At diagnosis, many patients present with a polyclonal population of myeloid blasts harbouring different genetic and morphological characteristics.

The heterogeneity of AML can make it more difficult to treat. Up to 70% of AML patients achieve complete remission after chemotherapy however the majority of patients eventually relapse (Rowe *et al.*, 2005; Ravandi, 2013). There is accumulating evidence of the importance of CD8 T-cells in the immune systems defence against AML. Knaus *et al* demonstrated a positive correlation between the upregulation of co-stimulatory molecules and down-regulation of apoptosis and inhibitory proteins in CD8 T-cells to therapeutic success in AML patients (Knaus *et al.*, 2018). Furthermore, CD8 T-cells have been identified in peripheral blood of AML patients specific for leukaemia-associated antigens Wilms' tumour protein 1 (WT1) and proteinase-3 (PR3) which are highly expressed in AML tumour cells (Flörcken *et al.*, 2013). These T-cells were also highly effective at killing AML tumour *in vivo* (Flörcken *et al.*, 2013). A recent clinical trial prophylactically treated AML patients with a high risk of relapse after HCT with a high affinity WT1-specific TCR, 12/12 patients treated were relapse free at the end of the trial (Chapuis *et al.*, 2019). However, perhaps the most convincing evidence of the role of T-cells in the control of AML is the graft vs leukaemia effect seen after allogenic stem cell transplantation. Transplanted cells are able to eradicate AML tumour, resulting in complete and lasting remission in some patients (Anguille, Van Tendeloo and Berneman, 2012).

5.1.3. Aims

The work in **Chapter 3** highlighted the ability of a super-agonist peptide, **MTSA**IGIL**P**V, to elicit large populations of melan-A reactive CD8 T-cells from HLA A2 healthy donors and two melanoma patients. **Chapter 4** demonstrated that **MTSA**IGIL**P**V can also elicit CD8 T-cells That recognise novel tumour associated epitopes derived from BST2 and IMP2, which are commonly over-expressed in many cancer types. I therefore sought to extend my studies on **MTSA**IGIL**P**V-primed T-cells to three other cancer types: RCC, CLL and AML.

This chapter sought to achieve the following aims:

- Demonstrate that **MTSA**IGIL**P**V super-agonist peptide can elicit multipronged CD8 T-cells from patients with cancers other than melanoma
- Demonstrate that **MTSA**IGIL**P**V-primed T-cells are superior at killing autologous tumour in non-melanoma cancers
- Interrogate the cross-reactivity of individual **MTSA**IGIL**P**V-reactive TCR clonotypes from non-melanoma patients

5.2. Results

5.2.1. Successful priming with super-agonist peptide MTSAIGILPV of CD8 T-cells from renal cell carcinoma (RCC) patient samples

Chapter 4 demonstrated that **MTSA**IGIL**P**V-primed CD8 T-cells could also recognise other tumour associated epitopes from BST2 and IMP2 in addition to the established melanoma antigen melan-A. Furthermore, it was shown that priming CD8 T-cells from healthy HLA A2 positive donors with super-agonist peptide **MTSA**IGIL**P**V elicited T-cells that saw all three epitopes (multi-pronged T-cells). It was therefore hypothesised that **MTSA**IGIL**P**V could be a promising pan-cancer peptide vaccine candidate. I decided to explore this hypothesis by assessing the capacity of super-agonist peptide **MTSA**IGIL**P**V to prime CD8 T-cells in a non-melanoma cancer setting. In **Chapter 4**, I demonstrated that the TAA BST2 and IMP2 are expressed in renal cancer cell lines. Through an ongoing collaboration with the CCIT, Copenhagen, I was able to obtain PBMC from two HLA A2+ renal cell carcinoma (RCC) patients who are enrolled in an ongoing clinical trial (Andersen *et al.*, 2015; Rikke Andersen *et al.*, 2018).

Initially, I wanted to see if it was possible to prime **MTSA**IGIL**P**V-reactive cells from a RCC patient sample. CD8 T-cells were isolated from RCC patient 2 and primed for 28 days or cultured alone (**Figure 5.1**). Success of peptide priming was determined by staining with **MTSA**IGIL**P**V or preproinsulin tetramers. Peptide priming led to 0.84% **MTSA**IGIL**P**V-specific CD8 T-cells compared to 0.37% in absence of peptide (**Figure 5.1**). The percentage of tetramer positive cells is lower than previously seen when priming healthy donor T-cells in **Chapter 3,** this effect could be a result of T-cell exhaustion in patient T-cells. This result confirms that is it possible to elicit **MTSA**IGIL**P**V-specific CD8 T-cells from RCC patient samples.

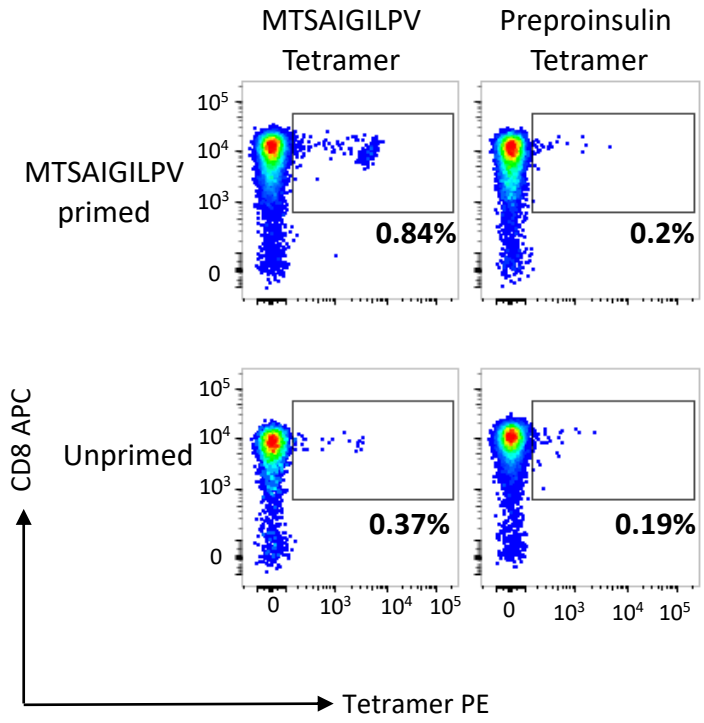


Figure 5.1: Successful priming with super-agonist peptide MTSAIGILPV of CD8 T-cells from RCC patient 2

*CD8 T-cells isolated from renal cell carcinoma patient 2 were primed for 28 days with super-agonist peptide **MTSA**IGIL**P**V or cultured alone. Success of peptide priming was confirmed using **MTSA**IGIL**P**V or ALWGPDPAAA tetramers using an optimised protocol (PKI + tetramer + I◦ PE antibody). Results are expressed as the percentage of CD8 + tetramer + cells.*

I next wanted to look closer into the ability of **MTSA**IGIL**P**V-primed CD8 T-cells from RCC patient samples to kill autologous tumour. CD8 T-cells were isolated from RCC patient 12 and primed for 28 days with **MTSA**IGIL**P**V peptide or cultured alone. Success of peptide priming was assessed by **MTSA**IGIL**P**V peptide reactivity through a TAPI-O activation assay (**Figure 5.2A**). 3 x10^4 T-cells were co-incubated with 6 x10^4 T2 APC and **MTSA**IGIL**P**V peptide at 10^{-6} M for 4 h and activation measured as TNF release. In response to **MTSA**IGIL**P**V peptide 2.12% of the **MTSA**IGIL**P**V-primed CD8 T-cells activated whilst only 0.15% of the CD8 T-cells cultured without peptide responded to the **MTSA**IGIL**P**V peptide. The ability of **MTSA**IGIL**P**V-primed CD8 T-cells from RCC patient 12 to kill an autologous tumour line was assessed by chromium release assay (**Figure 5.2B**). CD8 T-cells were incubated with autologous tumour at a ratio of 50 T-cells to 1 tumour cell for 4 h, **MTSA**IGIL**P**V-primed CD8 T-cells killed more autologous tumour than the T-cells cultured without peptide.

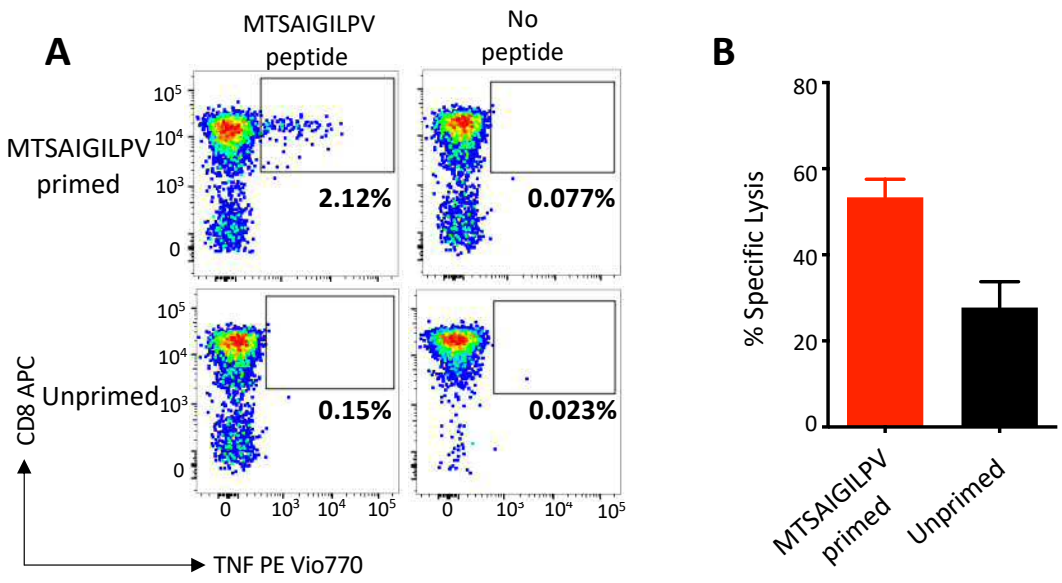


Figure 5.2: Successful priming of MTSAIGILPV-reactive CD8 T-cells capable of killing autologous tumour from RCC patient 12.

***A**) CD8 T-cells isolated from renal cell carcinoma patient 12 were primed for 28 days with super-agonist peptide **MTSA**IGIL**P**V or cultured alone. Success of peptide priming was confirmed as peptide reactivity using a TAPI-O activation assay. 3 x10^4 T-cells were co-incubated with 6 x10^4 T2 APC and **MTSA**IGIL**P**V peptide at a concentration of 10^{-6} M. Cells were stained with VIVID LIVE DEAD pacific blue and CD8 APC to allow the identification of alive CD8 + cells. Results are expressed as the percentage of CD8 + TNF + cells. **B**) Killing of autologous tumour by **MTSA**IGIL**P**V-primed CD8 T-cells was assessed by chromium release assay following 4 h incubation. T-cells and tumour cells were co-incubated at a ratio of 50 T-cells to 1 tumour cell. Results are expressed as the mean of two replicates, the error is shown as standard deviation.*

These data highlight the capacity of **MTSA**IGIL**P**V super-agonist peptide to prime tumour reactive CD8 T-cells in RCC patient samples. I was limited by the amount of PBMC sample I could obtain from the RCC patients, therefore I was unable to assess the success of priming BST2 or IMP2 T-cells with **MTSA**IGIL**P**V peptide. I next decided that I needed to expand this study to look at **MTSA**IGIL**P**V in other cancer types that were more easily accessible. Blood cancers provided an ideal model as both the cancer and the autologous CD8 T-cells could be obtained from a single, minimally invasive blood donation.

5.2.2. Expression of TAA BST2 and IMP2 in leukaemia tumour samples

The Sewell Laboratory has an existing collaboration with the haematology department at the University Hospital of Wales. I therefore began by looking to see if the TAAs melan-A, BST2

or IMP2 were over-expressed in leukaemia using data from established leukemic cell lines available. The expression data could have been generated in the laboratory using qPCR, monoclonal antibody staining or western blot however in the interest of time I decided to use the TRON Online Cell Portal (Scholtalbers *et al.*, 2015) and is expressed as the Log_2 reads per kilobase per million (RPKM). The expression of melan-A, BST2 and IMP2 was compared to the expression of Wilms Tumour antigen 1 (WT1). WT1 has been identified as leukaemia associated antigen (LAA), due to its overexpression in leukaemia cells when compared to non-malignant hematopoietic cells so acted as a good positive control (Ariyaratana and Loeb, 2007). The identification of WT1 as a LAA, has resulted in attempts to target WT1 using peptide vaccines (Oka *et al.*, 2004; Keilholz *et al.*, 2009; Maslak *et al.*, 2010) and transduced TCR-T therapy (Tawara *et al.*, 2017; Chapuis *et al.*, 2019). The TAA BST2 is expressed at high levels in all tumour cell lines apart from Kasumi-1 where it was only expressed at a low level (**Figure 5.3**). BST2 is also expressed at higher levels than WT1 in 10/12 of the leukaemia cell lines assessed, including 4/5 AML cell lines (THP-1, Kasumi-6, mono-mac-1, KG-1). IMP2 was expressed at high levels in the AML cell lines THP-1, Kasumi-1, Kasumi-6, mono-mac-1, KG-1, the chronic myeloid leukaemia cell line, K562 and Jurkat, an acute lymphoid leukaemia cell line (**Figure 5.3**). In all these cell lines IMP2 was expressed at a higher level than WT1. IMP2 was also expressed in 2/5 of the CLL cell lines included in this study, BCP-1 and RS4-11, these cell lines also had low expression of WT1 (**Figure 5.3**). All cell lines included in this cohort expressed minimal levels of the TAA melan-A (**Figure 5.3**). Overall, the results shown in **Figure 5.3** suggest that both BST2 and IMP2 would make better leukaemia antigens than WT1, the most commonly used TCR target to date. Multipronged T-cells that target *both* BST2 and IMP2 might be especially good for leukaemia therapies. I next wanted to see if I could induce such BST2/IMP2 multipronged T-cells from the blood of a leukaemia patient. The blood of such patient is often massively dominated by tumour cells leaving very little niche space for CD8 T-cells so priming of T-cells from the blood of leukaemia patients (**Figure 5.4** and **Figure 5.10**) was considered to be more difficult than for healthy donors of melanoma patients as attempted in **Chapter 3**.

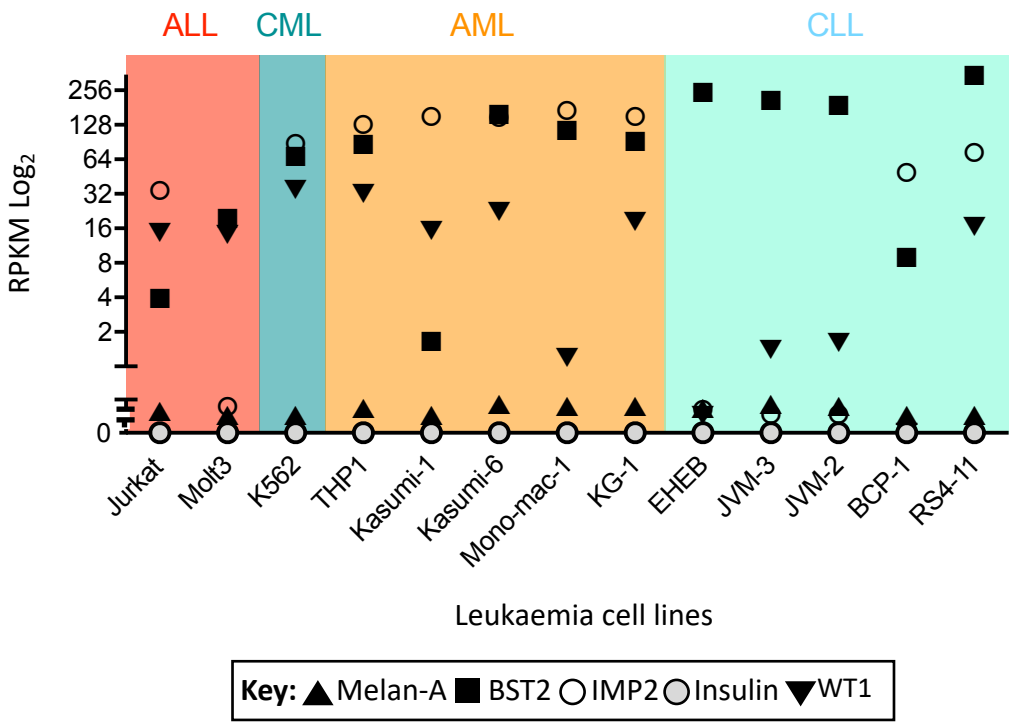


Figure 5.3: Expression of BST2 and IMP2 in leukaemia tumour samples

Expression of tumour associated antigens melan-A, BST2, IMP2 and WT1 in established ALL, CML, AML and CLL cell lines was determined using the TRON Online Cell Portal (Scholtalbers *et al.*, 2015)*. Results are expressed as the Log$_2$ reads per kilobase per million (RPKM). The expression of insulin was included as a negative reference gene.*

5.2.3. Successful priming with super-agonist peptide MTSAIGILPV of CD8 T-cells from CLL patient samples

I accessed samples from patients with chronic lymphocytic leukaemia (CLL) undergoing routine monitoring of their disease through Prof Steve Man and Dr Chris Pepper Cardiff University. Initially, I wanted to assess whether it would be possible to prime **MTSA**IGIL**P**V-specific T-cells from the blood of a CLL patient sample given that such blood was only available in low volume as was likely to be dominated by cancer cells. As already stated leukaemia samples were ideal for my studies due to the presence of both T-cells and tumour cells in the same blood sample. However, it is important to note that in most cases the majority (sometimes over 90+%) of the cells in the blood sample are CD19+ B-cell leukaemia, meaning that there were very few CD8 T-cells. **Figure 5.4** shows example staining of CLL patient blood for CD3 and CD19 and demonstrates that less than 4% of the cells in patient blood were T-cells. Given that CD4 T-cells are usually the numerically dominant population this suggests than less than 1 in 50 of the cells in this patient's blood were CD8 T-cells.

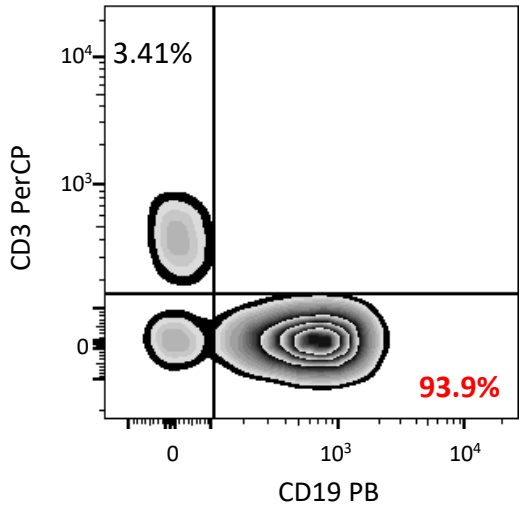


Figure 5.4: Phenotype of CLL patient PBMC sample

Analysis of the cellular composition of PBMC from a CLL patient sample was carried out using flow cytometry. PBMC were stained with LIVE/DEAD VIVID to allow the exclusion of dead cells, CD3 to allow the identification of T-cells and CD19 to identify CLL tumour cells. Results are displayed as percentage positive cells.

As a preliminary experiment, I used 10 mL of blood from CLL patient A276. Patient A276 T-cells were primed for 28 days with super-agonist peptide **MTSA**IGIL**P**V or cultured alone. Results were assessed using HLA A2-**MTSA**IGIL**P**V tetramers with a preproinsulin tetramer as a control (**Figure 5.5**). Interestingly, a small population of T-cells (0.23%) stained with **MTSA**IGIL**P**V in the absence of any priming. Priming with **MTSA**IGIL**P**V peptide increased this population to almost 5% of CD8 T-cells.

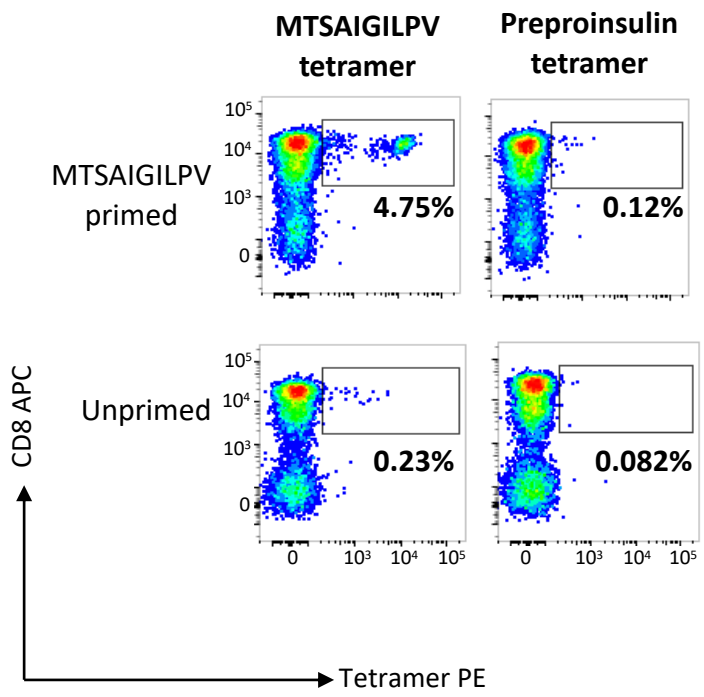


Figure 5.5: Successful priming of MTSAIGILPV-reactive CD8 T-cells from chronic lymphocytic leukaemia (CLL)

*CD8 T-cells were isolated from PBMC obtained from chronic lymphocytic leukaemia (CLL) patient, A276 who attended routine monitoring of their disease at the University Hospital of Wales (UHW). Isolated CD8 T-cells were co-incubated with autologous CD14, CD16, CD11b and CD11c positive cells and **MTSA**IGIL**P**V peptide at a concentration of 25 µM for 28 days. Success of **MTSA**IGIL**P**V peptide priming was assessed by peptide-MHC tetramer staining using **MTSA**IGIL**P**V and ALWGPDPAAA tetramers. An optimised tetramer staining protocol was used (PKI + tetramer + 1° PE antibody) and results are depicted as the percentage of CD8 + tetramer + cells.*

5.2.4. MTSAIGILPV-priming of CD8 T-cells from CLL patient samples kill autologous cancer cells

Once I had established that it was possible to induce **MTSA**IGIL**P**V-specific T-cells via peptide priming of leukaemia blood I next wanted to assess whether these cells could kill the autologous cancer. These experiments required that I could keep the cancer cells alive *ex vivo* so that they could be used in T-cell killing assays. This was achieved by culturing the CLL tumour cells on a bed of irradiated CD40L+ fibroblast cells, in the presence of IL-4 (Reid *et al.*, 2014). T-cells were primed from CLL patient A626 using the protocol I developed for patient A276. After 28 days 7.86% of T-cells from this donor stained with **MTSA**IGIL**P**V tetramer (**Figure 5.6**). I next compared how these T-cell lines killed the HLA A2+ autologous leukaemia line by chromium release assay, whereby tumour cells were incubated with T-cells at a T-cell: tumour ratio of 25:1 for 4 h. The percentage killing was then determined relative

to maximum and spontaneous release of tumour alone conditions. **MTSA**IGIL**P**V-primed T-cells demonstrated superior killing of autologous tumour than the not primed T-cells (**Figure 5.6B**).

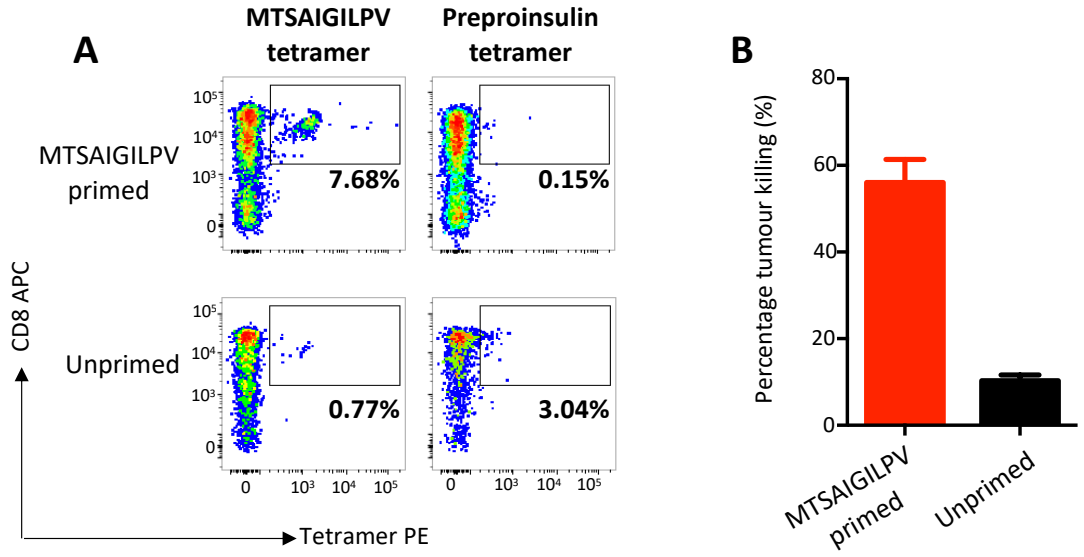


Figure 5.6: MTSAIGILPV-primed CD8 T-cells from CLL patient A626 kill autologous tumour in a chromium release assay

*CD8 T-cells were isolated from patient A626 PBMC and co-incubated with autologous CD14, CD16, CD11b and CD11c positive cells and **MTSA**IGIL**P**V peptide at a concentration of 25 µM for 28 days. Success of **MTSA**IGIL**P**V peptide priming was assessed by peptide-MHC tetramer staining using **MTSA**IGIL**P**V-tetramers and a preproinsulin tetramer as a control. An optimised tetramer staining protocol was used (PKI + tetramer + 1° PE antibody) and results are depicted as the percentage of CD8 + tetramer + cells. **B**) Killing of autologous tumour by **MTSA**IGIL**P**V-primed CD8 T-cells from CLL patient A626 was assessed by chromium release following 4 h of incubation. CD8 T-cells and tumour cells were co-incubated at a ratio of 25 T-cells:1 tumour cell. Results show standard deviation from the mean of two replicates.*

I repeated this experiment with CD8 T-cells from CLL patient U386 (**Figure 5.8**). Almost 7% of the T-cell line primed with the **MTSA**IGIL**P**V peptide stained with **MTSA**IGIL**P**V tetramer compared to 0.25% of the cells that were not primed. The ability of these T-cell lines to kill the U386 autologous cancer line was assessed by a flow cytometry based killing assay. This flow-based assay allowed me to use less T-cells and to run the experiment over a longer time. T-cells were incubated with tumour cells, at a ratio of 5 T-cells to 1 tumour cell, and killing was assessed after 6 days. As demonstrated in **Figure 5.4**, CLL patient PBMC is dominated by

CD19+ CLL tumour cells, therefore in my flow cytometry staining panel I included a CD19 antibody conjugated to PE to allow identification of CLL tumour cells (**Figure 5.7**).

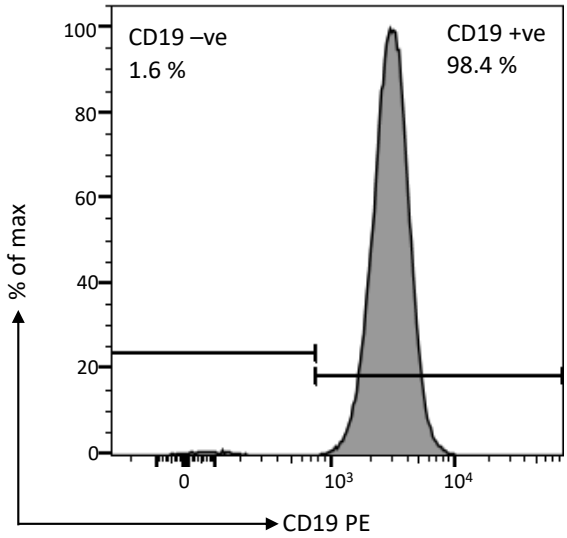


Figure 5.7: CD19 staining of CLL U386 tumour

CLL tumour cells isolated from CLL patient U386 were stained with CD19 PE and LIVE DEAD VIVID pacific blue to allow the identification of alive, CLL tumour cells. Results are depicted as the number of CD19+ cells.

MTSAIGIL**P**V-primed CD8 T-cells demonstrated more killing of autologous tumour after 6 days of co-incubation than unprimed T-cells (**Figure 5.8**). Killing of autologous tumour is depicted as remaining tumour cells in the unprimed T-cell wells relative to the reference cells used to calculate killing. This result infers that **MTSA**IGIL**P**V-primed T-cells from CLL patient U386 killed at least 50% more autologous tumour cells than the unprimed CD8 T-cells. The results are shown in this way as tumour cells cultured alone died during incubation, potentially due to a lack of stimulus or the duration of the killing assay, therefore it was not possible to calculate percentage tumour cell lysis (**Figure 5.8**).

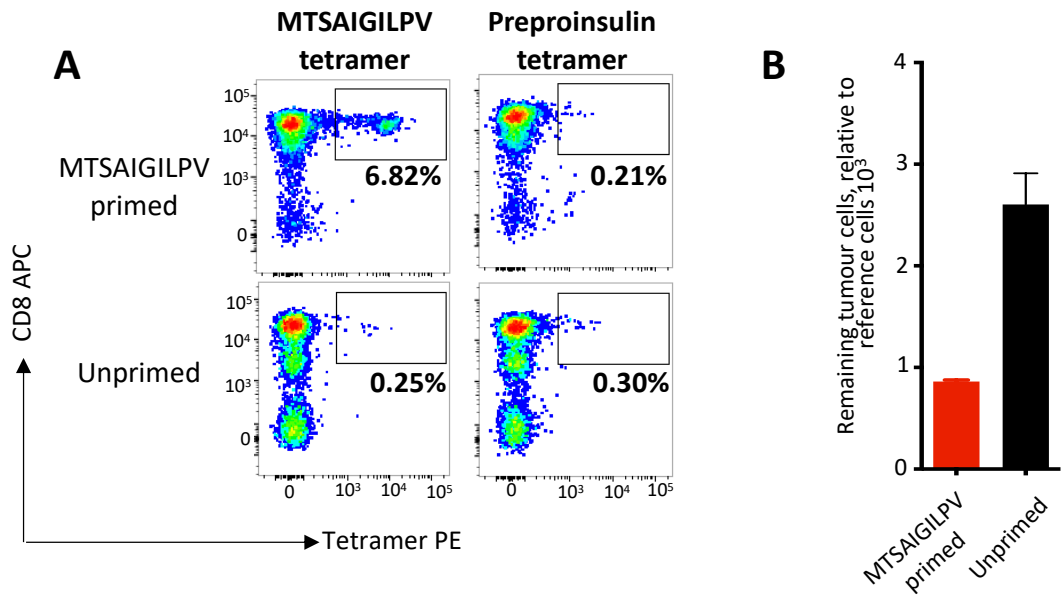


Figure 5.8: MTSAIGILPV-primed CD8 T-cells from CLL patient U386 kill autologous tumour in a flow cytometry assay

***A**) CD8 T-cells were isolated from patient U386 PBMC and co-incubated with autologous CD14, CD16, CD11b and CD11c positive cells and **MTSA**IGIL**P**V peptide at a concentration of 25 µM for 28 days. Success of **MTSA**IGIL**P**V peptide priming was assessed by peptide-MHC tetramer staining using **MTSA**IGIL**P**V-tetramers and a preproinsulin tetramer as a control. An optimised tetramer staining protocol was used (PKI + tetramer + 1° PE antibody) and results are depicted as the percentage of CD8 + tetramer + cells. **B**) Killing of autologous tumour by **MTSA**IGIL**P**V -primed CD8 T-cells isolated from CLL patient U386 was determined by a flow cytometry based killing assay. T-cells were co-incubated with tumour cells for 6 days before analysis, a CFSE-labelled reference cell population was included to allow calculation of tumour cell killing. Cells were stained with CD8 APC Vio 770 to allow identification of CD8 T-cells, and CD19 PE to allow identification of tumour cells. LIVE/DEAD vivid pacific blue was included to allow exclusion of dead cells. Results are shown as the number of tumour cells remaining at the end of the assay in the **MTSA**IGIL**P**V primed and unprimed conditions, relative to the reference cell population. Results are depicted as the mean of two replicates, and standard deviation is shown.*

A flow cytometry based killing assay was used as an alternative method to determine autologous tumour cell killing of U386 **MTSA**IGIL**P**V-primed CD8 T-cells. It was chosen over a chromium release assay as it allowed for longer term monitoring of tumour cell killing, allowing use of less T-cells. Furthermore, flow cytometry based killing also allowed for the separation of the leukaemia tumour cells, from any remaining CD14, CD16, CD11b and CD11c cells using CD19 staining. Flow cytometry based killing also allowed for calculation of total cell numbers, leading to more accurate assessment of tumour cell killing.

The results shown in **Figure 5.6** and **Figure 5.8** show that **MTSA**IGIL**P**V-primed CD8 T-cells from patient blood could kill the patient's own tumour cells. I next wanted to formally prove that the **MTSA**IGIL**P**V-primed CD8 T-cells from these patients contained T-cells that bound to the new IMP2 epitope. I also wanted to compare whether priming with the **MTSA**IGIL**P**V peptide produced more NLSALGIFST-specific T-cells than those primed with the natural NLSALGIFST sequence.

5.2.5. MTSAIGILPV peptide primes CD8 T-cells specific for TAA epitopes from leukaemia patient samples

I next wanted to understand whether priming with super-agonist peptide **MTSA**IGIL**P**V could elicit TAA-specific T-cells from CLL patient samples (**Figure 5.9**). CD8 T-cells isolated from CLL patients A276 and U386 were primed for 28 days with EAAGIGILTV, NLSALGIFST, super-agonist peptide **MTSA**IGIL**P**V and cultured alone. Priming was assessed by staining with HLA A2-EAAGIGILTV, HLA A2-NLSALGIFST and HLA A2-ALWGPDPAAA tetramers. In both donors, priming with **MTSA**IGIL**P**V peptide elicited more EAAGIGILTV and NLSALGIFST tetramer positive cells than priming with the EAAGIGILTV or NLSALGIFST peptides (**Figure 5.9**). Indeed, the NLSALGIFST sequence only primed populations of 0.51% and 0.43% of T-cells from patient A276 and U386 respectively compared to the 2.49% and 8.42% populations primed with the **MTSA**IGIL**P**V peptide. These results confirm that the **MTSA**IGIL**P**V peptide is superior at inducing IMP2 and melan-A T-cells compared to the wildtype sequences in CLL patient samples.

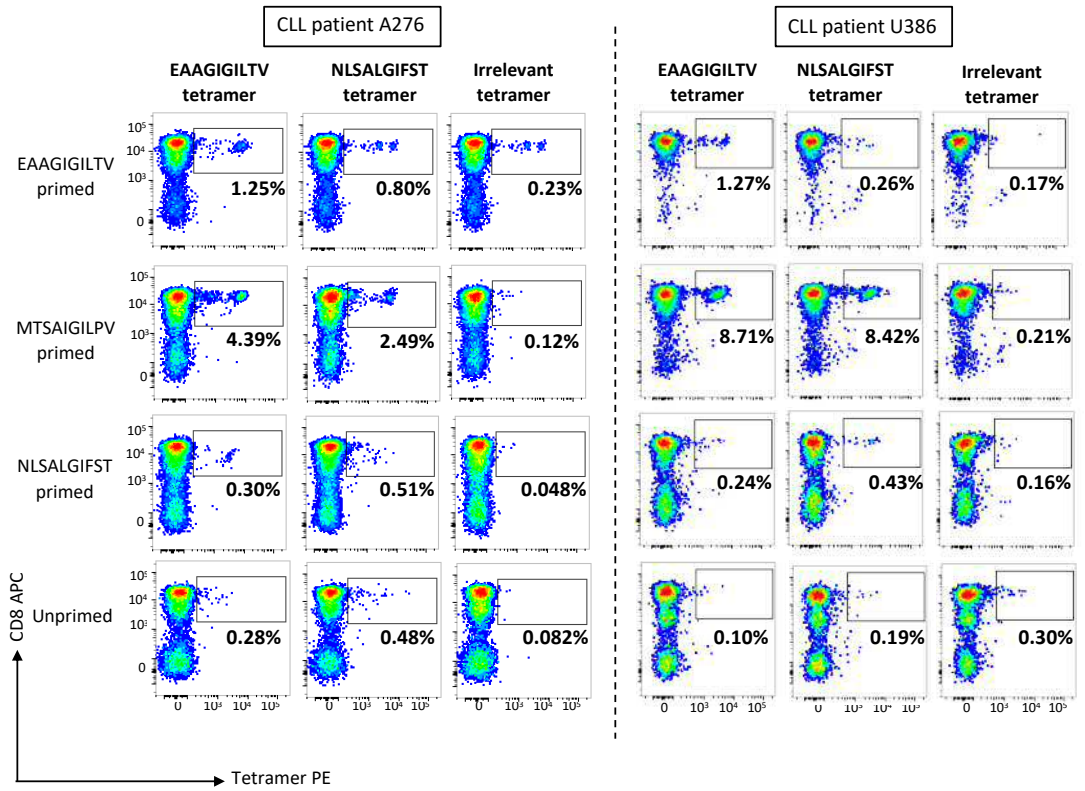


Figure 5.9: MTSAIGILPV peptide priming of EAAGIGILTV and NLSALGIFST reactive CD8 T-cells from CLL patient samples

CD8 T-cells isolated from PBMC obtained from chronic lymphocytic leukaemia (CLL) patients A276 and U386 were primed for 28 days with super-agonist peptide MTSAIGILPV, tumour associated peptide EAAGIGILTV and NLSALGIFST or alone. Subsequently, polyclonal CD8 T-cell populations were stained with EAAGIGILTV, NLSALGIFST or an irrelevant preproinsulin (ALWGPDPAAA) pMHC tetramers using an optimised protocol (PKI + tetramer + anti-PE 1◦ antibody), percentage of tetramer positive cells shown. Cells were also stained with VIVID live/dead (PB), CD8 (APC) and CD3 (PerCP) to allow the identification of live, CD8 T-cells.

Anti-CD19 CAR-T therapy is now given as a salvage treatment for CLL on the NHS and this treatment of last resort is saving the lives of lymphoid leukaemia patients. There is an urgent need for T-cell therapy that allows safe targeting of *myeloid* leukaemia. Given the success of super-agonist peptide **MTSA**IGIL**P**V priming on CD8 T-cells from CLL patients I wanted to determine whether the same pattern could be replicated using AML patient samples.

5.2.6. MTSAIGILPV-priming of multipronged CD8 T-cells from a AML patient sample

I obtained samples from patients presenting in A&E with AML through a collaboration with Prof Oliver Ottmann, Cardiff University. As with the CLL patient samples, AML patient PBMC populations are dominated by leukemic cells, and therefore the number of CD8 T-cells

available from each sample is limited. To confirm the amount of AML tumour cells in the patients PBMC I used a panel of markers recently published by Chapuis *et al* to isolated AML tumour cells from PBMC of patients (Chapuis *et al.*, 2019). Staining of AML patient CH160486 PBMC with this antibody panel confirmed that 96% of the patients PBMC was CD3 and CD19 negative. The CD3/CD19- population expressed markers associated with AML blasts including; CD15, CD34, CD38, CD45, CD96, CD117 and CD123 (**Figure 5.10**).

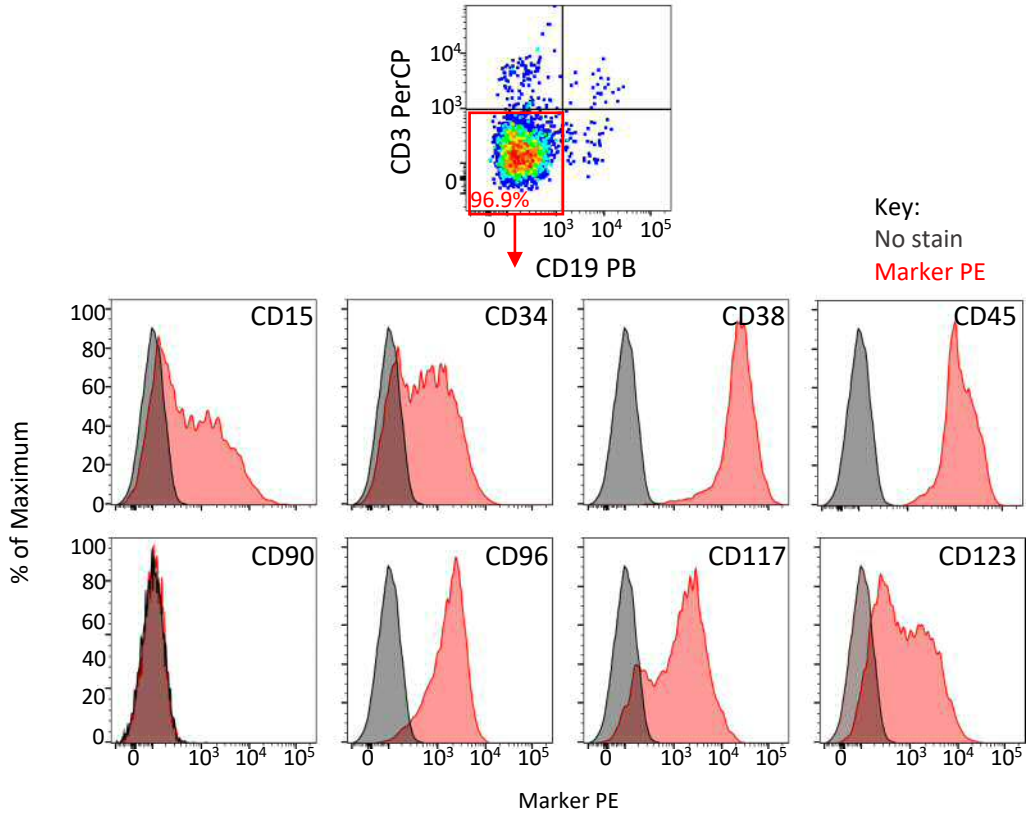


Figure 5.10: Phenotyping of PBMC from AML patient CH160486

Phenotyping of AML patient CH160486 PBMC using an established AML tumour antibody panel. Cells were also stained with LIVE/DEAD AQUA, CD3 PerCP and CD19 PB to allow the exclusion of dead cells and of T and B lymphocytes. Histograms depict a no stain control (grey) and staining with PE-conjugated marker (red).

Using what I learned from the CLL patient experiments above, CD8 T-cells isolated from HLA A2+ AML patient CH160486 PBMC were primed for 28 days with peptide EAAGIGILTV, LLLGIGILVL, NLSALGIFST, super-agonist peptide **MTSA**IGIL**P**V or cultured alone. Success of priming was determined by tetramer staining with HLA A2-EAAGIGILTV, LLLGIGILVL, NLSALGIFST or **MTSA**IGIL**P**V tetramers (**Figure 5.11**). After 28 days of peptide priming, the **MTSA**IGIL**P**V peptide primed population had greater numbers of EAAGIGILTV-, LLLGIGILVL-,

NLSALGIFST- and **MTSA**IGIL**P**V-tetramer positive CD8 T-cells compared to priming with the wild type antigens (**Figure 5.11**). The percentage of **MTSA**IGIL**P**V- and NLSALGIFST-tetramer positive cells was almost identical, 5.4% and 5.8% respectively, inferring that **MTSA**IGIL**P**V peptide priming may always elicit cells that respond to the NLSALGIFST IMP2-derived epitope. **MTSA**IGIL**P**V peptide priming elicited 1.4% LLLGIGILVL- and 2.7% EAAGIGILTV-tetramer positive cells, inferring that not all **MTSA**IGIL**P**V-primed cells recognise these antigens.

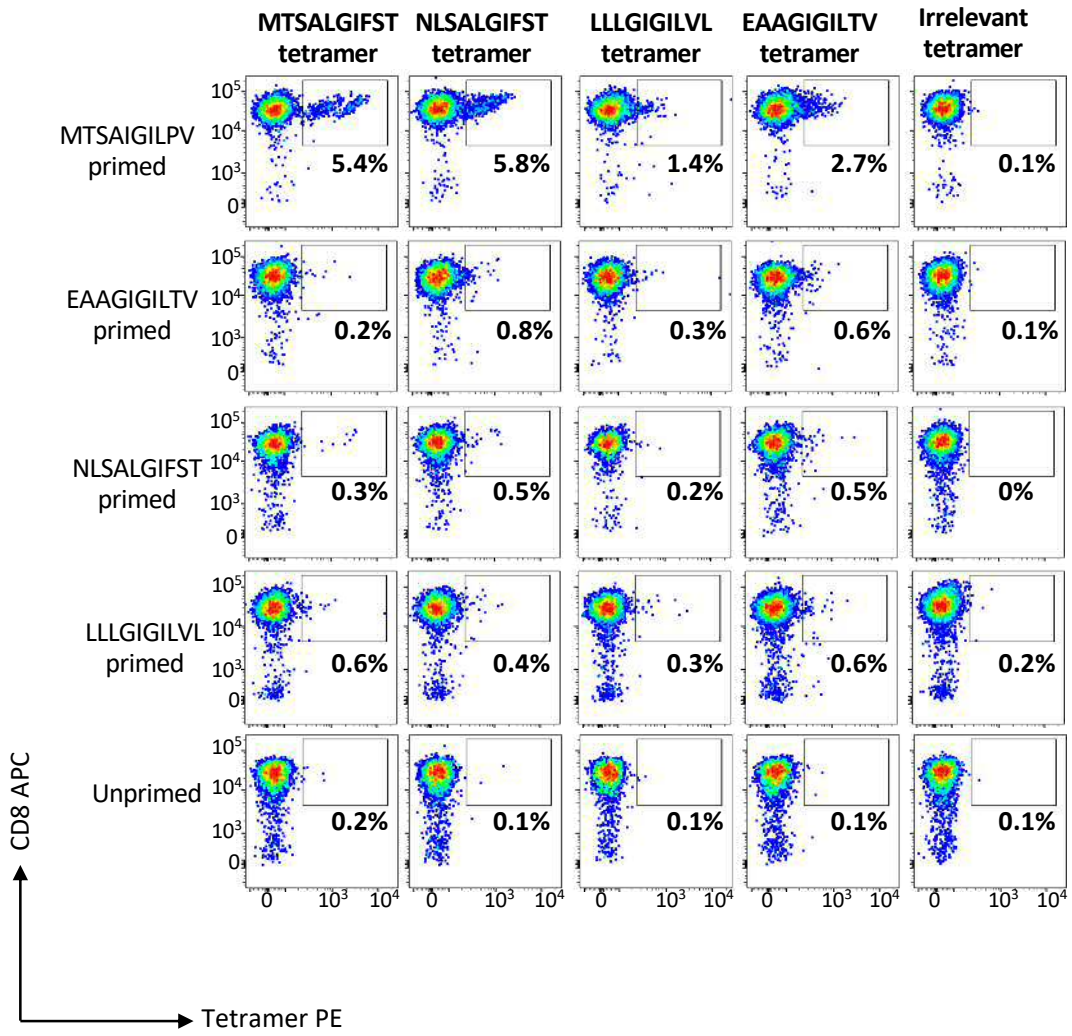


Figure 5.11: Successful priming of EAAGIGILTV, LLLGIGILVL and NLSALGIFST reactive CD8 T-cells in AML patient CH160486 samples by super-agonist peptide MTSAIGILPV

CD8 T-cells isolated from PBMC obtained from acute myeloid leukaemia patients CH160486 were primed for 28 days with super-agonist peptide MTSAIGILPV, tumour associated peptides EAAGIGILTV, LLLGIGILVL and NLSALGIFST or alone. Subsequently, polyclonal CD8 T-cell populations were stained with EAAGIGILTV, NLSALGIFST or an irrelevant (ALWGPDPAAA) pMHC tetramer using an optimised protocol (PKI + tetramer + anti-PE 1◦ antibody), percentage of tetramer positive cells shown. Cells were also stained with VIVID live/dead (PB), CD8 (APC) and CD3 (PerCP) to allow the identification of live, CD8 T-cells.

5.2.7. MTSAIGILPV peptide primed CD8 T-cells from AML patient CH160486 kill AML tumour cells

Having established in **Figure 5.11** that priming CD8 T-cells from AML patient CH160486 with **MTSA**IGIL**P**V elicits T-cells specific for TAA melan-A, BST2 and IMP2, I next wanted to assess autologous tumour cell killing by **MTSA**IGIL**P**V primed cells (**Figure 5.12**). Killing of autologous tumour cells by peptide primed CD8 T-cells from AML patient CH160486 was assessed using

a flow cytometry based killing assay (**Figure 5.12**). **MTSA**IGIL**P**V primed T-cells killed over 80% of the autologous tumour after 3.5 days. T-cells primed with EAAGIGILTV and LLLGIGILVL failed to induce tumour cell death, in fact the tumour grew over the culture period giving rise to negative percentage killing values. T-cells primed with NLSALGIFST failed to kill tumour cells above the level of T-cells not primed with any peptide (**Figure 5.12**).

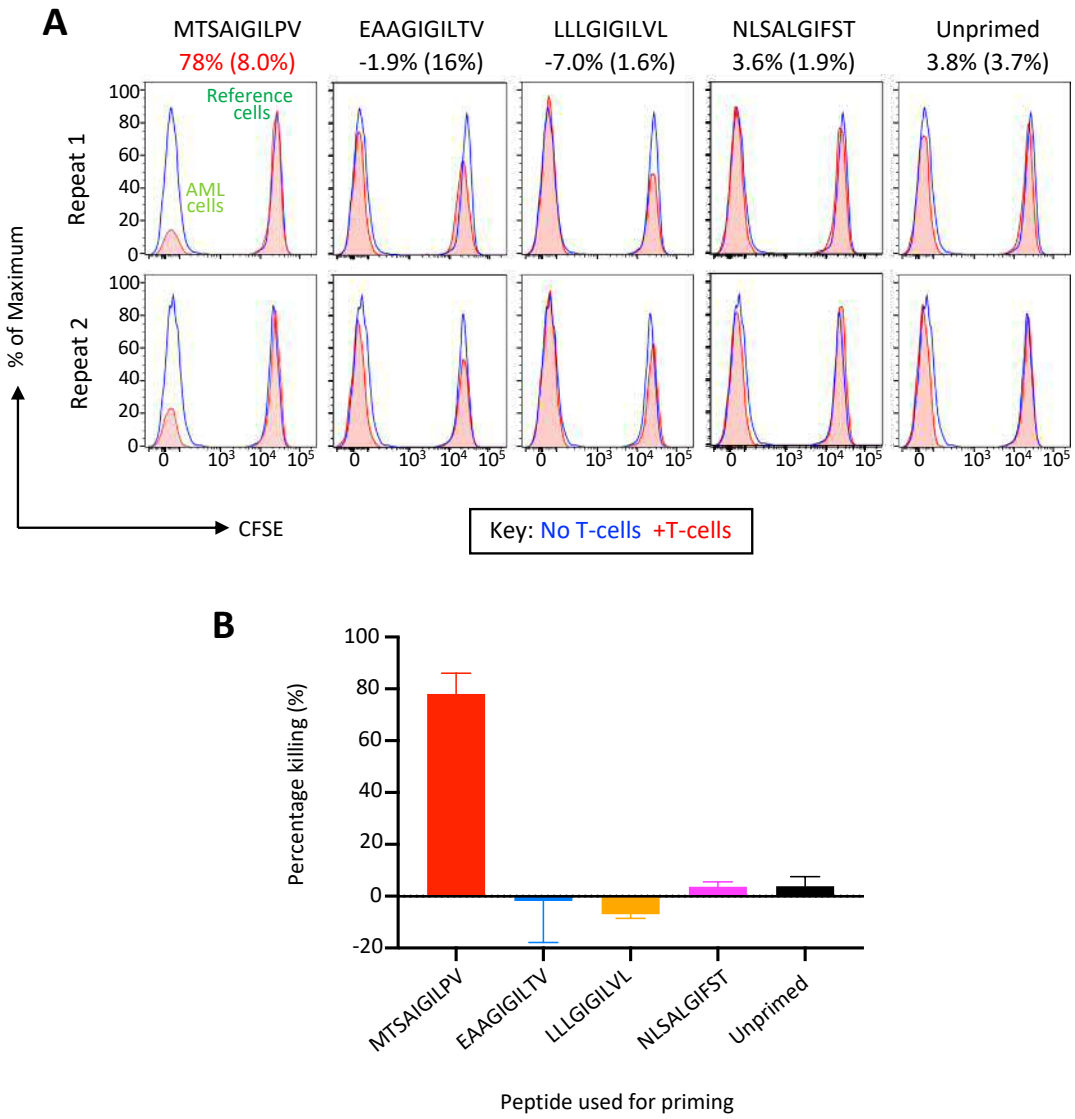


Figure 5.12: Improved killing of autologous AML tumour by MTSAIGILPV-primed T-cells from AML patient CH160486

CD8 T-cells isolated from AML patient CH160486 were primed for 28 days with EAAGIGILTV, LLLGIGILVL, NLSALGIFST, super-agonist peptide MTSAIGILPV or cultured alone. ***A****) Killing of CH160486 autologous tumour was assessed using a flow cytometry based killing assays at a T-cell: tumour cell ratio of 10:1 after 3.5 days of co-culture. A CFSE-reference cell population was included in the analysis to allow the calculation of percentage tumour cell killing. Killing by CH160486 peptide primed T-cells (red line) is compared to tumour alone (blue line).* ***B****) Killing of autologous tumour by AML patient CH160486 peptide primed T-cells as in* ***A****, displayed in a bar chart. Results are displayed as an average of two replicates with the error represented as standard deviation.*

I next wanted to confirm that killing of AML tumour cells by **MTSA**IGIL**P**V-primed T-cells from AML patient CH160486 was HLA A2 restricted. To do this I first generated an HLA A2 negative version of the AML cell line THP-1, using CRISPR/Cas9. KO of HLA A2 was confirmed using

flow cytometry (**Figure 5.13A**). Killing of HLA A2+ and HLA A2- THP-1 tumour cell line was assessed using a flow cytometry based killing assay after 3.5 days of incubation at a T-cell:tumour cell ratio of 10:1. HLA A2+ and HLA A2- tumour cells were combined in the well (50% of each) to reduce the number of T-cells required for the assay. **MTSA**IGIL**P**V primed T-cells killed 80% and 84% of HLA A2+ THP-1 cells in replicate assays (**Figure 5.12B**). EAAGIGILTV, LLLGIGILVL, NLSALGIFST primed T-cells failed to mediate killing of THP-1 cells.

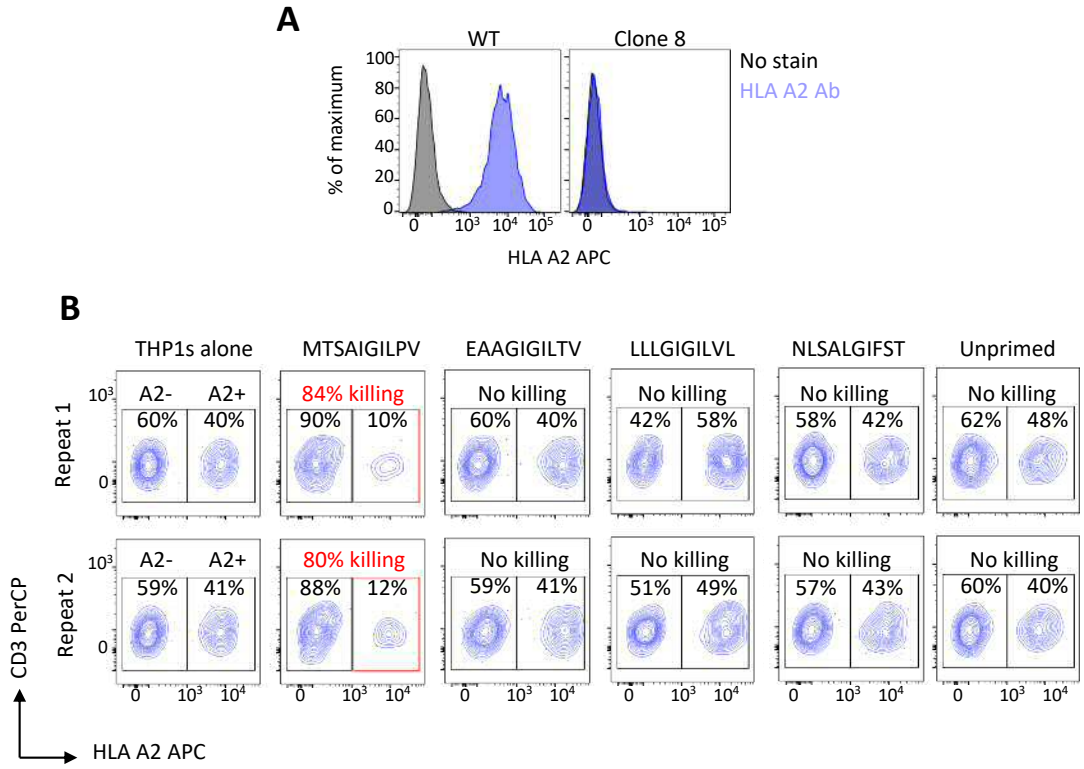


Figure 5.13: Superior killing of AML cell line THP-1 by MTSAIGILPV-primed T-cells from AML patient CH160486

***A**) Flow cytometry histograms of tumour cell lines used depicting HLA A2 status. Cell line THP-1 is naturally A2 positive, THP-1 A2- line was generated using CRISPR/cas9 system with a HLA A2 gRNA. Tumour lines were stained with VIVID live/dead (to allow exclusion of dead cells) and HLA A2 FITC. **B**) Killing of HLA A2 positive and negative AML cell line, THP-1, by these T-cell lines was assessed by a flow cytometry based killing assay. T-cells and tumour cells were co-incubated at a ratio of 10 T-cells to 1 tumour cell for 3.5 days before percentage killing was assessed. Tumour cells were made up of 50% A2+ and 50% A2- cells. Remaining tumour cells were gated based on size (FSC-A vs SSC-A) and viability using VIVID LIVE/DEAD pacific blue. CD8 T-cells were gated out using CD3 PerCP, CD8 PE and CD4 FITC. Results depict two replicates and is displayed as the percentage or remaining HLA A2+ and HLA A2- tumour cells.*

These results show that T-cells primed by **MTSA**IGIL**P**V peptide and that stain with IMP2 and BST2 tetramers kill AML. Results also suggest that **MTSA**IGIL**P**V-primed T-cells were

multipronged and recognised multiple AML-presented epitopes. In order to formally prove this possibility, I set about growing a monoclonal T-cell population from CLL patient U386.

5.2.8. Isolation of a MTSAIGILPV-reactive multi-pronged CD8 T-cell from CLL patient U386

Since the sample size from patients was small, the scope to assess the tumour reactivity of CD8 T-cells was limited. To aid interrogation of the CD8 T-cells present in the polyclonal population I sought to isolate single clonotypes from the polyclonal **MTSA**IGIL**P**V-primed populations from leukaemia patient samples (**Figure 5.14**). Previous studies have demonstrated the importance of the quality of the individual TCR clonotypes on the anti-tumour immune response (Alexander-Miller *et al.,* 1996; Seder *et al.,* 2008; Bangham, 2009; Ekeruche-Makinde *et al.*, 2012). The study of individual TCR clonotypes in **Chapter 3** and **Chapter 4** allowed the identification of super-agonist peptide **MTSA**IGIL**P**V and a more detailed understanding of multipronged TCRs. I therefore thought it was important to attempt to generate individual TCR clonotypes from **MTSA**IGIL**P**V-primed CD8 T-cells isolated from leukaemia patient samples for further analysis. Tetramer-positive cells were isolated by magnetic separation and single cell cloned by limiting dilution. Once obvious growth was seen, individual clones were tested for reactivity to **MTSA**IGIL**P**V peptide and to autologous tumour. Only **MTSA**IGIL**P**V-restricted, autologous tumour reactive CD8 T-cell clones were maintained in culture for further testing.

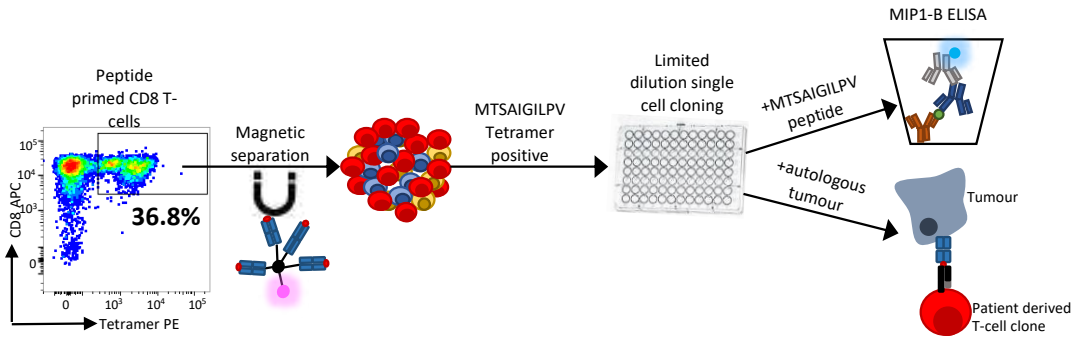


Figure 5.14: Schematic of CD8 T-cell clone isolation from MTSAIGILPV-primed polyclonal T-cell populations

CD8 T-cells isolated from the PBMC of CLL and AML patients were primed for 28 days with MTSAIGILPV super-agonist peptide. Tetramer staining of MTSAIGILPV-primed populations using an optimised protocol (PKI + tetramer + anti-PE 1◦ antibody) identified MTSAIGILPV tetramer positive CD8 T-cell populations. MTSAIGILPV-tetramer positive CD8 T-cells were isolated by MACS magnetic separation using MTSAIGILPV tetramers and anti-PE microbeads. CD8 T-cell clones were generated though single cell cloning of the MTSAIGILPV-tetramer sorted population and expansion using irradiate allogenic feeders isolated from buffy coats supplied by the Welsh Blood Service (WBS) and PHA. Following expansion, clones were identified by obvious growth in the well. Each clone was then tested for MTSAIGILPV-reactivity though an overnight activation assay via a MIP1β ELISA and for reactivity to autologous tumour though TAPI-O activation assay or flow cytometry based killing assay.

I generated over 40 T-cell clones by single cell cloning of the **MTSA**IGIL**P**V-primed T-cell population of CLL patient U386. Clone CLL 9 T-cell expanded to sufficient numbers in culture to allow testing of whether it recognised the IMP2-derived peptide NLSALGIFST and the super-agonist peptide **MTSA**IGIL**P**V. Furthermore, since **MTSA**IGIL**P**V was initially found to elicit melan-A reactive T-cells, I included EAAGIGILTV in the analysis. The ability of CLL 9 to recognise EAAGIGILTV, NLSALGIFST and **MTSA**IGIL**P**V peptides was first assessed using peptide-MHC tetramer staining, CLL 9 bound all three pMHC tetramers (**Figure 5.15A**). Staining with pMHC tetramers can give an indication of the affinity of a TCR for its antigens (Laugel *et al.*, 2007; Tungatt *et al.*, 2015; Rius *et al.*, 2018). Since self-peptide restricted TCRs tend to have a lower affinity for their cognate antigen, tetramer staining 'tricks' are required to improve the fluorescent staining, allowing identification of low affinity TCRs (Dolton *et al.*, 2015; Tungatt *et al.*, 2015). The affinity of CLL 9 for the three peptide epitopes was assessed by stained using tetramer alone, tetramer plus protein kinase inhibitor (PKI) which reduces TCR internalisation (Lissina *et al.*, 2009) and tetramer + PKI plus a primary anti-PE antibody to intensify the fluorescent staining (Tungatt *et al.*, 2015). CLL 9 bound to **MTSA**IGIL**P**V and NLSALGIFST without the need for any tetramer 'tricks' protocols, inferring that CLL 9 has a

high affinity for these epitopes. CLL 9 only stained with HLA A2-EAAGIGILTV tetramer when tricks were included suggesting that the CLL 9 TCR has a low affinity (K_D > 50 μM) for this antigen (Dolton *et al.*, 2018).

Peptide titrations at concentrations 10^{-5} M to 10^{-11} M, of EAAGIGILTV, NLSALGIFST and **MTSA**IGIL**P**V further confirmed reactivity of CLL 9 to these peptides (**Figure 5.15B**). It was also important to assess the ability of CLL 9 to kill the autologous tumour *in vitro.* Killing of autologous tumour, U386, by CLL 9 was determined using a flow cytometry based killing assay, CLL 9 efficiently killed tumour U386 after 36 hours at a T-cell to tumour ratio of 5:1 (**Figure 5.15C**).

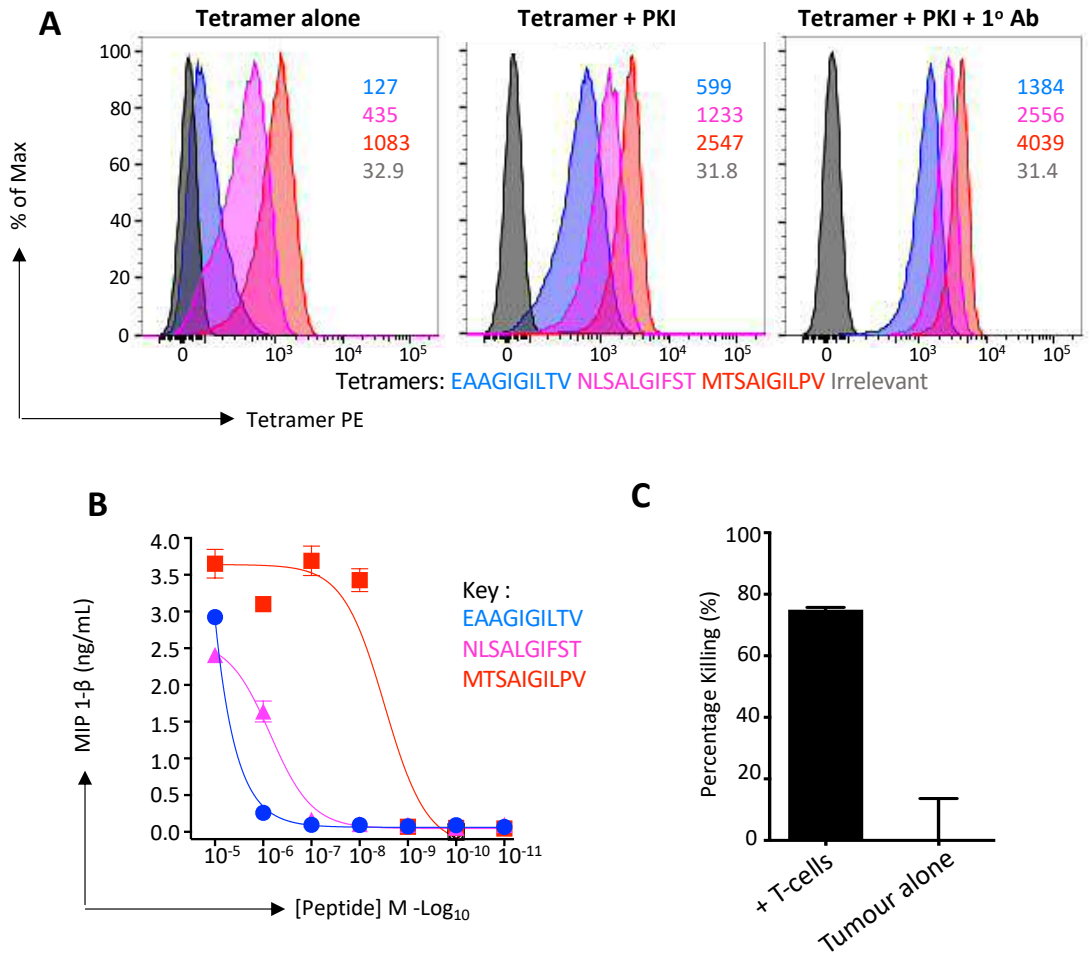


Figure 5.15: CD8 T-cell clone, CLL 9, is multi-pronged and kills autologous tumour

*CLL 9 was isolated from the MTSAIGILPV-primed CD8 T-cell population of CLL patient U386 by single cell cloning **A**) Tetramer staining of CLL 9 with MTSAIGILPV, EAAGIGILTV, NLSALGIFST and ALWGPDPAAA (irrelevant) tetramers using different protocols; tetramer alone, tetramer + PKI and an optimised protocol (PKI + tetramer + anti-PE 1◦ antibody). Mean fluorescence intensity of tetramer-PE is shown. **B**) Sensitivity of CLL 9 to MTSAIGILPV, EAAGIGILTV and NLSALGIFST measured though peptide titrations. CLL 9 was co-incubated overnight with peptides at the concentrations indicated (10^{-5} M -10^{-11} M) at 37◦C. The supernatants were harvested the following day and MIP1β production assessed via an ELISA. Results are depicted as an average of 2 replicated and the standard deviation (SD) is shown **C**) Percentage killing of autologous tumour by CLL 9. CLL 9 was co-incubated with autologous tumour for 36 hours at a ratio of 5 T-cells to 1 tumour cell, which were seeded on a bed of CD40L fibroblasts which are required for CLL tumour growth. Cells were then stained with VIVID live/dead pacific blue (to allow the exclusion of dead cells), CD8 APC (to allow identification of CLL 9) and HLA A2 PE (to allow identification of the CLL U386 tumour) and acquired using flow cytometry. A CFSE labelled reference cell population was used as a constant to allow the percentage of tumour killing to be quantified. Results are depicted as an average of 3 replicates, standard deviation is shown.*

5.2.9. T-cell clone CLL9 does not respond to healthy cells

I next tested CLL 9 on a range of cancer cell lines from different anatomical origin in an HLA A2 restricted manner. This required that I either use CRISPR/cas9 to remove HLA A2 from HLA A2+ cancer lines or transduce HLA A2 into cancer lines that lacked this allele using lentivirus. C1R, SKBR3, MM909.24 and PANC1 cells produced with and without HLA A2 as confirmed by staining with an HLA A2-specific antibody (**Figure 5.16**).

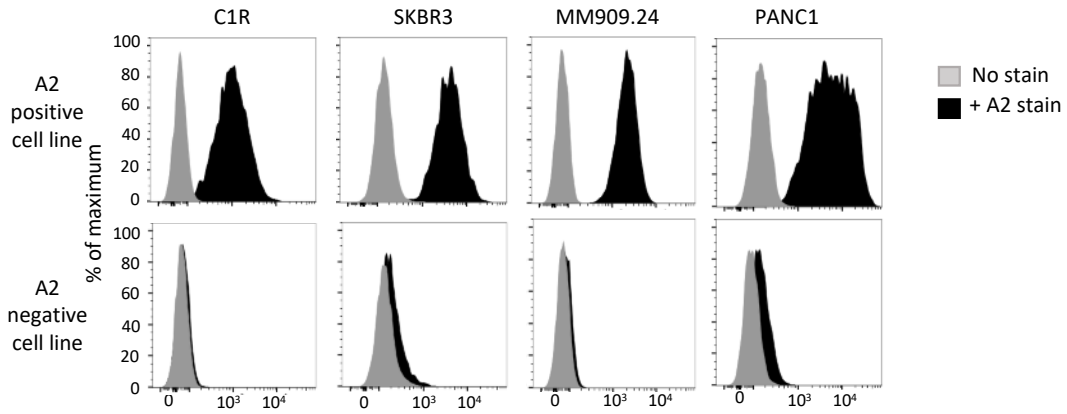


Figure 5.16: HLA A2 staining of tumour cell lines MM909.24, PANC1, SKBR3 and C1R

Flow cytometry histograms of tumour cell lines used depicting HLA A2 status. Cell lines MM909.24 and PANC1 are naturally A2 positive, MM909.24 A2- were generated using HLA A2 KO lentivirus and PANC1 using a CRISPR/cas9 system with a HLA A2 gRNA. C1R A2 and SKBR3 are naturally HLA A2 null and therefore were transfected with HLA A2 lentivirus. Tumour lines were stained with VIVID live/dead (to allow exclusion of dead cells) and HLA A2 FITC.

Initially, recognition of HLA A2+ and HLA A2- non-autologous tumour cell lines MM909.24 (skin), PANC1 (pancreas) and SKBR3 (breast) was assessed using a TAPI-O activation assay (**Figure 5.17**). CLL 9 showed substantially more reactivity to the HLA A2+ cell lines, as shown by increased production of TNF and CD107a (**Figure 5.17A**). I next wanted to determine whether the reactivity of CLL 9 to non-autologous tumour cell lines translated into specific tumour cell killing. Killing of HLA A2+ and HLA A2- tumour cell lines MM909.24, SKBR3 and C1R was assessed using a flow cytometry based killing assay at a T-cell:tumour cell ratio of 1:1 after 24 h. CLL 9 killed tumour cell lines from the skin, breast and blood in an HLA A2 restricted manner (**Figure 5.17**).

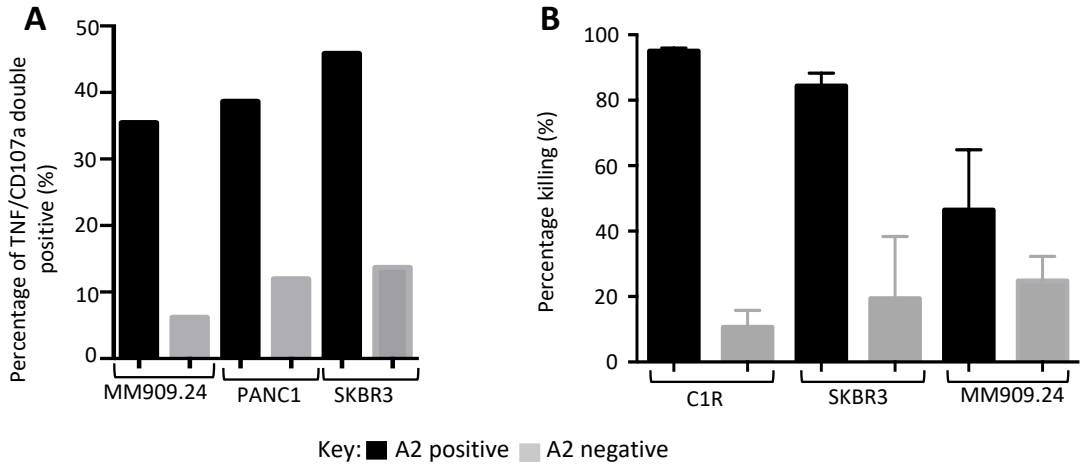


Figure 5.17: Recognition and killing of non-autologous tumour cell lines by CLL 9

***A**) Reactivity of CLL 9 to a panel of tumours from different anatomical locations; skin (melanoma MM909.24), pancreas (PANC1) and breast (SKBR3) measured though TAPI-O activation assay. Release of TNF and CD107a measured by flow cytometry. Percentage of TNF/CD107a double positive cells shown. **B**) Percentage killing of a panel of distinct tumour subtypes; C1r (lymphoma), SKBR3 (breast) and MM909.24 (melanoma) by CLL 9. T-cells and tumour targets were co-incubated at a ratio of 1 T-cell to 1 tumour cell for 24 hours and percentage of tumour cell killing measured by flow cytometry. A CFSE-labelled reference cell population was used as a constant to allow calculation of percentage killing. Results are shown as the mean to 3 replicates and the error is shown as standard deviation.*

I also felt that it was important to establish that any reactivity of CLL 9 to non-leukaemia malignancies was cancer specific and that the T-cell clone did not recognise healthy cell lines. It was important to show that priming with **MTSA**IGIL**P**V elicits cancer-specific CD8 T-cells as the first step in assessing the safety of an **MTSA**IGIL**P**V cancer vaccine. Reactivity of CLL 9 to a panel of HLA A2 positive healthy cell lines and immune cell subsets isolated from HLA A2 positive and negative PBMC was assessed by TNF ELISA (**Figure 5.18**). As a control, reactivity was compared to that of A2 positive and A2 negative cancer cell lines; blood (C1R), melanoma (MM909.24) and pancreatic (PANC1) (**Figure 5.18**). CLL 9 reacted strongly to melanoma, pancreatic, breast and blood cancer cell lines but did not produce TNF above baseline level (no targets) in response to any of the healthy cells assessed.

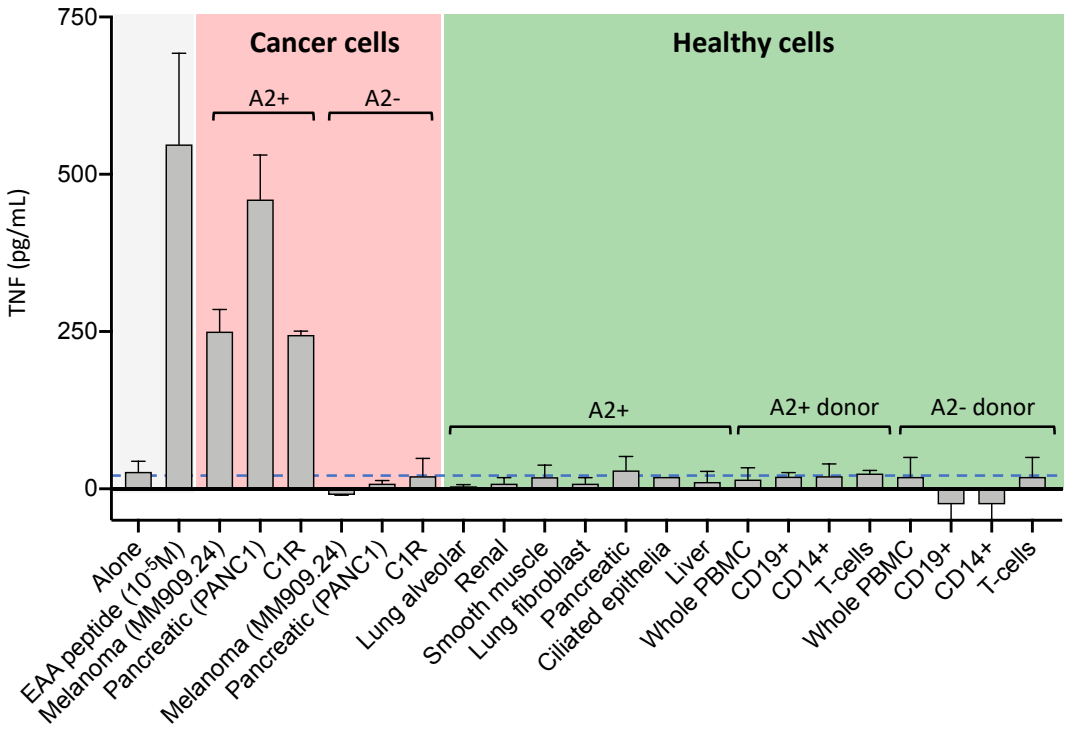


Figure 5.18: Multi-pronged CD8 T-cell clone, CLL 9, recognises tumours distinct from CLL but not healthy cells

Overnight activation assay with clone CLL9 versus cancer and healthy cells followed by a TNF ELISA. Cancer cells +/- HLA A2 (either transgene or CRISPR knock-out**), HLA A2+ healthy cells and whole PBMCs or blood derived cell subset from an HLA A2+ and HLA A2- donors were used. The B-cells (CD19+), monocytes (CD14+) and T-cells (pooled CD4+ and CD8+) were purified using magnetic beads to greater than 90% purity. 3 x10^4 T-cells and 6 x10^4 target cells were used per well and performed in duplicate. Error bars depict standard deviation. Dashed line set a mean TNF value for CLL9 clone alone.*

To further confirm the specificity of CLL 9, I next examined specific target cell killing by CLL 9 using a flow cytometry based killing assay (**Figure 5.19**). Killing of HLA A2 healthy cell lines was assessed after 24 h and compared to the killing of HLA A2 positive and negative cancer cell lines from the skin, pancreas and blood (**Figure 5.19B**). Whilst CLL 9 killed the tumour cell lines in an HLA A2 restricted manner, it did not kill any of the HLA A2 healthy cell lines included in the assay which originated from the liver, lung, pancreas and kidneys.

The data in **Figure 5.17**, **Figure 5.18** and **Figure 5.19** demonstrate that it is possible to elicit cancer-specific cross-reactive TCR clonotypes that do not react to healthy cells by priming with **MTSA**IGIL**P**V super-agonist peptide.

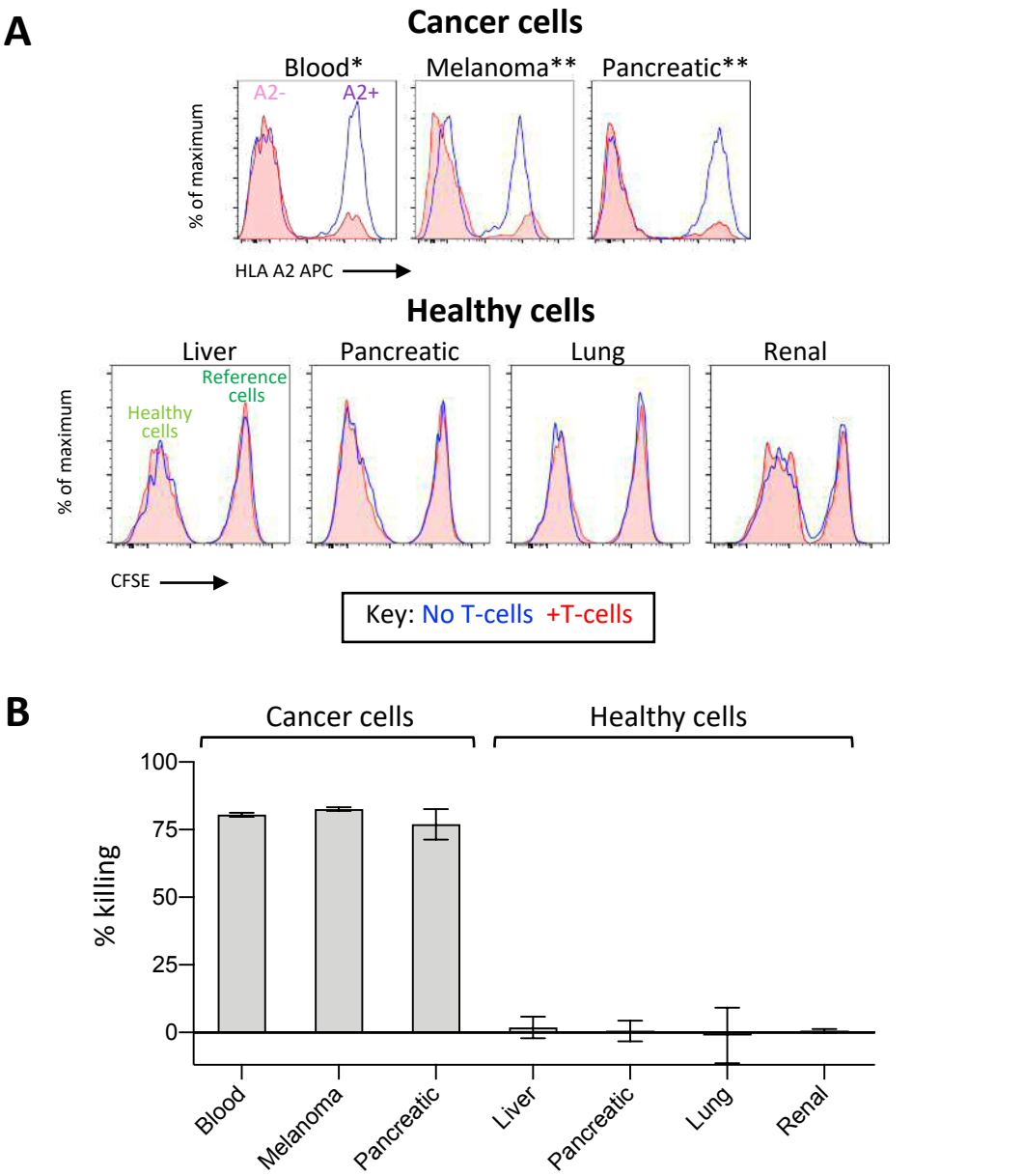


Figure 5.19: Tumour-reactive multi-pronged CD8 T-cell clone, CLL 9, does not kill healthy cells

24h flow-based killing assay at a 1:1 ratio of CLL9 versus cancer or healthy cells. Cancer cells +/- HLA A2 (either transgene or CRISPR knock-out**) co-incubated in the same well with and without CLL9. HLA A2+ healthy cells used in the same assay but with CFSE+ reference cells added to the wells prior to harvest and flow cytometry. Cells were stained with the viability dye VIVID, CD8-PE, CD3-PeCy7 and the cancer cells also with HLA A2-APC. Gated on viable CD3-CD8-HLA A2+/- for cancer cells or viable CD3-CD8-CFSE-/+ for healthy cells. (**A**) Performed in duplicate with one of the replicates shown. (**B**) The Percentage killing was calculated according to the materials and methods. The solid bars depict the mean and error bars the SD.*

5.3. Discussion

Tumour associated antigens can have a strong association with one malignancy or can be widely expressed in multiple different cancers. As demonstrated in **Chapter 4**, the TAA melan-A has a restricted expression to melanoma, whilst the TAAs BST2 and IMP2 have a varied expression, including in multiple cancer cell lines from different origins. In **Chapter 4**, I demonstrated that super-agonist peptide **MTSA**IGIL**P**V could elicit T-cells reactive to TAA melan-A, BST2 and IMP2. These results let me to hypothesize that successful peptide priming of anti-cancer T-cells with **MTSA**IGIL**P**V peptide could be possible in cancers distinct from melanoma.

In this chapter I wanted to interrogate the ability of **MTSA**IGIL**P**V to prime TAA-specific cells in non-melanoma cancers by focussing on three distinct malignancies where relevant samples were available, renal cell carcinoma (RCC), chronic lymphocytic leukaemia (CLL) and acute myeloid leukaemia (AML).

5.3.1. Successful MTSAIGILPV peptide priming in renal cell carcinoma (RCC) patient samples

I first sought to look at **MTSA**IGIL**P**V peptide priming in samples from renal cell carcinoma patients who were enrolled in clinical trials at the CCIT in Copenhagen. Small sample size prevented me from looking at priming with BST2 or IMP2 epitopes in these samples however I was able to assess the success of priming with **MTSA**IGIL**P**V peptide. In RCC patient 2, **MTSA**IGIL**P**V peptide priming for 28 days elicited more **MTSA**IGIL**P**V-tetramer positive T-cells than in T-cells cultured alone (**Figure 5.1**). Following on from this result, I wanted to assess the peptide reactivity of **MTSA**IGIL**P**V primed T-cells from RCC patient 12 (**Figure 5.2**). CD8 T-cells from RCC patient 12 cultured for 28 days with **MTSA**IGIL**P**V peptide were more reactive to **MTSA**IGIL**P**V peptide than T-cells cultured alone, assessed though TNF output following a 4 h activation assay. To further assess the outcome of **MTSA**IGIL**P**V peptide priming, I wanted to establish if T-cells primed with **MTSA**IGIL**P**V from RCC patient 12 killed autologous tumour (**Figure 5.2**). Killing was assessed using a chromium release assay and **MTSA**IGIL**P**V primed T-cells demonstrated enhanced killing of the autologous RCC 12 tumour in a chromium release assay. A study by Andersen *et al.* found that CD8 T-cells isolated from the tumour microenvironment of RCC patient 12 were poorly reactive to autologous tumour (Rikke Andersen *et al.*, 2018). The RCC patients in this study were enrolled in tumour infiltrating lymphocyte (TIL) clinical trials at the CCIT. The data presented here showing increased CD8

T-cell reactivity to tumour after **MTSA**IGIL**P**V peptide priming is promising and could suggest a way to enhance the potency of TIL infusion products in future. **MTSA**IGIL**P**V peptide priming of TIL infusion products prior to reinfusion could result in enhanced numbers of TAA specific, tumour-reactive CD8 T-cells being put back into the patient.

5.3.2. BST2 and IMP2 expression in leukaemia cell lines

As the number and size of RCC patient samples was very limiting, I decided to look at **MTSA**IGIL**P**V peptide priming in another cancer types that we had access to in Cardiff, potentially increasing the number of patients and ease of access to the samples. Through a collaboration with the Department of Haematology, University hospital of Wales I had access to two blood cancers, namely chronic lymphocytic leukaemia (CLL) and acute myeloid leukaemia (AML). Before initiating my experiments, I wanted to assess whether the TAAs BST2 and IMP2 were expressed in AML and CLL cell lines. To do this I used the TRON Online Cell Portal (Scholtalbers *et al.*, 2015) which displays the expression of TAA in cancer cell lines as log_2 reads per kilobase per million (RPKM). I compared the expression of melan-A, BST2 and IMP2 to insulin, as a negative control and to Wilms tumour antigen 1, an established leukaemia associated antigen (LAA) (Ariyaratana and Loeb, 2007). I felt it was important to compare the TAA BST2 and IMP2 expression to the expression of WT1 as WT1 has been extensively researched as a target for AML immunotherapies. It is important that an antigen target for immunotherapy is ubiquitously expressed, if the expression of BST2 and IMP2 did not match that of WT1 then BST2 and IMP2 would not make improved candidate targets for a leukaemia peptide vaccination. BST2 and IMP2 were expressed at higher levels in 10/12 and 12/12 of the leukaemia cell lines assessed respectively (**Figure 5.3**). This result was not expected due to the association of WT1 and leukaemia, furthermore it highlighted BST2 and IMP2 as potentially superior targets for leukaemia-focused immunotherapy than WT1. The high expression of BST2 and IMP2 in established leukaemia cell lines and the ability of **MTSA**IGIL**P**V-peptide priming to elicit T-cells specific for these antigens was encouraging as I sought to interrogate **MTSA**IGIL**P**V super-agonist peptide priming in CLL and AML patient samples.

5.3.3. Successful priming of MTSAIGILPV-tetramer positive cells in leukaemia patient samples that kill autologous tumour

I first wanted to establish whether I could prime **MTSA**IGIL**P**V-specific CD8 T-cells from CLL patient samples. CD8 T-cells isolated from CLL patient A276 were primed for 28 days with

MTSAIGIL**P**V or cultured alone. Success of **MTSA**IGIL**P**V priming was then assessed by staining with **MTSA**IGIL**P**V-tetramers, 4.75% **MTSA**IGIL**P**V-tetramer positive was seen in the **MTSA**IGIL**P**V-primed cells compared to 0.23% in the not primed cells (**Figure 5.5**). Following on from successful **MTSA**IGIL**P**V priming in patient A276, I wanted to assess whether this could be replicated in two further CLL patients, A626 and U386 (**Figure 5.6** & **Figure 5.8**). Again, priming was assessed using **MTSA**IGIL**P**V-tetramers and in both patients priming with **MTSA**IGIL**P**V peptide elicited more **MTSA**IGIL**P**V-tetramer positive cells than culturing T-cells without peptide. Given the success of priming with **MTSA**IGIL**P**V peptide, I then wanted to look at whether **MTSA**IGIL**P**V-primed T-cells could kill autologous tumour. In patient A626, killing was assessed using a chromium release assay and after 4 h of incubation **MTSA**IGIL**P**V primed T-cells killed almost 60% of autologous tumour compared to 10% in the unprimed cells (**Figure 5.6**). For patient U386, killing was assessed using a flow cytometry based killing assay, and after 6 days of incubation **MTSA**IGIL**P**V primed T-cells demonstrated significantly more autologous tumour killing relative to T-cells cultured alone (**Figure 5.8**). Studies have suggested that CLL tumours can alter the functional capacity of CD8 T-cells (Gonnord *et al.*, 2019) leading to T-cell dysfunction, reduced tumour cell killing and ultimately tumour escape (Ramsay *et al.*, 2008; van Bruggen *et al.*, 2019). My data shown here suggesting that **MTSA**IGIL**P**V priming can significantly increase autologous cell killing by CD8 T-cells and provide promise that dysfunction inflicted by tumour cells can be overcome.

I also attempted to prime **MTSA**IGIL**P**V-tetramer positive cells in samples from a patient presenting with emergent AML. I was able to gain access to a 9 mL blood sample from patient CH160486 through an ongoing collaboration with the haematology department at the University Hospital of Wales. Almost 97% of the cells in this small blood sample were shown to be tumour meaning that there were very few CD8 T-cells to work with. After 28 days peptide priming of CD8 T-cells from AML patient CH160486, was assessed by EAAGIGILTV, LLLGIGILVL, NLSALGIFST and **MTSA**IGIL**P**V-tetramer staining (**Figure 5.11**). **MTSA**IGIL**P**V peptide priming elicited greater EAAGIGILTV, LLLGIGILVL, NLSALGIFST-tetramer positive populations from AML patient CH160486 than priming with the WT peptides. Since I demonstrated that **MTSA**IGIL**P**V-primed T-cells from RCC and CLL patients were superior at killing autologous tumour I wanted to assess tumour cell killing by **MTSA**IGIL**P**V -primed T-cells from AML patient CH160486 (**Figure 5.12**). It was important to determine whether **MTSA**IGIL**P**V-primed cells had an improved ability to kill tumour cells as AML has been shown to cause T-cell dysfunction, resulting in a reduced capacity to kill autologous tumour (Le Dieu *et al.*, 2009; Knaus *et al.*, 2018). **MTSA**IGIL**P**V-primed T-cells from patient CH160486 killed

substantially more autologous tumour than T-cells primed with EAAGIGILTV, LLLGIGILVL or NLSALGIFST. Furthermore, **MTSA**IGIL**P**V-primed T-cells demonstrated superior killing of the HLA A2+ AML cell line THP-1, compared to the T-cells primed with the WT peptide antigens. Taken together, these data show that priming AML patient T-cells with **MTSA**IGIL**P**V peptide elicits multipronged T-cells with a superior ability to kill AML tumour.

5.3.4. Super-agonist peptide MTSAIGILPV elicits cross-reactive CD8 T-cells from CLL patient samples

In **Chapter 4**, I demonstrated that priming with **MTSA**IGIL**P**V peptide could elicit T-cells specific for TAA antigen epitopes EAAGIGILTV (melan-A), LLLGIGILVL (BST2) and NLSALGIFST (IMP2) from healthy donor PBMC. I therefore wanted to assess whether **MTSA**IGIL**P**V priming in cancer patient samples could also elicit T-cells specific for these antigens. Priming CD8 T-cells isolated from CLL patients A276 and U386 for 28 days with **MTSA**IGIL**P**V peptide elicited more EAAGIGILTV and NLSALGIFST tetramer positive cells than priming with the wild type antigens (**Figure 5.9**). There was not enough sample from patient A626 to expand peptide priming in this way. The identification of T-cells specific for multiple TAA in the **MTSA**IGIL**P**V primed population may explain the increased capacity of **MTSA**IGIL**P**V-primed CD8 T-cells from donor U386 to kill autologous tumour. Unfortunately, I did not have enough sample to include BST2 derived peptide LLLGIGILVL in my priming experiments. It would be interesting to assess the success of priming CLL patient derived CD8 T-cells with LLLGIGILVL as established CLL cell lines expressed a high level of BST2 (**Figure 5.3**). Peptide priming with **MTSA**IGIL**P**V also elicited sizable populations of EAAGIGILTV-, LLLGIGILVL- and NLSALGIFST-tetramer positive cells from AML patient CH160486 (**Figure 5.11**).

5.3.5. A T-cell clone isolated from MTSAIGILPV-primed population is multipronged

I wanted to further assess the peptide specificities and tumour killing of T-cells from **MTSA**IGIL**P**V-primed polyclonal T-cell populations. Since samples were limited in volume and the number of T-cells that could be isolated from each sample was small, I attempted to isolate individual TCR clonotypes from these patients. I started with CLL patient U386 and managed to isolate TCR clone CLL 9 that grew well in culture (**Figure 5.14**). Initially, I discovered that CLL 9 recognised peptides EAAGIGILTV, NLSALGIFST and MTSAIGILPV (**Figure 5.15**). Furthermore, CLL 9 is effective at killing autologous tumour. This data led me to believe that CLL 9 may be multipronged. As demonstrated in **Chapter 4**, some multipronged TCRs can recognise and kill many types of different cancer cell lines. Initially, I assessed the

reactivity and killing of cancer cell lines from the breast, skin, pancreas and blood and found that CLL 9 recognises and kills these tumours in an HLA A2 restricted manner (**Figure 5.17**). I next wanted to determine if this recognition and killing was cancer-specific. In the same experiment, it was shown that whilst CLL 9 recognised MM909.24, PANC1 and C1R tumour cell lines, it does not react to healthy cell lines and immune cells isolated from healthy PBMC (**Figure 5.18**). I then examined specific killing of tumour and healthy cell lines by CLL 9 (**Figure 5.19**). CLL 9 killed blood, melanoma and pancreatic cancer cell lines but did not kill healthy cells from liver, pancreas, lung or kidney (**Figure 5.19**).

This investigation has identified another example of a cross-reactive TCR clonotype that is capable of killing multiple different types of cancer cell including the autologous tumour from the patient the clone was isolated from. Importantly CLL 9 was shown to remain inert to healthy cells. This TCR clonotype originated from a polyclonal population of **MTSA**IGIL**P**V-primed CD8 T-cells and exhibits sensitive recognition of the **MTSA**IGIL**P**V peptide. This result highlights a link between the super-agonist peptide **MTSA**IGIL**P**V and potent, cross-reactive TCR clonotypes, providing promise that *in vivo* administration of **MTSA**IGIL**P**V might elicit effective TCR clonotypes, capable of mediating an anti-cancer immune response.

5.3.6. Summary

In this chapter, the super-agonist peptide **MTSA**IGIL**P**V was shown to be able to elicit CD8 T-cells from RCC, CLL and AML patient samples. In CLL and AML patient samples **MTSA**IGIL**P**V-primed CD8 T-cells were also specific for EAAGIGILTV (melan-A) LLLGIGILVL (BST2) and NLSALGIFST (IMP2). Furthermore, **MTSA**IGIL**P**V-primed polyclonal T-cell populations show enhanced killing of autologous tumour. Further interrogation of an individual TCR clonotype, CLL 9, from the **MTSA**IGIL**P**V-primed T-cell population from CLL patient U386 showed that it recognised multiple TAA epitopes and demonstrated killing of autologous CLL tumour in addition to multiple HLA A2+ cancer cell lines of various origin. Early stage safety testing of TCR clonotype CLL 9 demonstrated cancer-specific killing as CLL 9 did not react to or kill any of the healthy cell subsets it was exposed to. The data presented here further highlights the potential super-agonist peptide **MTSA**IGIL**P**V as a pan cancer vaccine candidate.

6. General discussion

6.1. Summary of work

The immune system has a well-established role in protecting against the development of malignant disease. With this increasing knowledge came research into how to harness patients own potent immune cells to treat cancer. This form of cancer treatment is termed immunotherapy and is considered to be a revolutionary breakthrough in the treatment of malignant disease (Couzin-Frankel, 2013). Immunotherapies attempt to improve the patient's own immune response to cancer by modulating the tumour microenvironment and/or harnessing cytotoxic cells. To date, effective cancer immunotherapies have revolved around harnessing cytotoxic CD8 T-cells, the focus of this thesis. Successful immunotherapeutic strategies include monoclonal antibodies that block key T-cell checkpoints to re-activate cytotoxic T-cells in the TME, causing tumour regression in some patients (Eggermont *et al.*, 2016; Du *et al.*, 2018). Patient-specific adoptive cell transfer immunotherapies such as TIL therapy have also shown some clinical success with some tumours, especially melanoma (Rikke Andersen *et al.*, 2016; R Andersen *et al.*, 2018).

Peptide vaccines are a form of immunotherapy that have failed to live up to expectations. Poor choice of peptide targets, flawed delivery strategies and inability to overcome T-cell tolerance have led to poor clinical outcomes. Peptide targets for vaccines are generally derived from self-proteins with varied expression patterns during malignancy, otherwise known as tumour associated antigens (TAA). Generally, TCRs have low affinity for epitopes derived from self-proteins, providing a potential problem when attempting to use these epitopes for immunotherapy. Activation of low affinity TCRs, can lead to ineffective responses to malignant disease (Kawakami *et al.*, 1994; Morgan *et al.*, 2006). My study sought to use a TCR clonotype isolated from a metastatic melanoma patient who successfully entered remission after TIL therapy to design better peptide ligands for anti-cancer peptide vaccination. These peptide ligands are referred to as super-agonist peptides as they should activate T-cells with higher potency than the cognate ligand. The TCR clonotype ST8.24 was originally isolated from the TIL infusion product of HLA A2+ melanoma patient MM909.24. ST8.24 is specific for the TAA melan-A which has a restricted expression in melanocytes and over-expression in most malignant melanomas. The dominant HLA A2 epitope from melan-A, EAAGIGILTV, is poorly immunogenic and therefore is unable to elicit potent anti-melanoma T-cells *in vivo* (Kawakami *et al.*, 1994).

6.1.1. Identification of super-agonist peptide MTSAIGILPV

In order to identify a panel of candidate super-agonist peptides I used a combinatorial peptide library (CPL) screen (**Chapter 3**). CPL screening exposes a given TCR clonotype to all possible combinations of peptides of a given length, allowing a hierarchy of recognition of amino acids at each position of the peptide to be identified (**Figure 6.1**). I believed that using a CPL would be the optimal method for identifying super-agonist peptides as CPLs provide an unbiased method to identify peptide ligands. Using a patient derived, persistent TCR clonotype to design candidate super-agonist peptides was important as it provided a clinical context to the work. ST8.24 TCR clonotype was identified in the TIL infusion product and PBMC 6 months post cure so it can be assumed that it did not elicit harmful cytotoxicity to melanoma patient MM909.24. Using a patient derived TCR provides some initial hope for design of safe candidate peptides.

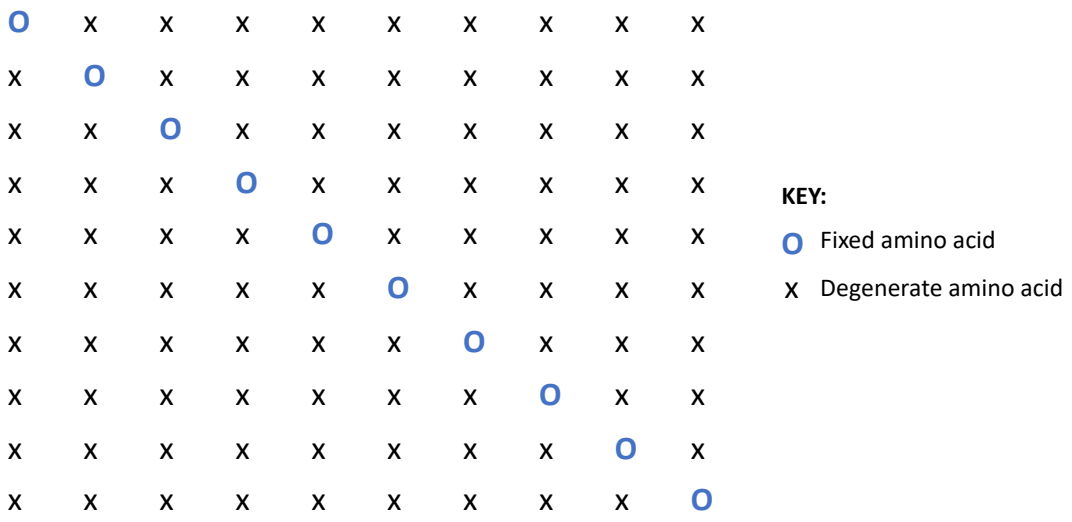


Figure 6.1: Combinatory peptide library (CPL) screen

*A combinatorial peptide library (CPL) screen exposes a T-cell clone to all possible combinations of amino acids in a peptide of a given length. In each pool of peptides, the amino acid is fixed at one position (depicted as **O**) whilst the rest of the amino acids in the sequence are degenerate (depicted as x). For each position of the peptide there are 20 pools corresponding to each of the 20 naturally occurring amino acids. CPL screens allow a map to be generated, highlighting the preferred amino acids at each position for the T-cell clone of interest. CPL screens are typically conducted using an ELISA, with an overnight activation assay and T-cell activation measured as MIP-1β release.*

CPL data generated from ST8.24 CPL was used to generate a panel of ten candidate super-agonist peptides. It is important to note that there is a lack of conservation between the ten candidate super-agonist peptides and the cognate antigen EAAGIGILTV, which highlights the

importance of an unbiased screening method when designing candidate super-agonist peptides.

Initially, candidate super-agonist peptides were screened using individual EAAGIGILTV-specific TCR clonotypes, and the **ITS**GIG**V**L**P**V peptide appeared to be superior. However, further screening of candidate peptides by peptide priming of polyclonal T-cell populations from healthy HLA A2+ donors and subsequent EAAGIGILTV-tetramer staining resulted in the identification of **MTSA**IGIL**P**V as the optimal super-agonist peptide in terms of T-cell priming. Priming with **MTSA**IGIL**P**V elicited more EAAGIGILTV-tetramer positive cells than the other nine candidate peptides and EAAGIGILTV itself. Furthermore, comparison of **ITS**GIG**V**L**P**V and **MTSA**IGIL**P**V priming across multiple healthy donors confirmed **MTSA**IGIL**P**V as the optimal candidate super-agonist peptide as it routinely primed more EAAGIGILTV-tetramer positive T-cells than **ITS**GIG**V**L**P**V.

Priming with super-agonist peptide **MTSA**IGIL**P**V elicited more potent T-cells capable of enhanced tumour cell killing compared to priming with the natural EAAGIGILTV peptide. This was a critical finding as the EAAGIGILTV peptide is poorly immunogenic and had little effect when administered to melanoma patients (Kawakami *et al.*, 1994). This finding led me to wonder why **MTSA**IGIL**P**V was so superior to EAAGIGILTV. My first thought was to assess the relationship of **MTSA**IGIL**P**V to HLA A2 as the binding of a peptide to HLA molecule can affects its capacity to elicit effective T-cell responses.

6.1.2. Biophysical analysis of the superiority of MTSAIGILPV

In order to interrogate why **MTSA**IGIL**P**V was superior to EAAGIGILTV, I initially wanted to look at the binding of **MTSA**IGIL**P**V to HLA A2. A T2 binding assay, which provides a crude measurement of the interaction of a peptide for a given HLA molecule, demonstrated that **MTSA**IGIL**P**V exhibited improved binding to HLA A2 when compared to EAAGIGILTV. It has been previously demonstrated that peptides that exhibit stronger binding to HLA molecules are present at higher density at the target cell surface and provide a stronger signal to peptide-specific T-cells, eliciting more TAA-specific T-cells (Valmori, Fonteneau, Lizana, Gervois, Liénard, Rimoldi, Jongeneel, Jotereau, Cerottini, Romero, *et al.*, 1998; Ekeruche-Makinde *et al.*, 2012; Smith, Rekoske and McNeel, 2014; Kolawole *et al.*, 2018). Valmori *et al.* designed the heteroclitic peptide E**L**AGIGILTV, to specifically improved binding to HLA A2 due to the inclusion of the preferred anchor residue, L, at P2. E**L**AGIGILTV has been widely used as an alternative peptide to EAAGIGILTV as it elicits more EAAGIGILTV-specific T-cells

than priming with EAAGIGILTV itself (Valmori, Fonteneau, Lizana, Gervois, Liénard, Rimoldi, Jongeneel, Jotereau, Cerottini, Romero, *et al.*, 1998). However, E**L**AGIGILTV has had limited success clinically and studies have suggested that it elicits inferior TCR clonotypes, that are not able to recognise the cognate antigen (Cole *et al.*, 1997; Wieckowski *et al.*, 2009). To further understand **MTSA**IGIL**P**V and its interaction with HLA A2 the crystal structure of HLA A2-**MTSA**IGIL**P**V was resolved. Surprisingly, EAAGIGILTV and **MTSA**IGIL**P**V are close structural mimics of each other when bound to HLA A2, despite differing by 5/10 amino acids. The structural similarity of EAAGIGILTV and **MTSA**IGIL**P**V provided some explanation as to how **MTSA**IGIL**P**V-primed T-cell populations cross-recognised EAAGIGILTV.

Another possible explanation for the increase expansion of EAAGIGILTV-specific T-cells following **MTSA**IGIL**P**V peptide priming could be an increased affinity of TCRs for the **MTSA**IGIL**P**V peptide. Increased affinity of a TCR for a pMHC molecule can result in increased proliferation and improved functional capacity, which has been seen through **MTSA**IGIL**P**V peptide priming. The affinity of T-cells for HLA A2-**MTSA**IGIL**P**V was assessed using pMHC-tetramers for EAAGIGILTV and **MTSA**IGIL**P**V. Affinity in this instance was shown as fluorescent intensity of the pMHC-tetramer staining. **MTSA**IGIL**P**V-tetramers bound with increase fluorescent intensity than EAAGIGILTV-tetramers in matched clonal and polyclonal populations, inferring increased TCR affinity for **MTSA**IGIL**P**V.

The biophysical data helped to somewhat explain the superior peptide priming elicited by super-agonist peptide **MTSA**IGIL**P**V but failed to explain why the individual clonotypes induced by **MTSA**IGIL**P**V exhibited improved recognition of melanoma compared to T-cell populations primed by the natural antigen. In **Chapter 4**, I sought to explain why **MTSA**IGIL**P**V peptide primed more effective T-cells.

6.1.3. Super-agonist peptide MTSAIGILPV and T-cell cross-reactivity

The structural mimicry of EAAGIGILTV and **MTSA**IGIL**P**V peptides when bound to HLA A2 and increased affinity of T-cells for **MTSA**IGIL**P**V somewhat explained the superiority of priming with **MTSA**IGIL**P**V super-agonist peptide. However, I wanted to determine why **MTSA**IGIL**P**V primed T-cells were functionally better than EAAGIGILTV primed T-cells (**Chapter 4**).

Cross-reactivity underlies the ability to design super-agonist peptides using EAAGIGILTV-specific ST8.24 TCR clonotype, as ST8.24 is reactive to many different peptides. I next considered whether such cross-reactivity of **MTSA**IGIL**P**V-primed CD8 T-cells could underlie their superior functional capacity. To assess this aspect, I used the CPL data generated using

the ST8.24 clone to screen the human genome for potential agonist peptides. Three of the top four hits from this exercise were EAAGIGILTV, LLLGIGILVL and NLSALGIFST, derived from melan-A, BST2 and IMP2 respectively. My colleague, Dr Cristina Rius, showed that the BST2 and IMP2 epitopes were genuinely processed and presented on the surface of target cells expressing both HLA A2 and the TAA (Rius Rafael, 2019).

It was hypothesised that ST8.24 could recognise these TAA on the same tumour cell, reducing the risk of tumour escape by antigenic drift. The Sewell laboratory has coined the term 'multipronged' for such multiepitope cross-reactive T-cells that see multiple antigens on the same tumour cell. If super-agonist peptide **MTSA**IGIL**P**V could elicit multipronged T-cells this would further explain the superior functional capacity seen by **MTSA**IGIL**P**V-primed T-cells. A melan-A KO in the autologous tumour MM909.24 confirmed that ST8.24 could target the autologous tumour through another epitope (i.e. it was multipronged). Without melan-A, ST8.24 still showed HLA A2 restricted reactivity to MM909.24, albeit reduced, indicating that it is recognising other TAA on the same tumour cell. Furthermore, ST8.24 could recognise EAAGIGILTV, LLLGIGILVL and NLSALGIFST peptides at suboptimal concentrations when they were in combination, this was important as it showed that multipronged T-cells require a lesser amount of any given antigen on the surface of the tumour cell to become activated. These results infer that multipronged T-cells like ST8.24 could prevent tumour escape *in vivo* via loss of any one antigen.

T-cell cross-reactivity is not a simple entity. There are many mechanisms by which T-cells ability to see multiple peptide-MHC complexes (reviewed in (Sewell, 2012)). Conserved residues, or 'hotspots' in peptides can underlie T-cell cross-reactivity (Cole *et al.*, 2016). The peptides recognised by ST8.24, and elicited by **MTSA**IGIL**P**V, EAAGIGILTV, LLLGIGILVL and NLSALGIFST, all possess a glycine (G) residue at position 6 of the peptide and the CPL screen of ST8.24 shows a strong conservation at that residue. All good agonists also possess an isoleucine residue at position 7 suggesting that positions 6 and 7 might form a binding hotspot. Coles *et al.* describe three TCRs which have the same cognate peptide, two TCRs bind in a similar way and have similar peptide pools whilst the third binds in a 'flipped' conformation and so is able to recognise the cognate antigen and a vastly different pool of peptides (Coles *et al.*, 2020). Furthermore, peptide structural mimicry can result in cross-reactive T-cell responses, a recent study described how molecular mimicry between bacterial antigens and gladin antigens underpins T-cell cross-reactivity in celiac disease (Petersen *et al.*, 2020). Molecular mimicry of EAAGIGILTV and **MTSA**IGIL**P**V documented here, can somewhat explain the ability of T-cells to recognise both antigens. Understanding how

individual TCRs can bind to HLA A2 presenting EAAGIGILTV, LLLGIGILVL and NLSALGIFST peptides would require comparison of the co-complex structures. My colleague, Aaron Wall, has been examining this aspect and has solved the structure of another multipronged TCR, MEL5 in complex with EAAGIGILTV-, LLLGIGILVL-, NLSALGIFST- or MTSAIGILPV-HLA A2. Overlay of these structures shows that they bind HLA A2 in a remarkably similar way, despite differing amino acid sequences (**Figure 6.2A**). Furthermore, comparison of crystal structures of the MEL5 TCR in complex with HLA A2-EAAGIGILTV, HLA A2-LLLGIGILVL and HLA A2-NLSALGIFST highlights a hotspot of recognition with the TCR preferentially interacting with P 4-7 of the peptide, in particular the glycine (G) residue at position 6 of each peptide (**Figure 6.2B-D**).

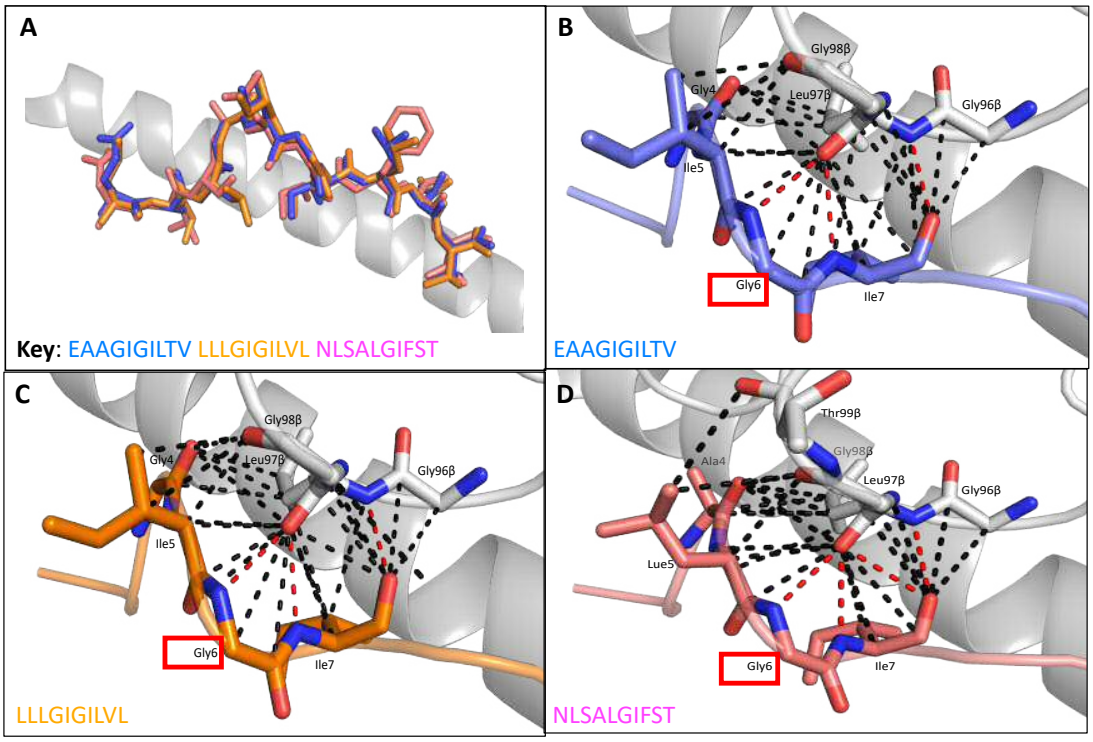


Figure 6.2: TCR clonotype MEL5 interacts with cancer derived epitopes from melan-A, BST2 and IMP2, in a conserved manner.

Crystallographic structures of MEL5 TCR in complex with HLA A2 restricted cancer derived epitopes EAAGIGILTV (PDB:4qok, blue), LLLGIGILVL (orange) and NLSALGIFST (pink), at resolutions of 3Å, 2.25Å and 2.55Å respectively. ***A****) Overlay of epitopes presented by HLA A2.* ***B-D****) Interactions between MEL5 CDR loops (white sticks) and 'hotspot' regions (P4-P7) of cancer derived epitopes EAAGIGILTV (****B****), LLLGIGILVL (****C****) and NLSALGIFST (****D****). Van der Waal interaction (black dotted lines) and hydrogen bond (red dotted lines) are shown, cut offs are 4Å and 3.4Å respectively. Data provided by Aaron Wall.*

6.1.4. Super-agonist peptide MTSAIGILPV as a pan-cancer vaccine candidate

Given the potency of cross-reactive, multipronged T-cells to prevent tumour escape and mediate effective tumour killing *in vitro*, it could be advantageous to elicit such cells by vaccination. In **Chapter 3**, **MTSA**IGIL**P**V-primed T-cells from a melanoma patient sample were shown to be superior at killing autologous tumour. In **Chapter 4**, multipronged TCR clonotypes were shown to be better at killing tumour cells, from different anatomical origins, than single pronged TCRs and that **MTSA**IGIL**P**V could prime T-cells that recognised TAAs BST2 and IMP2. These data led me to hypothesise that **MTSA**IGIL**P**V-primed T-cells might enhance autologous tumour cell killing in non-melanoma cancer settings (**Chapter 5**). To test this hypothesis, I was able to access samples from patients with renal cell carcinoma (RCC), chronic lymphocytic leukaemia (CLL) and acute myeloid leukaemia (AML). Peptide priming with **MTSA**IGIL**P**V successfully induced T-cells from the PBMC of T RCC, CLL and AML patients. Where it was possible to test these T-cells, they were found to be capable of enhanced killing of autologous tumour. With these data in mind, I wanted to further explore the tumour reactivity and peptide specificities of T-cells primed by **MTSA**IGIL**P**V in leukaemia patients. The cell numbers in direct *ex vivo* samples were limited, so I decided to isolate individual TCR clonotypes from CD8 T-cell populations primed using **MTSA**IGIL**P**V and a blood sample from a CLL patient. CLL 9 TCR clonotype was isolated from CLL patient U386 and was found to be potent at killing autologous tumour. Furthermore, CLL 9 recognised **MTSA**IGIL**P**V, as well as EAAGIGLTV and NLSALGIFST peptides. CLL 9 also demonstrated potent killing of non-autologous tumour cells, whilst leaving healthy cells untouched. This was an important outcome as off-target toxicity is a risk factor when eliciting cross-reactive T-cells for immunotherapy.

Taken together these data highlight the CPL screens as an effective method of designing super-agonist peptides, capable of eliciting T-cells specific for multiple TAA epitopes that mediate effective tumour killing *in vitro*. These data are a promising step towards designing optimal peptides for cancer vaccination however more research is required to fully understand **MTSA**IGIL**P**V-specific T-cells and determine whether **MTSA**IGIL**P**V is safe to use therapeutically.

6.2. Future work

Melan-A has become the prime model TAA for immunotherapy as the most frequent HLA in the population, HLA*0201, presents an immunodominant epitope EAAGIGILTV to which many T-cells and TCRs can respond. Most T-cells that recognise HLA*0201-EAAGIGILTV use

a TRAV12-2 TCR and unusually, the germline-encoded TRAV-12-2 CDR1 loop plays a dominant role in peptide recognition (Cole *et al.*, 2009b). In addition, the form of melan-A expressed in the thymus lacks the EAAGIGILTV epitope (Pinto *et al.*, 2014). These two factors combined mean that VDJ rearrangement produces lots of TCRs with potential to interact with HLA*0201-EAAGIGILTV that are not deleted by central tolerance mechanisms ensuring that naïve T-cells with the capacity to respond to the EAAGIGILTV peptide are hundreds of times more frequent than those responding to other TAA (Pittet *et al.*, 1999; Zippelius *et al.*, 2002b). The dominance of the melan-A model and the high frequency of naïve T-cells with a capacity to respond to this antigen made it an obvious first choice model for studying peptide vaccination. Now that I have shown that APL can be used to prime more effective T-cells *in vitro* an obvious next step would be to apply the same approach to other TAA.

The TAA carcinoembryonic antigen (CEA) is upregulated in 90% of pancreatic tumours, a notoriously difficult malignancy to treat (Yamaguchi, Enjoji and Tsuneyoshi, 1991). As HLA A2+ CEA antigens, such as CAP1, are self-antigens and poorly immunogenic, a recent study attempted to design an altered peptide ligand (APL), CAP1-6D, to improve T-cell responses *in vivo* (Geynisman *et al.*, 2013). This study saw dose dependant responses in pancreatic patients treated with CAP1-6D, however overall clinical outcomes were poor. CEA and pancreatic cancer provide an alternative system in which to design super-agonist peptides using the methodology described in this thesis. It is important to note that, melan-A and CEA are both expressed at low levels in healthy tissues, and targeting them using immunotherapy has resulted in side effects (L. A. Johnson *et al.*, 2009). Future studies using the methodology described in this thesis might look to design super-agonist peptides for TAA expressed exclusively by tumour cells. This should reduce the risk of severe off target effects of any therapy.

In **Chapter 4**, I showed that the DMF4 TCR, which was used to treat metastatic melanoma patients, did not recognise multiple TAA epitopes and was less efficient at killing melanoma tumour when compared to a multipronged TCR, MEL5. To build on this work I would like to compare the antigen recognition and tumour killing of other TCR clonotypes used in clinical trials, such as the DMF5 TCR, to multipronged TCR clonotypes in this thesis; ST8.24, MEL5 and CLL9. These analyses may begin to explain the overall disappointing clinical efficacy of TCR-T therapy for metastatic melanoma to date.

In **Chapter 4**, I showed that a multipronged TCR clonotype ST8.24 was reactive to Melan-A KO melanoma tumour MM909.24, all be it with reduced activation. To further explore the

multipronged capacity of ST8.24 and the other T-cell clones discussed in this thesis, and to confirm that they were indeed recognising multiple tumour epitopes on the surface of tumour cells it would be interesting to knockout the IMP2 and BST2. Furthermore, it would be interesting to assess the gradient of activation by generating tumour cell lines expressing one, two or all three of melan-A, IMP2 and BST2. The generation of cell lines with and without IMP2 and BST2 was work in progress however it was prematurely halted due to the commencement of lockdown during the COVID-19 pandemic.

6.3. Future perspectives

Super-agonist peptide **MTSA**IGIL**P**V primes T-cells specific for epitopes derived from the TAA melan-A, BST2 and IMP2, which mediate enhanced killing of tumour cells *in vitro*. My preliminary data are promising, and provide hope that immunotherapy using **MTSA**IGIL**P**V could mediate effective tumour regression *in vivo*. Direct administration of peptides as a form of immunotherapy has been unsuccessful due to rapid degradation of peptides by proteases, inhibiting trafficking to dendritic cells and T-cell activation. Therefore, harnessing of **MTSA**IGIL**P**V-peptide priming to elicit of potent cytotoxic T-cells *in vivo* may require other immunotherapeutic strategies (**Figure 6.3**).

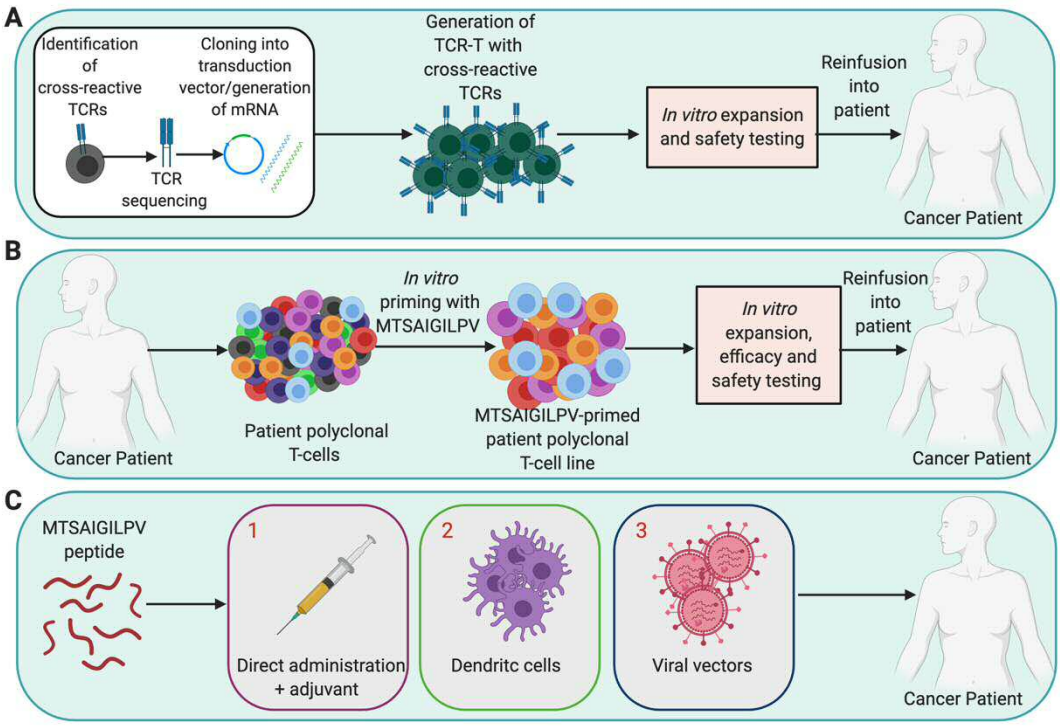


Figure 6.3: Taking super-agonist peptide MTSAIGILPV to the clinic

A) *TCR-T therapy with* ***MTSA****IGIL****P****V-specific cross-reactive TCRs provides a possible therapeutic strategy. Upon the identification of cross-reactive TCRs, such a ST8.24, MEL5 or CLL9, TCR sequencing can then take place following by the cloning of TCR sequences into vectors or the generation of mRNA for electroporation. The patient's own T-cells can then be transduced with cross-reactive TCRs, safety tested in vitro and then expanded to large numbers before reinfusion back into the patient.* ***B)*** ***MTSA****IGIL****P****V-peptide priming can elicit cross-reactive T-cells, specific for multiple TAA-derived epitopes.* ***MTSA****IGIL****P****V-specific T-cells can be preferentially expanded from polyclonal T-cell populations isolated from cancer patients in vitro. The* ***MTSA****IGIL****P****V-specific T-cell line can then be tested for reactivity to autologous tumour before being expanded and reinfused back into the patient.* ***C)*** ***MTSA****IGIL****P****V peptide could also be administered to patients using conventional vaccine technology. This could be through direct administration of a more stable* ***MTSA****IGIL****P****V peptide, through co-administration with viral vectors such as oncolytic viruses or by dendritic cell vaccination.*

6.3.1. Clinical application of **MTSA**IGIL**P**V peptide and multipronged TCRs

6.3.1.1. TCR-T therapy

The administration of tumour-specific TCRs as a form of adoptive cell therapy has been attempted to treat metastatic melanoma. TCRs are chosen due to specificity to a commonly expressed TAA. Melan-A is commonly overexpressed by melanoma tumours (Kawakami *et al.*, 1994). The DMF4 and DMF5 are specific for the melan-A derived epitope EAAGIGILTV and have been used to treat melanoma patients. DMF4 and DMF5 both failed to mediate effective tumour regression (Morgan *et al.*, 2006; L. A. Johnson *et al.*, 2009), in fact, patients treated with the DMF5 TCR experienced severe side effects (L. A. Johnson *et al.*, 2009). In **Chapter 4**, I showed that the DMF4 TCR was specific for the melan-A derived peptide, EAAGIGILTV but did not recognise epitopes derived from BST2 or IMP2. Furthermore, I showed that T-cells transduced with the MEL5 TCR, which recognises all three peptide antigens, mediated enhanced killing of melanoma tumour cell lines compared to T-cells transduced with the DMF4 TCR. The fact that the DMF4 TCR only targets melan-A, could explain its lack of efficacy. The DMF5 TCR was chosen as it has a higher affinity for EAAGIGILTV than the DMF4 TCR therefore it would be interesting to ascertain whether DMF5 has capacity to recognise BST2 or IMP2-derived epitopes. In this thesis, the TCR clonotypes ST8.24, CLL 9 and MEL5 were all shown to recognise more than one TAA derived epitope and to effectively kill tumour cells *in vitro*. This data suggests that administering cross-reactive TCRs as adoptive cell transfer by TCR-T could mediate tumour regression and improve clinical outcomes. Importantly, use of a melan-A/BST2/IMP2-specific multipronged TCR as a TCR-T therapy would allow the TCR to be screened by the FDA-approved TCR safety programme to fully check for any unwanted self-reactivities. While expensive, this approach would allow for the testing of both safety and efficacy before attempts to roll out the approach in the form of a vaccine.

It has been previously reported that some TCRs can recognise over one million peptide antigens (Wooldridge *et al.*, 2012). Therefore, it is quite possible that TCRs specific for **MTSA**IGIL**P**V, EAAGIGILTV, LLLGIGILVL and NLSALGIFST could also recognise additional peptide antigens. Whilst this could be a good thing for mediating cancer regression, it is important to bear in mind that some cross-reactivity can have negative consequences, and has resulted in unexpected fatalities to patients receiving TCR-T therapy (G. P. Linette *et al.*, 2013; Richard A Morgan *et al.*, 2013). Whilst I begun to interrogate the safety of one **MTSA**IGIL**P**V-reactive TCR clonotype, CLL 9, which did not react to healthy cell lines or

immune cells isolated from healthy HLA A2+ donors. This is only one TCR clonotype, and any off-target effects of cross-reactive, **MTSA**IGIL**P**V-specific CD8 T-cell must be further assessed before their use *in vivo* could be considered. There have been recent improvements in screening techniques which have resulted in increased safety profiling for TCT-T therapy. For example an HLA A2 restricted affinity enhanced MAGE A4 TCR underwent an enhanced screening protocol before administration to patients and no negative cross-reactivity was detected (Sanderson *et al.*, 2020). This TCR is now part of two clinical trials (NCT03132922, NCT04044768). Alternatively, genetic engineering methods have been employed to improve the safety of TCR-T therapy. TCR therapies have been designed with druggable 'suicide switches' to allow control over the persistence of TCR transduced cells *in vivo*. The use of safety switches in adoptive cell transfer was pioneered to combat graft vs host disease (GvHD) resulting from allogeneic stem cell transplants. The HSV-TK gene renders lymphocytes sensitive to the anti-herpes drug ganciclovir (Lupo-Stanghellini *et al.*, 2010). In patients treated with lymphocytes expressing the HSV-TK suicide gene, administration of ganciclovir controlled the development of acute GvHD (Ciceri *et al.*, 2009). Alternative safety switches have also been developed, Stasi *et al.* developed a safety switch that utilises the pro-apoptotic protein caspase 9 (Stasi *et al.*, 2011). Activation of caspase 9 in this system *in vivo* resulted in elimination of 90% of transduced T-cells within 30 minutes (Stasi *et al.*, 2011). T-cells have also been engineered to express surface markers, such as CD20 or EGFR, to allow targeting by monoclonal antibodies post-transfusion (Griffioen *et al.*, 2009; Wang *et al.*, 2011). The safety of TCR-T therapy can also be improved by inducing transient expression of the TCR using mRNA electroporation (Campillo-Davo *et al.*, 2018; Mensali *et al.*, 2019). TCR mRNA electroporation can also be combined with dicer-substrate small interfering RNAs (DsiRNA) which supress the expression of the endogenous TCR, reducing the risk of TCR chain mispairing (Campillo-Davo *et al.*, 2018).

6.3.1.2. Polyclonal adoptive cell transfer (ACT) therapy

Tumour infiltrating lymphocyte (TIL) therapy involves expanding the patient's own T-cells *ex vivo* before reinfusing them back into the patient. TIL therapy has demonstrated remarkable success in some patients with metastatic melanoma (Rikke Andersen *et al.*, 2016). However, success in non-melanoma tumours has been limited. In order to improve the cancer-specificity of this treatment two phase I studies sought to preferentially expand TAA-specific T-cells in the TIL infusion product (Yee *et al.*, 2002; Mackensen *et al.*, 2006). These studies saw some tumour regression and persistence of activated antigen-specific T-cells however

they also reported antigen loss by the patient tumour, possibly due to selection pressures applied by monospecific T-cells. Antigen loss can lead to tumour escape from the immune system and progression of malignant disease. **MTSA**IGIL**P**V peptide priming can elicit T-cells capable of recognising multiple TAA epitopes, and capable of potent tumour killing *in vitro*. Furthermore, **MTSA**IGIL**P**V-primed polyclonal T-cells lines from four distinct cancer types; melanoma, RCC, CLL and AML, mediated enhanced autologous tumour killing. It could therefore be possible to use **MTSA**IGIL**P**V peptide priming to preferentially expand a **MTSA**IGIL**P**V-specific T-cell line, which could then be reinfused back into the patient. The eliciting of cross-reactive T-cells by **MTSA**IGIL**P**V-priming could eliminate the risk of tumour escape through loss of single antigens. This methodology would allow screening of T-cells destined for reinfusion before initiation of the treatment. T-cells could be assessed for reactivity to autologous tumour, as well as any off-target effects, increasing the safety and specificity of the treatment. The adoptive transfer of **MTSA**IGIL**P**V-primed T-cells would again form a nice stepping stone towards the use of this peptide in a vaccine by demonstrating both safety and efficacy aspects of the approach.

6.3.1.3. Conventional vaccine technology

The **MTSA**IGIL**P**V peptide could be administered to patients using established vaccine technology. Since the **MTSA**IGIL**P**V peptide is not found in the human proteome it would first have to be established whether it could be processed and presented by antigen presenting cells *in vivo*. Direct delivery of peptide antigens has failed to elicit potent immune responses, studies have sought to improve delivery of synthetic peptide antigens by utilising adjuvants such as TLR 9 ligand CpG (Khan *et al.*, 2007). Activation of TLRs have been shown to increase the immunogenicity of peptide antigens, resulting in efficient vaccine delivery (Ni *et al.*, 2020). Due to the immunosuppressive tumour microenvironment commonly associated with malignant disease, it is important to consider eliciting responses from multiple different immune cells in the vicinity of a tumour cell. With this in mind, peptide-based immunotherapeutic strategies comprising epitopes for MHC class I and II have been designed with the hope of eliciting both CD8 and CD4 T-cell responses. Eliciting CD8 and type 1 CD4 T-cell responses is important for cytotoxicity to a broad range of epitopes and may help to prevent tumour escape. Strategies comprising multiple epitopes include those using synthetic long peptides (SLP), traditionally 25-35 aa in length, containing a mix of CD4 and CD8 T-cell epitopes which have been shown to successfully activate cancer-specific immune responses (Melief and Van Der Burg, 2008; Kenter *et al.*, 2009; Rabu *et al.*, 2019). *Rosalia et*

al. showed that SLPs are more rapidly and efficiently processed and presented by DCs, allowing for increased presentation to CD8 and CD4 T-cells (Rosalia *et al.*, 2013). Clinical trials using SLPs to treat patients with HPV-related malignancies resulted in 47% of patients experiencing a complete response (Kenter *et al.*, 2009). In addition, RNA vaccines which have been adapted to include multiple neoantigen epitopes spanning MHC class I and II epitopes have resulted in complete responses in treated patients (Ott *et al.*, 2017). It might therefore be possible to combine super-agonist peptide **MTSA**IGIL**P**V with additional peptide antigens to recruit not only multipronged CD8 T-cells but cytotoxic CD4 T-cells as well, further reducing the chance of tumour escape. Furthermore, peptide antigens have been conjugated to DC targeting antibodies which promote uptake of the peptide antigen by DCs, leading to enhanced MHC class I presentation *in vivo* (Swee *et al.*, 2013). In addition, **MTSA**IGIL**P**V could be delivered to patients using established immunotherapeutic vaccine strategies. Dendritic cell (DC) vaccines involve the removal of autologous DCs, which are then loaded with peptide antigen before being reinfused into the patient. The use of DCs increases the possibility of processing and presentation of the peptide onto MHC class I molecules and subsequent activation of cytotoxic T-cells. Murine studies have shown benefit of combining peptides which are targeted to dendritic cells and anti-PD-1 monoclonal antibodies (Mizumoto *et al.*, 2020). Peptides have also been administered alongside oncolytic viruses, providing a 'two-pronged' strategy. The oncolytic virus causing rupturing of tumour cells and non-specific T-cell activation in the TME (Koske *et al.*, 2019), whilst the attached peptide can elicit antigen-specific T-cell responses (Scheibenbogen *et al.*, 2003; Cerullo *et al.*, 2010; Capasso *et al.*, 2016).

6.4. Summary

Cancer peptide vaccines have the potential to provide an effective, low cost immunotherapy. Furthermore, peptide vaccines could provide a generic treatment for many people with the same HLA type. In order for a vaccine to be effective, it must elicit potent T-cells capable of mediating effective tumour regression *in vivo*. Recent attempts to treat malignant disease with peptide vaccines have been disappointing. This lack of clinical efficacy has resulted from poorly immunogenic peptides which failed to overcome T-cell tolerance.

I believe that super-agonist peptides could be the future of cancer peptide vaccination, given their ability to break T-cell tolerance and elicit potent CD8 T-cells. My investigation led to the discovery of **MTSA**IGIL**P**V peptide which elicits potent TAA-specific T-cells, capable of mediating enhanced tumour cell killing *in vitro*. I demonstrated that **MTSA**IGIL**P**V binds strongly to HLA A2 and TCRs specific for **MTSA**IGIL**P**V are high affinity, overcoming T-cell

tolerance. Furthermore, I showed that **MTSA**IGIL**P**V could elicit T-cells capable of recognising multiple TAAs. Finally, **MTSA**IGIL**P**V priming of CD8 T-cells in melanoma, RCC, CLL and AML resulted in enhanced tumour cell killing. These data make super-agonist peptide **MTSA**IGIL**P**V a candidate for a pan-cancer peptide vaccine.

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Appendix

HLA A2

TCTAGAGCCGCCACCTAGAGGATGGCCGTCATGGCGCCCCGAACCCTCGTCCTGCTACTCTC
GGGGGCTCTGGCCCTGACCCAGACCTGGGCGGGCTCTCACTCCATGAGGTATTTCTTCACAT
CCGTGTCCCGGCCCGGCCGCGGGGAGCCCCGCTTCATCGCAGTGGGCTACGTGGACGACACG
CAGTTCGTGCGGTTCGACAGCGACGCCGCGAGCCAGAGGATGGAGCCGCGGGCGCCGTGGAT
AGAGCAGGAGGGTCCGGAGTATTGGGACGGGGAGACACGGAAAGTGAAGGCCCACTCACAGA
CTCACCGAGTGGACCTGGGGACCCTGCGCGGCTACTACAACCAGAGCGAGGCCGGTTCTCAC
ACCGTCCAGAGGATGTATGGCTGCGACGTGGGGTCGGACTGGCGCTTCCTCCGCGGGTACCA
CCAGTACGCCTACGACGGCAAGGATTACATCGCCCTGAAAGAGGACCTGCGCTCTTGGACCG
CGGCGGACATGGCAGCTCAGACCACCAAGCACAAGTGGGAGGCGGCCCATGTGGCGGAGCAG
TTGAGAGCCTACCTGGAGGGCACGTGCGTGGAGTGGCTCCGCAGATACCTGGAGAACGGGAA
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CAGCGGGATGGGGAGGACCAGACCCAGGACACGGAGCTCGTGGAGACCAGGCCTGCAGGGGA
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GCCATGTGCAGCATGAGGGTTTGCCCAAGCCCCTCACCCTGAGATGGGAGCCGTCTTCCCAG
CCCACCATCCCCATCGTGGGCATCATTGCTGGCCTGGTTCTCTTTGGAGCTGTGATCACTGG
AGCTGTGGTCGCTGCTGTGATGTGGAGGAGGAAGAGCTCAGATAGAAAAGGAGGGAGCTACT
CTCAGGCTGCAAGCAGTGACAGTGCCCAGGGCTCTGATGTGTCTCTCACAGCTTGTAAAGTG
TGAGCGGCCGCCCTCGAG

Melan-A

TCTAGAGCCGCCACCATGCCCAGGGAGGACGCCCACTTTATCTACGGCTACCCCAAGAAGGG
CCACGGCCACAGCTATACCACCGCCGAGGAGGCCGCCGGAATTGGCATCCTGACCGTGATCC
TGGGCGTGCTGCTGCTGATCGGCTGCTGGTATTGCAGGAGAAGGAACGGCTACAGGGCCCTG
ATGGACAAGAGCCTGCACGTGGGCACACAGTGCGCCCTGACCAGAAGATGCCCCCAGGAGGG
CTTCGACCACAGGGACAGCAAGGTGAGCCTGCAGGAAAAGAACTGCGAGCCTGTGGTGCCCA
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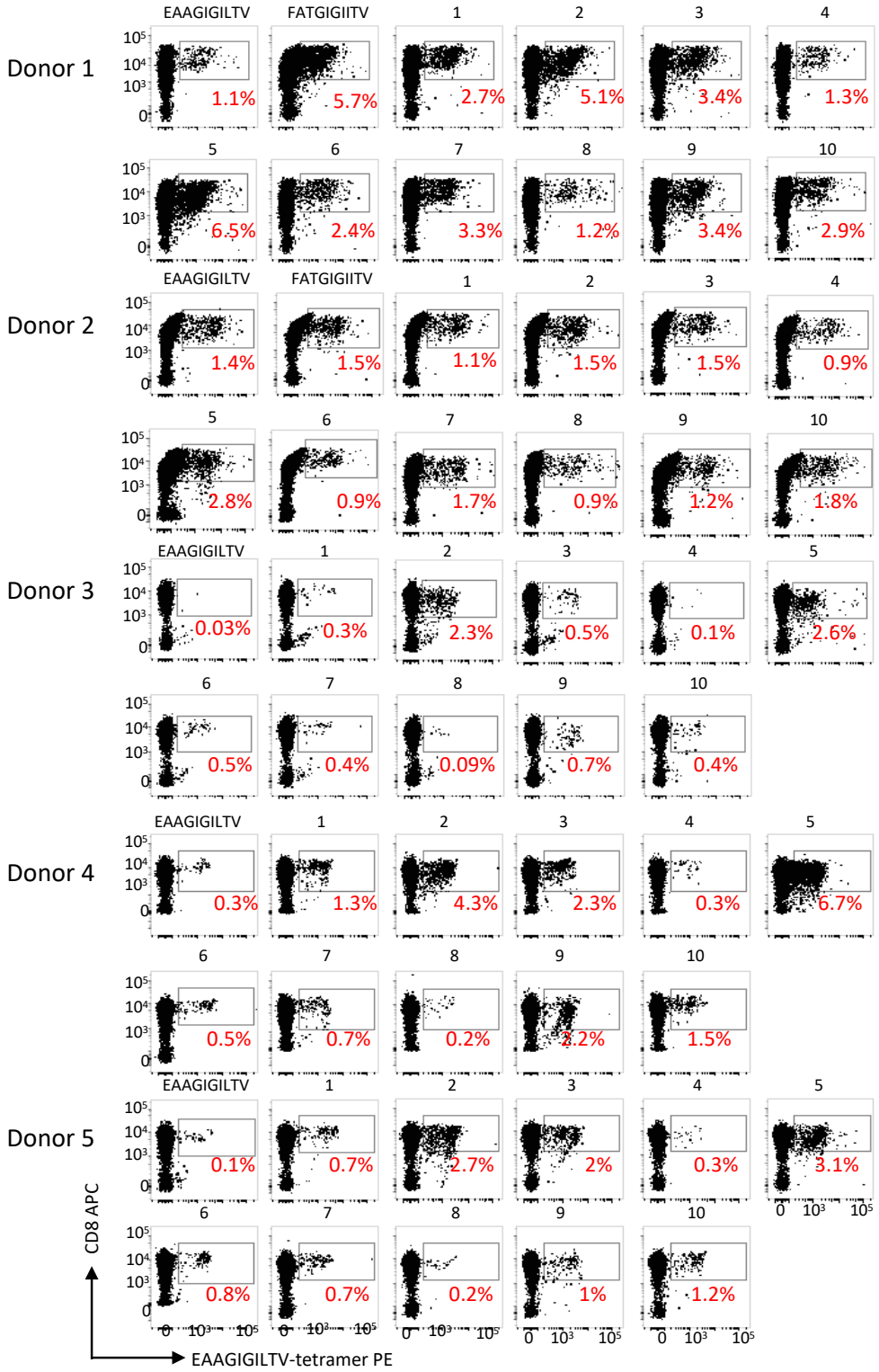
GAG

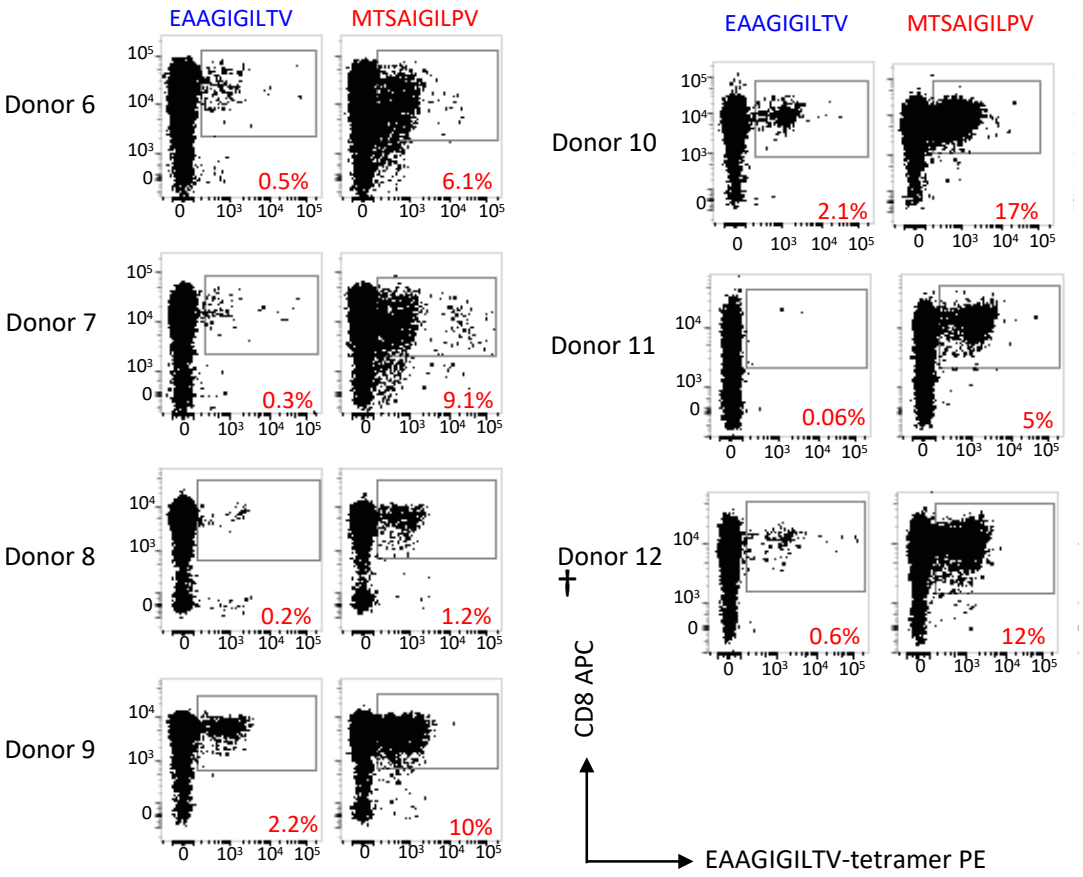
Appendix Figure 1: Sequences cloned into lentiviral vectors

Xba1, Xho 1, kozak sequence



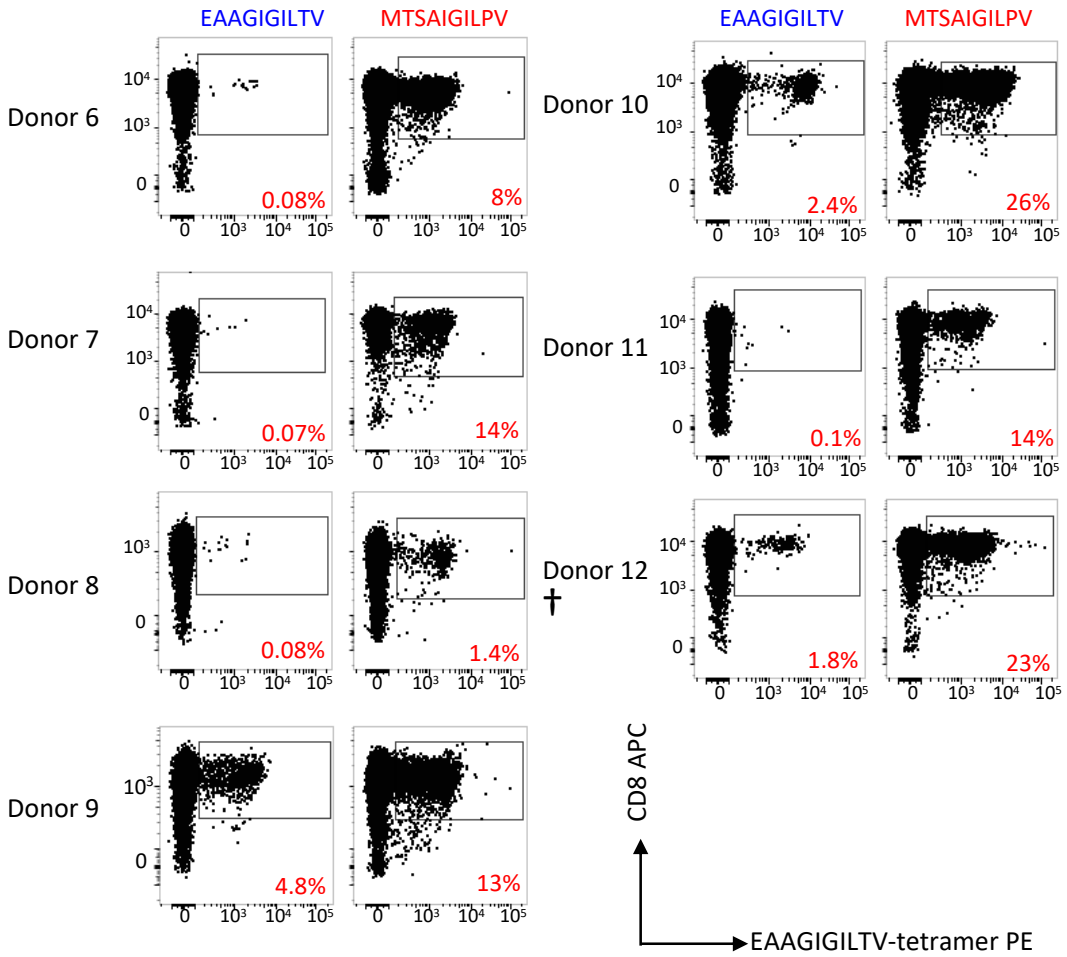
Appendix Figure 2: Associated flow cytometry plots super-agonist peptide test

Associated flow cytometry plots from Melan-A tetramer staining of CD8+ T-cells primed with candidate super-agonist peptides. PBMC were extracted from healthy buffy coat obtained from the Welsh Blood Service (WBS), using SepMate protocol. CD8-positive T-cells were extracted and co-incubated, at a density of 3 x10^6 per mL, with 7 x10^6 irradiated autologous CD8-negative cells pulsed for 1 hour indicated peptide for two weeks. EAAGIGILTV tetramer-positive cells generated by each candidate super-agonist is expressed as a percentage.



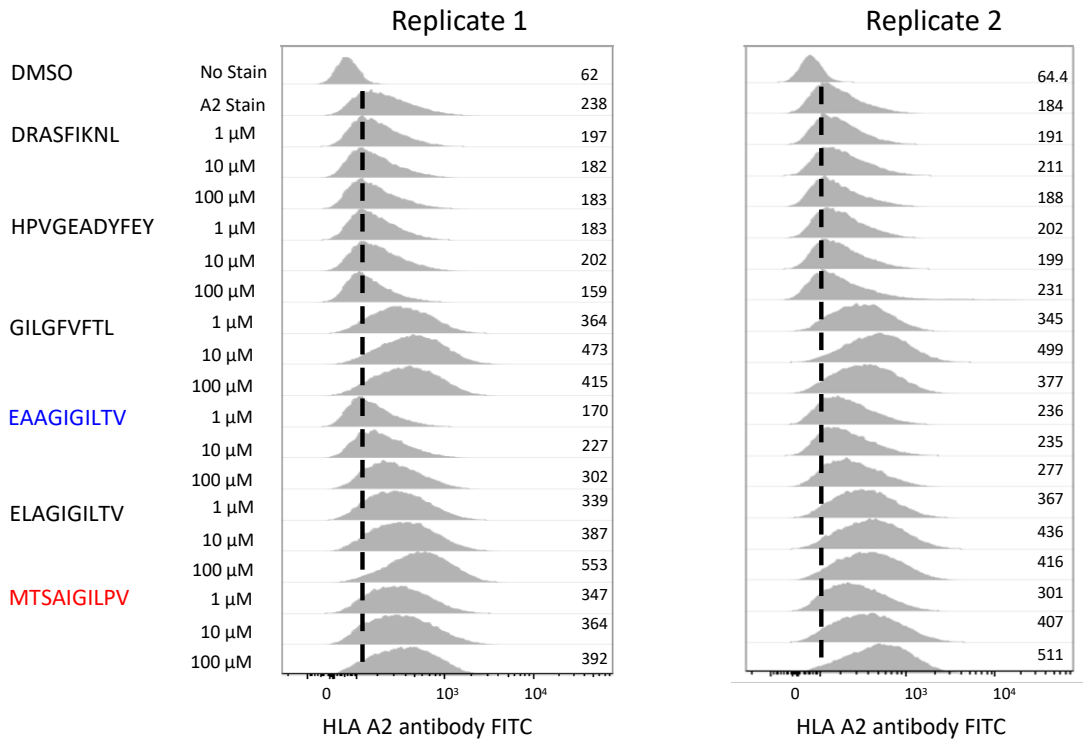
Appendix Figure 3: Associated peptide priming flow cytometry plots after 14 days

*Associate flow cytometry plots of EAAGIGILTV-tetramer stained CD8 T-cells primed with super-agonist peptide **MTSA**IGIL**P**V and wild type peptide EAAGIGILTV for 14 days. CD8-T-cells from 7 healthy HLA-A2 donors were isolated from healthy PBMC were then cultured for 14 days alongside autologous and primed with EAAGIGILTV or **MTSA**IGIL**P**V peptides. The number of CD8 positive, HLA A2-EAAGIGILTV tetramer+ cells from each condition is expressed as a percentage. The plots for donor 12 also appear in **Figure 3.9**.*



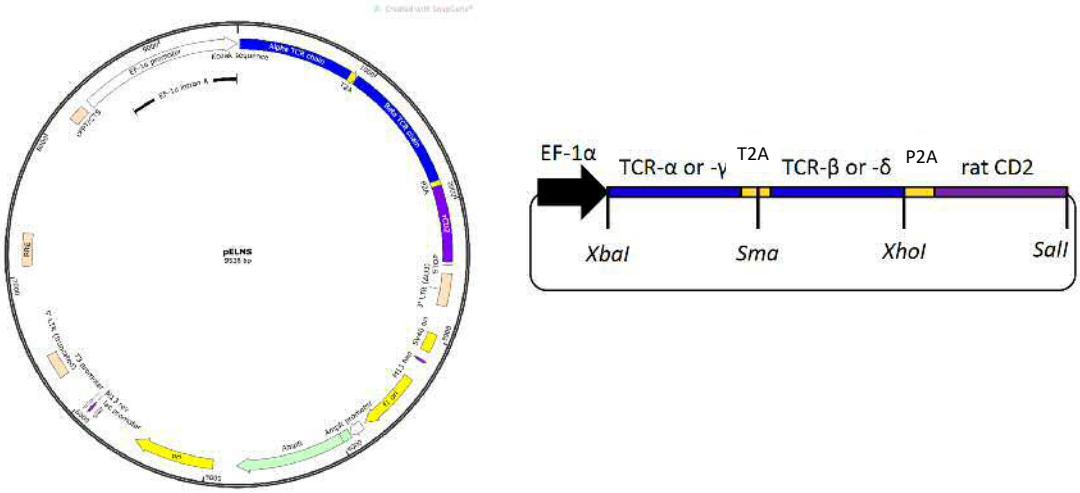
Appendix Figure 4: Associated peptide priming flow cytometry plots after 28 days

As Supplementary Figure 2 but after 28 days. The plots for donor 12 also appear in ***Figure 3.9****.*



Appendix Figure 5: Associated flow cytometry plots T2 binding assay

*Supporting flow cytometry data of HLA-A2 staining from T2 cell binding assays shown in Figure 5A. 5 x10^4 T2 cells (TAP deficient HLA A*0201+) were cultured in AIM-V serum-free media with indicated peptides at concentrations of 1 µM, 10 µM and 100 µM. Assays performed in duplicate. The following day, cells were stained with FITC-conjugated HLA-A2 antibody and LIVE/DEAD vivid (to allow exclusion of dead cells). Results are expressed as the MFI of HLA A2 expression, with the dashed line indicating base line HLA A*0201 expression of T2 cells untreated with peptide.*

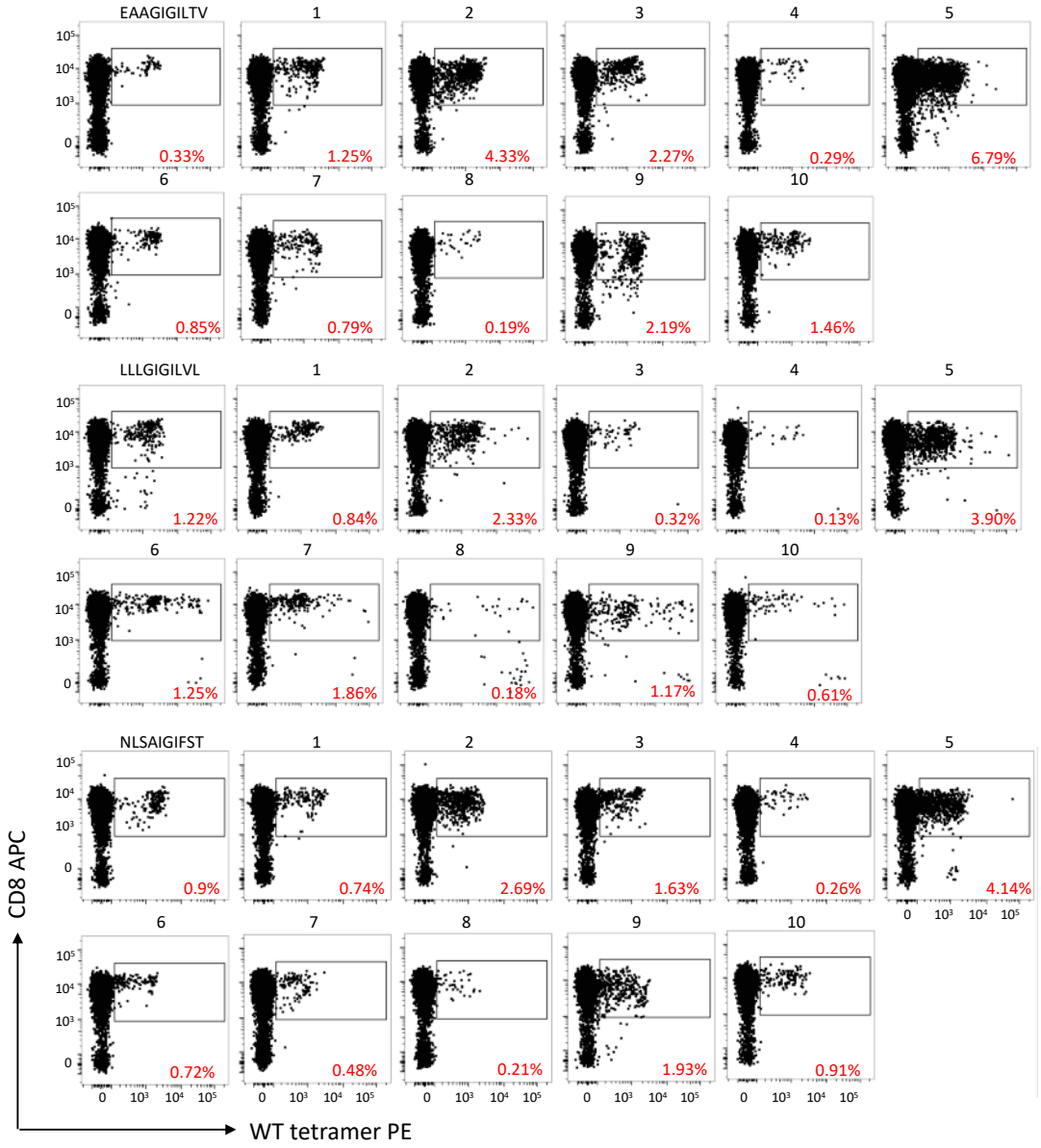


Appendix Figure 6: pELNS plasmid for TCR transduction

Transduction of TCRs MEL5 and DMF4 into CD8 T-cells from a healthy HLA A2+ donor was carried out using lentivirus. The appropriate constructs containing MEL5 or DMF4 TCR α and β chain sequences were cloned into the pELNS plasmid. The contruct contains rat CD2 which acts as a marker for successful lentivirus transfection.

Appendix Figure 7: Associated flow cytometry plots of super-agonist peptide priming healthy donor 1

CD8 T-cells isolated from healthy donor PBMC obtained from buffy coats supplied by the Welsh Blood Service (WBS) and primed for 14 days with candidate super-agonist peptides and tumour associated antigen peptides EAAGIGILTV (melan-A), NLSALGIFST (IMP2) and LLLGIGILVL (BST2). Polyclonal CD8 T-cell populations were then stained with EAAGIGILTV, NLSALGIFST and EAAGIGILTV pMHC tetramers using an optimised protocol (PKI + tetramer + anti-PE 1◦ antibody). Cells were also stained with VIVID live/dead, CD8 (APC) and CD3 (PerCP) to allow the identification of live CD8 T-cells by flow cytometry. Results are expressed as a percentage of CD8 tetramer positive cells where each set of primings was stained with the tetramer corresponding to the wild type peptide displayed.



Appendix Figure 8: Associated flow cytometry plots of super-agonist peptide priming healthy donor 2

As appendix figure 6 but with healthy donor 2.

Appendix Figure 9: Associated flow cytometry plots of super-agonist peptide priming healthy donor 3

As appendix figure 6 but with healthy donor 3.