

Peripheral T cell responses to immunotherapy treatment in metastatic melanoma

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I would like to dedicate this thesis to my late supervisor Professor Vincenzo Cerundolo,
whose passion and dedication to science was a true inspiration.

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Abstract

Immune checkpoint inhibitor treatment has revolutionised treatment for cancers such as malignant melanoma, producing durable responses in patients with metastatic malignancy. Despite this progress, responses are still only seen in a subset of patients and the phenotype and specificity of the T cells responsible for determining efficacy remain poorly characterised. A better understanding of T cell behaviour during immunotherapy treatment is required to identify markers of response and resistance and to predict effective combination strategies. To address this, we performed longitudinal peripheral immune monitoring of metastatic melanoma patients receiving checkpoint inhibitor therapy. By integrating a variety of single-cell analysis approaches including mass cytometry, MHC Class I tetramer staining, single-cell fluorescence activated cell sorting and single-cell RNA-sequencing, we were able to track and analyse in detail tumour-specific T cell responses in the periphery over time. Accumulation of activated effector CD8⁺ T cells expressing CD38 and HLA-DR was observed in response to treatment, was more pronounced with combination anti-CTLA-4/anti-PD-1 treatment than anti-PD-1 monotherapy, and was correlated with treatment outcome. Activated cells were enriched in tumour-specific compared to microbial-specific T cells, with diversification and expansion of the tumour but not microbial-specific response observed over the course of treatment. This was accompanied by transient alterations in the functional avidity of T cell clones specific for the common tumour-associated antigen MART-1. Single-cell RNA-seq revealed substantial heterogeneity in the transcriptional profile of tumour-specific T cells during therapy with transient activation and persistence of specific clonotypes observed. These results demonstrate both the feasibility of monitoring tumour-specific T cells mobilised in response to immunotherapy in the periphery, and provide insights into the mechanisms underpinning therapeutic efficacy of combination checkpoint blockade. The results also have implications for the identification of T cells and TCR clonotypes with maximum therapeutic potential for other therapeutic strategies such as adoptive cell therapies.

List of abbreviations

ACT	Adoptive cell therapy
APC	Antigen presenting cell
β2M	Beta 2 microglobulin
C2	Cycle 2
C3	Cycle 3
C4	Cycle 4
CAR	Chimeric antigen receptor
CB	Clinical benefit
CDR3	Complimentary determining region
CLA	Cutaneous lymphocyte antigen
CMV	Cytomegalovirus
CR	Complete response
CSB	Cell staining buffer
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
CyTOF	Cytometry by time-of-flight
dNTP	Deoxyribonucleotide triphosphate
DMSO	Dimethyl sulfoxide
EC ₅₀	Half maximal effective concentration
EBV	Epstein-Barr virus
ERCC	External RNA control consortium
FACS	Fluorescence-activated cell sorting
FBS	Foetal bovine serum
FC	Fold change
FcγR	Fcγ receptor
gp100	Glycoprotein 100
HLA	Human leucocyte antigen
HS	Human serum
ICB	Immune checkpoint blockade
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
IMC-Durv	IMCgp100 plus Durvalumab
IMC-Durv-Treme	IMCgp100 plus Durvalumab plus Tremelimumab
ImmTAC	Immune mobilising monoclonal TCR against cancer
Ipi	Ipilimumab
MAGE	Melanoma-associated antigen
MART-1	Melanoma antigen recognised by T cells-1

MCSP	Melanoma-associated chondroitin sulfate proteoglycan
MDSC	Myeloid derived suppressor cells
MHC	Major Histocompatibility Complex
mTCR	Monoclonal TCR
NCB	No clinical benefit
Nivo	Nivolumab
NK cell	Natural killer cell
NSCLC	Non-small-cell lung cancer
NY-ESO-1	New York Esophageal Squamous Cell Carcinoma-1
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PD	Progressive disease
PD-1	Programmed cell death-1
PD-L1	Programmed cell death ligand-1
Pembro	Pembrolizumab
PFS	Progression-free survival
PHA	Phytohemagglutinin
PMA	Phorbol myristate acetate
PR	Partial response
q2w	Two-weekly
q3w	Three-weekly
q4w	Four-weekly
RECIST	Response Evaluation Criteria in Solid Tumours
RNA-seq	RNA-sequencing
RPMI	Roswell Park Memorial Institute media
ScRNA-seq	Single-cell RNA-sequencing
SD	Stable disease
TAA	Tumour-associated antigen
T _{CM}	Central memory T cell
TCR	T cell receptor
T _{EM}	Effector memory T cell
T _{EMRA}	Effector memory RA T cell
TGFβ	Transforming growth factor beta
TIL	Tumour infiltrating lymphocyte
TNF	Tumour necrosis factor
TPM	Transcripts per million
TRA	T cell receptor alpha gene
TRAC	T cell receptor alpha constant gene

TRAV	T cell receptor alpha variable gene
TRB	T cell receptor beta gene
TRBC	T cell receptor beta constant gene
Treg	Regulatory T cell
TRG	T cell receptor gamma gene
T _{RM}	Tissue resident memory T cell
TSO	Template switching oligonucleotide
UMAP	Uniform manifold approximation and projection
UV	Ultraviolet
WES	Whole exome sequencing

Chapter 1. Introduction

1.1 The immune system and cancer

The primary function of the immune system, and its component effector cells and molecules, is to provide protection against infectious agents and their consequences. It is now well recognised that in addition to this, the immune system also plays an important protective role against cancer, through a process of immunosurveillance which was first proposed in the 1950s¹⁻⁵. Although it was established that tumour elimination could occur upon transplantation of tumours between non-inbred strains of mice, the demonstration that this was mediated by allograft rejection rather than tumour-specific rejection initially cast doubt on the concept⁶. The first data supporting the existence of tumour-specific rejection came from experiments showing that protection against challenge with tumours in inbred mice could be achieved by prior injection with irradiated tumour cells of the same cancer⁷, however conclusive evidence for the absence of such responses in mice deficient in T cells was not obtained until more recently. Carcinogenesis experiments in RAG-2^{-/-} mice, lacking lymphocytes bearing antigen receptors, showed higher rates of chemically induced sarcomas but also higher rates of spontaneous epithelial tumours than wild-type mice⁸. In support of this concept, immunodeficiency in humans is associated with an increased incidence of cancers, whether this be primary⁹ or acquired such as following organ transplantation¹⁰. Whilst a large number of these are cancers linked to the failure of the immune system to protect from oncogenic viruses such as human herpes virus 8 (Kaposi Sarcoma), Epstein-Barr virus (Hodgkin's lymphoma) and human papilloma virus (cervical cancer), increased rates of spontaneous and carcinogen induced cancers such as melanoma¹¹ point to a failure of immunosurveillance. In addition, for a number of tumours, most consistently in cutaneous malignant melanoma, positive correlations between the presence of intra-tumoural lymphocytes and increased patient survival have been shown¹² further supporting the importance of the immune system in the anti-cancer response.

1.1.1 T cell recognition of antigens

This ability of the immune system to recognise cancer cells as foreign and eliminate them relies on the ability to discriminate antigens that are abnormally expressed in cancer as non-self. T cells are the primary mediator of cell-mediated antigen-specific immunity, recognising peptide fragments that are displayed on the surface of cells by host cell glycoproteins termed MHC (major histocompatibility complex) molecules. Two different classes of MHC molecules exist (MHC class I and class II), with differing structures, expression patterns and peptide binding capabilities.

MHC class I molecules are expressed on nearly all nucleated cells at varying levels and consist of a membrane spanning 3 domain α polypeptide chain complexed with a β_2 microglobulin chain, with a peptide binding cleft formed by the folding of the α_1 and α_2 domains of the alpha chain. This is able to bind short (8-10 amino acid) peptides derived from intracellular pathogens and endogenous proteins found in the endoplasmic reticulum, and to present them to CD8+ T cells. Upon recognising a peptide specific for their T cell receptor (TCR), these cytotoxic T cells release lytic granules including perforin, granzymes and granulysin which result in destruction of the target cell. MHC class II molecules consist of two membrane spanning chains (α and β) with the α_1 and β_1 domains forming a peptide binding cleft that is more open-ended than that of MHC Class I, allowing it to incorporate a greater variety of peptides, typically 13 amino acids or longer in length. Whilst MHC class II can be upregulated on all cells, its expression is generally restricted to antigen presenting cells such as macrophages and dendritic cells, which take up exogenous foreign antigens, process them, and then display them to CD4+ helper T cells. Expression of both MHC class I and II is modulated by cytokines such as interferon alpha and beta, which upregulate MHC Class I, and interferon gamma (IFN- γ) which can upregulate expression of MHC class I and II.

As well as displaying peptides, MHC proteins act as co-receptors through associations of the invariant site on the MHC molecule with the T cell receptor during antigen recognition.

This enhances the sensitivity of the T cell to the antigen through recruitment of intracellular tyrosine kinases which help mediate intracellular signalling induced by antigen recognition. The human leucocyte antigen (HLA) genes which encode MHC are highly polymorphic with many different allelic variations of both class I and class II genes present in the population. Each MHC molecule is able to bind a wide variety of different peptides, with different MHCs preferentially binding different peptides. This generates diversity both within and between individuals in the spectrum of peptides that are able to be presented and recognised by the immune system, although a single individual will have only a small selection of these possible variants.

The specificity of a given T cell is determined by the T cell receptor (TCR), which is a membrane-anchored heterodimer consisting of two polypeptide chains linked by a disulfide bond. Most T cells bear one T cell receptor alpha (TCR α) and one T cell receptor beta (TCR β) chain each consisting of a constant (C) region and a variable region (V), although a small minority of T cells express alternative gamma(γ) and delta(δ) chains (termed gamma-delta T cells). These paired chains form a single antigen binding site, with each T cell expressing multiple copies of this TCR. Diversity amongst TCRs is generated by variable regions in the respective chains, resulting from three hypervariable regions or complementarity determining regions (CDR3). These CDR3 loops mediate contact with the antigenic peptide and can therefore be considered to determine the fine specificity. The V region of each chain is encoded by different gene segments that are recombined during development of T cells in the thymus to produce a vast array of unique variable region coding sequences and subsequent T cell receptors. Each chain locus is comprised of a constant region and multiple variable (V) and joining (J) regions, and in the case of the beta chain, diversity (D) segments. Fifty-two different variable segments are known for the human β chain, and around 70 for the α chain, with 2 possible D segments and 13 possible J segments for the β chain, and 61 possible J segments for the α chain¹³. For a given individual, the spectrum of expressed TCRs constitutes the TCR repertoire and can be

characterised by CDR3 sequence variation. An estimated 10^{15} - 10^{18} possible different TCRs could theoretically be produced as a result of VDJ rearrangement, although the exact number of different TCR clones (populations of T cells carrying an identical $\alpha\beta$ TCR) found amongst the approximately 10^{12} T cells in a typical individual is unknown. More than 10^8 distinct TCRs have been found in the naïve T cell pool, indicating that the naïve repertoire is highly diverse, whilst the memory compartment, accounts for less than 1% of the total diversity¹⁴.

During the process of recombination in $\alpha\beta$ T cells, the TCR β locus is recombined first, followed by proliferation before the TCR α locus is recombined. Cells derived from a single progenitor before this proliferative expansion will therefore all carry the same TCR β but will each generate a different random TCR α , meaning that it is possible for a single TCR β to be found with multiple different TCR α . In addition, recombination commonly results in non-productive genes, due to stop codons or frame-shifts, and subsequent recombination of the β chain on the second chromosome may occur if the initial recombination is unsuccessful at producing a functional receptor. In the case of the α chain, recombination occurs on both chromosomes simultaneously. As a result, up to 4 recombinants (2 α and 2 β) can be produced in each cell. Whilst historically it was thought that the process of allelic exclusion (only one allele of a gene being expressed while the other is silenced) meant that a single T cell could bear only a single paired TCR, data from various studies has demonstrated the existence of T lymphocytes expressing two different beta chains^{15,16} and two different alpha chains¹⁷. Recent advances in single-cell sequencing and computational analysis approaches have provided further support for this, with estimates for the frequency of T cells having two productive β chains in the region of 6-7%¹⁸⁻²⁰, two productive α chains between 6-35%^{13,18-21} and 1.6% for two productive α and two productive β chains²⁰.

After generation of a TCR these T cell precursors, expressing both CD8 and CD4, are subject to a process of positive and negative selection within the thymus. T cells which are able to recognise self-antigens presented on self-MHC molecules, and therefore which

could potentially recognise foreign antigen presented on self-MHC, are positively selected. However, those which bind self-peptide-MHC complexes too strongly and which are potentially autoreactive are negatively selected and undergo apoptosis. The cells that survive the process of thymic selection then differentiate into single-positive T cells, bearing either CD8 or CD4 depending on whether they recognised an antigen presented by self-MHC Class I or Class II respectively. There is some evidence to suggest that this expression of a second T cell receptor might prevent cells with auto-specific receptors from being deleted in the process of thymic selection allowing their exit into the periphery²² and that such cells may play a role in autoimmunity²³.

1.1.2 Types of tumour antigen

As discussed, T cells recognise their targets by interaction of the TCR with peptides presented by MHC. Tumour antigens that are naturally processed and presented by MHC Class I on the surface of human cancers can be categorised as either non-self-antigens or self-antigens. Non-self-antigens are antigens that are not normally encoded by the host genome and can arise either from oncogenic viral proteins, or as a result of somatic mutation during carcinogenesis resulting in the formation of neoantigens. The later can be either driver mutations, responsible for initiating malignant transformation, or more commonly passenger mutations that arise as a consequence of the genomic instability that accompanies this process²⁴⁻²⁶. If these resultant mutant proteins are expressed, processed and presented on the cell surface they can potentially be recognised as foreign to the immune system and can elicit a potent immune response as they are unlikely to be subject to immunological tolerance. Despite the characterisation of numerous neoantigens, the vast majority (99.6%) of neo-antigen reactive T cells recognise unique mutations not shared between patients²⁷ and can therefore be considered 'private'.

Self-antigens, also termed tumour-associated antigens (TAA), are those which are expressed at low levels in some normal tissues, but that are over-expressed in malignancy.

Cancer-testis antigens such as NY-ESO-1²⁸ and MAGE-A3²⁹ are proteins expressed during foetal development with limited expression in normal adult tissues that are re-expressed in some cancers. Differentiation antigens such as Melan-A/MART-1^{30,31} and gp100³² are expressed in normal adult tissue, in this case melanocytes, as well as in tumours derived from that tissue. Finally, overexpressed antigens such as carcinoembryonic antigen are proteins that are over expressed in malignant cells relative to normal tissue. Whilst these different tumour-associated antigens may be shared between many patients with the same type of malignancy, resulting in shared or 'public' T cell responses against these antigens, central tolerance is thought to lead to elimination of cells with high-avidity TCRs for these proteins³³. Despite this, tumour infiltrating lymphocytes (TILs) specific for these shared antigens are frequently found within tumours. In melanoma, T cells reactive for melanosomal proteins are detected at high frequencies amongst TIL cultures^{34,35} and have been demonstrated to be capable of causing in vivo tumour regression upon adoptive transfer to autologous patients³⁴. The most frequently observed of these amongst most patients are T cells capable of recognising the antigen MART-1. These can be detected in both tumours and in the peripheral blood at much higher frequencies than for other tumour-associated antigens and, in melanoma patients, display an effector memory phenotype³⁶.

MART-1 however appears to be unusual in that high frequencies of MART-1-specific cells (0.07% by tetramer staining) can be found in a large proportion of healthy HLA-A*0201 individuals³⁷, as well as in HLA-A*02:01 negative individuals³⁸. In both melanoma patients and healthy donors, MART-1-specific cells have also been shown to have a strong bias towards the T cell receptor alpha variable (TRAV) 12-2 TCR segment usage^{38,39}. However, unlike the MART-1-specific cells found in melanoma patients, these T cells display a naïve phenotype. Why such a high frequency of naïve, antigen-specific T cells should be produced despite known thymic expression of the *MLANA* gene encoding this protein in medullary thymic epithelial cells⁴⁰ has been the subject of investigation for some time⁴¹. Accumulating evidence suggests that this is due a combination of mis-initiated transcription

of the *MLANA* gene in the thymus, leading to the expression of a truncated epitope lacking the most immunodominant part and hence escape from central tolerance, together with the TRAV 12-2 CDR1 region being unusually permissive in conferring MART-1-specificity in the context of different TCR $\alpha\beta$ chain pairings⁴².

1.1.3 Antigen-specific T cell responses

As discussed in Section 1.1.1, each individual carries a large circulating repertoire of naïve T cells, each with a different specificity. These cells, characterised by the expression of the chemokine receptor CCR7 and CD45RA, recirculate between the blood and lymph nodes until they encounter an antigen their TCR is capable of recognising, presented on the surface of an antigen presenting cell (APC) as a peptide-MHC complex. Engagement of the TCR with its cognate peptide, together with co-stimulatory signals provided by ligation of the CD4/8 co-receptor and the interaction of CD28 on the T cell with B7 on the surface of the APC, initiates 'priming' of the T cell causing activation and triggering proliferation and differentiation to form a clonal population of effector T cells bearing the same TCR. This process is accompanied by transient upregulation of the high affinity interleukin(IL)-2 receptor (CD25), loss of expression of CD45RA and increased expression of CD45RO. In the case of CD8 T cells, differentiation is to cytotoxic effector T cells capable of killing other host cells presenting their cognate antigen. CD4 differentiation is more complex, with the resultant effector subsets depending on the class of pathogen encountered and the presence of different cytokines, each characterised by distinct cell surface molecules and with different functions and cytokine production (Table 1-1).

Table 1-1. CD4+ T cell subsets

Subset	T_H1	T_H2	T_H17	T_{FH}	Treg
Chemokines expressed	CXCR3+ CCR4-CCR6- CCR5+	CCR4+ CXCR3- CCR6-	CCR6+ CCR4+ CXCR3-	CXCR5+	CD25+
Function	Activate macrophages to destroy intracellular pathogens	Recruit and activate eosinophils, mast cells and basophils, promote enhanced barrier immunity at mucosal sites	Activate epithelial cells to produce cytokines that attract neutrophils	B cell help	Suppress T cell activity
Cytokines produced	IFN- γ	IL-4, IL-5, IL-13	IL-17, IL-22	IL-21, IFN- γ , IL-4, IL-17	

The site of the lymph node in which a T cell has been primed imprints effector T cells with homing receptors which determine their ability to traffic to different tissues. For example, T cells primed in lymph nodes draining the skin express the cutaneous lymphocyte antigen (CLA)⁴³ as well as chemokine receptors CCR10 and CCR4. These effector T cells migrate to sites of infection/inflammation where, upon encountering their specific antigen, they can be activated without the need for further co-stimulation. After a successful adaptive immune response and clearance of the antigen, most effector cells undergo apoptosis resulting in a clonal contraction. This is mediated via loss of CD25 and CD127 after antigenic stimulation, reducing the ability of the T cell to respond to IL-2 or IL-7 signalling resulting in loss of the expression of the anti-apoptotic survival factor Bcl-2⁴⁴. Apoptosis can also be triggered directly by signalling via Fas (CD95). Some effector cells however, maintain sensitivity to IL-7, continue to express Bcl-2, and survive and persist as memory T cells. Different subsets of memory cells are recognised and can be classified according to the expression of different cell surface markers^{45,46}.

Central memory T cells (T_{CM}) express the lymph node homing receptor CCR7, but although they have high proliferative potential, they lack immediate effector function. They can however rapidly differentiate into CCR7⁻ effector memory (T_{EM}) cells which express

receptors such as CXCR3 allowing them to traffic to peripheral tissues upon re-stimulation⁴⁶. These cells have limited proliferative potential but immediate effector function. A third memory subset, T_{EMRA}⁴⁷ re-express CD45RA but lack CCR7, express markers of senescence including CD57, and have low proliferative and functional capacity, indicating terminal differentiation⁴⁸. Tissue-resident memory T cells (T_{RM}) have also been described^{49,50}, lacking CCR7 but expressing CXCR3. These cells typically do not recirculate, but become resident in epithelial sites such as the dermis where TGF- β production by epithelial cells induces these tissue resident memory cells to express integrin α_E (CD103): β_7 required for tissue retention.

Whilst different models of T cell differentiation have been proposed⁵¹⁻⁵⁴ and remain incompletely resolved, it is known that these different memory populations are heterogeneous, differing in their expression of other markers such as chemokine receptors. Furthermore, T cell proliferation during the immune response appears to be important in determining long-lasting immunity, with the magnitude of the initial clonal burst having been shown to determine memory T cell numbers⁵⁵.

1.1.4 Regulation of the antigen-specific immune response

In order to maintain homeostasis and prevent autoimmunity, the antigen-specific immune response is tightly regulated by a fine balance of co-stimulatory and negative regulatory molecules and immune checkpoints⁵⁶. Two of the most well studied regulatory immune checkpoints, and those which are the focus of this work, are cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and programmed cell death-1 (PD-1).

Following T cell priming, CTLA-4 is translocated to the T cell surface membrane where it competes with a higher affinity than CD28 for the B7.1/B7.2(CD80/86) costimulatory ligand, inhibiting proliferation⁵⁷ and IL-2 secretion by T cells⁵⁸⁻⁶⁰. CTLA-4 is also constitutively expressed on regulatory T cells (CD4+CD25+CD127-FoxP3+)⁶¹. Removal of the second activation signal by CTLA-4 binding results in T cell anergy and tolerance through cell cycle arrest⁶². The importance of this homeostatic mechanism is demonstrated by the

observation of fatal lymphoproliferative disorders in the absence of CTLA-4 in *Ctla4*^{-/-} mice⁶³⁻⁶⁵.

In contrast to CTLA-4, which limits T cell activation, PD-1 is a negative regulator of the effector function of T cells in the periphery. PD-1 expression is induced upon T cells, B cells and monocytes during activation⁶⁶. The ligands for PD-1, PD-L1 (B7-H1)⁶⁷ and PD-L2 (B7-DC)⁶⁸, have distinct expression patterns. While PD-L2 is upregulated on dendritic cells and macrophages in response to pro-inflammatory cytokines such as IL-4⁶⁸, PD-L1 is expressed both on haematopoietic cells as well as many epithelial and endothelial cell types in response to IFN- γ release in the context of inflammation⁶⁹. Engagement of PD-1 by its ligand inhibits T cell mediated lymphocyte proliferation and cytokine secretion⁶⁷ and serves as a negative regulator to prevent sustained damage to peripheral tissues following activation of an immune response.

1.1.5 Mechanisms of cancer immune escape

Given that the immune system is capable of recognising cancer antigens as foreign and eliminating them, the question arises as to why cancers develop in immunocompetent individuals. Whilst the regulatory checkpoints mentioned above can be beneficial in the setting of an acute infection, where the immune response is typically successful in eliminating the antigen, in the setting of cancer these can be deleterious to the ability to mount an ongoing anti-tumour immune response and can contribute to immune escape. Anergy induced by CTLA-4 expression may prevent sufficient and sustained T cell activation, whilst PD-L1 upregulation on tumour cells in response to IFN- γ secretion by cytotoxic lymphocytes⁷⁰, can further suppress the anti-tumour immune response.

Although these negative regulatory mechanisms may help to explain immune escape in the context of an existing anti-tumour response, not all cancers are recognised by the immune system. This can be secondary to a lack of antigenicity⁷¹ of the tumour, due either to a paucity of immunogenic antigens as a result of a low underlying somatic mutation rate or the absence of high-affinity T cells against tumour-associated antigens following thymic

selection³³, or because of defective antigen presentation through mechanisms such as HLA class I loss and/or downregulation which is observed in between 16-60% of common solid cancers⁷². However, the mutational status alone is not necessarily a good predictor of the antigenicity of the tumour. Indeed, it is known that there is wide variation in the somatic mutation rates of cancers⁷³ and whilst some cancers that are considered immunogenic such as melanoma have a high somatic mutation rate, others such as renal cancer do not. Furthermore, there is evidence that even where T cells capable of recognising the tumour exist, they may sometimes be excluded from the tumour microenvironment by other factors⁷⁴, such as for example the presence of myeloid-derived suppressor cells (MDSC)⁷⁵. Secretion of various factors by cells in the tumour microenvironment may also impair the anti-tumour immune response. Cytokines such as transforming growth factor beta (TGF- β) can mediate the conversion of CD4+CD25-FoxP3- T cells to CD25+FoxP3+ T regulatory cells (Tregs) within the tumour microenvironment⁷⁶, which can inhibit T cell proliferation and function. Increased expression of enzymes such as indoleamine 2,3-dioxygenase (IDO), which catalyses the breakdown of the amino-acid tryptophan to kynurenine thus suppressing T cell function⁷⁷, can also be found within the tumour microenvironment⁷⁸.

The interaction between the immune system and a developing or established cancer can therefore be considered a dynamic process, and one in which selection pressure exerted by the immune system upon the tumour may result in survival of variants that are more resistant to immune attack, with those being highly immunogenic being eliminated, and those which are less immunogenic or which have acquired mechanisms to evade immune attack surviving, resulting in tumour escape⁶.

1.2 Therapeutic strategies to overcome immune escape

Despite recognition of the role of the immune system in the control of cancer, attempts to utilise this for therapeutic benefit have historically had only limited success. Recent advances in the field of immunotherapy have however revolutionised the treatment of

metastatic malignancy, with durable treatment responses being observed in patients with a wide range of cancers. Various strategies have been employed to improve the ability of T cells to target cancer (Figure 1-1). Vaccination, adoptive T cell therapy (ACT) and use of chimeric antigen receptors (CAR) focus on increasing the frequency of T cells capable of recognising tumour, whilst checkpoint inhibitors aim to prevent the negative regulation of T cell activation and effector function. A full description of all of these various approaches is beyond the scope of this thesis, but those which form the basis of the experimental work will be discussed in further detail.

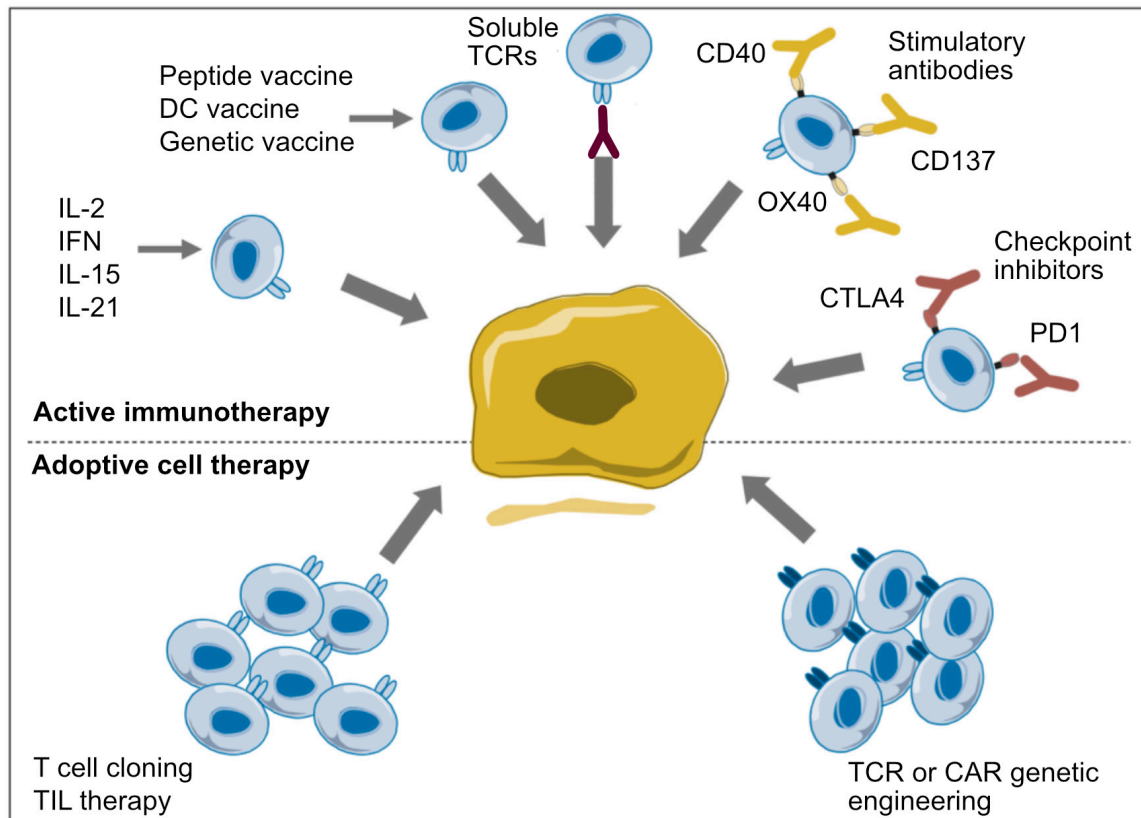


Figure 1-1. Immunotherapeutic approaches to targeting cancers. Adapted from McArthur *et al.*⁷⁹ Reprinted with permission. © 2012 American Society of Clinical Oncology.

1.2.1 Checkpoint inhibition

Given the importance of immune checkpoints in the regulation of the immune response, blockade of these targets to augment an otherwise ineffective anti-tumour response was investigated. Much of the initial work and most mature data come from patients with

metastatic melanoma, a highly aggressive form of skin cancer, which historically had a dismal prognosis of around 6 months with chemotherapy treatment⁸⁰.

1.2.1.1 CTLA-4 inhibition

Following the demonstration that blockade of CTLA-4 in vitro resulted in enhanced T cell activation⁵⁹ and resulted in rejection of established tumours in mouse models⁸¹, antibodies targeted against CTLA-4 (Ipilimumab⁸² and Tremelimumab⁸³) were developed clinically. Ipilimumab produced dramatic improvements in overall survival in Phase III trials in metastatic melanoma⁸⁴ resulting in long-term survival rates of approximately 20%⁸⁵. This led to regulatory approval of Ipilimumab in 2011 for advanced melanoma and subsequently, following clinical trials which demonstrated it could reduce the risk of recurrence and improve overall survival in patients with locally advanced melanoma⁸⁶, as an adjuvant therapy.

1.2.1.2 PD-1/PD-L1 inhibition

Similarly, the clinical development of antibodies against PD-1 and PD-L1 was pursued after it was shown that blockade of the PD-1 pathway could partially reinvigorate exhausted T cells in mouse models⁸⁷, and enhance cytolytic activity of human tumour-specific cytotoxic T cells and cytokine production of tumour-specific T helper cells in vitro⁸⁸. These agents produced impressive results in clinical trials⁸⁹⁻⁹² with durable responses in approximately 40% of treatment-naïve patients in the metastatic melanoma setting⁹³. As a result, two anti-PD-1 antibodies (Pembrolizumab and Nivolumab) and three anti-PD-L1 antibodies (Atezolizumab, Durvalumab and Avelumab) have received approval by the US Food and Drug Administration (FDA) for treating a wide range of malignancies including metastatic melanoma, non-small cell lung cancer, urothelial cancer and relapsed Hodgkin's lymphoma. As well as displaying greater efficacy than CTLA-4 inhibition in the metastatic melanoma setting, toxicity rates were also lower with blockade of the PD-1/PD-L1 axis⁹² than with CTLA-4 blockade.

1.2.1.3 Combination immune checkpoint inhibitor therapy

Despite the success of anti-CTLA-4 and anti-PD-1 based treatments as monotherapies, a substantial fraction of patients still failed to respond. Given the non-overlapping mechanisms of action, with CTLA-4 blockade promoting T cell priming and PD-1 blockade promoting T cell effector functions, combining the two treatments was also explored in clinical trials. In patients with metastatic melanoma, five year survival rates of 52% were seen for patients treated with Nivolumab plus Ipilimumab and 44% for patients treated with Nivolumab alone, compared with 26% in patients receiving Ipilimumab alone⁹⁴. However, rates of Grade 3 or 4 immune-related toxicity were higher in the combination regime (59%) than in either of the monotherapy regimes (23% and 28% for Nivolumab and Ipilimumab respectively).

Whilst checkpoint inhibitor therapies can help to overcome loss of immunogenicity, alternative approaches to deal with other mechanisms of immune evasion and maximise benefit from checkpoint inhibitors whilst minimising toxicity remain necessary and are under investigation.

1.2.2 Bispecifics

As discussed previously, a significant number of antigens displayed by tumours are self-antigens for which high affinity T cells are removed during thymic selection. Combined with antigen down-regulation by tumours, this can result in loss of antigenicity of these potential targets of the anti-tumour immune response. Releasing the inhibition on T cell function by blockade of immune checkpoints may therefore have limited benefit in the absence of tumour-specific T cells with a sufficient level of affinity for these tumour antigens. One approach which has been taken to address this, is investigating the use of bispecific monoclonal antibodies which combine two or more different specific antigen binding elements in a single construct. One such class of bispecific are ImmTACs (Immune mobilising monoclonal TCRs against cancer). These consist of a high-affinity tumour-

associated epitope specific monoclonal TCR (mTCR) fused to a humanised CD3 specific single-chain antibody fragment⁹⁵. These have the potential to redirect T cells, regardless of their specificity to kill cancer cells expressing the relevant tumour-associated epitope (Figure 1-2), with the high affinity TCR allowing T cells to recognise tumour cells even when HLA has been down-regulated.

IMCgp100, the first of these agents to enter clinical development is an ImmTAC bearing a mTCR with specificity for the HLA-A*02:01 restricted tumour-associated epitope gp100₂₈₀₋₂₈₈YLEPGPVTA⁹⁵. In vitro this ImmTAC was able to enhance the killing of Mel624 cells by a T cell clone specific for MART-1 and also able to re-direct the activity of a CD8⁺ T cell clone specific for an epitope BRLF1 derived from Epstein-Barr virus (EBV). High levels of lysis were observed at low effector: target (E:T) ratios indicating that single CD8⁺ T cells were serially killing several target cells over the course of the incubation period. This was shown to occur effectively even with low surface epitope densities and was able to suppress tumour growth in vivo in mouse xenograft models. Using peptide pulsed peripheral blood mononuclear cells (PBMCs), CD8⁺ T cells from the various memory compartments were activated in the presence of ImmTAC in a dose-dependent manner to elicit effector functions including degranulation and production of IFN- γ , tumour necrosis factor (TNF) and IL-2. Furthermore, when PBMCs were incubated with ImmTAC and Mel526 melanoma cells, many of the responding T cells showed a terminally differentiated phenotype known to be associated with high cytotoxic potential. Activation was also seen within the CD4⁺ compartment resulting in production of TNF and IL-2⁹⁵.

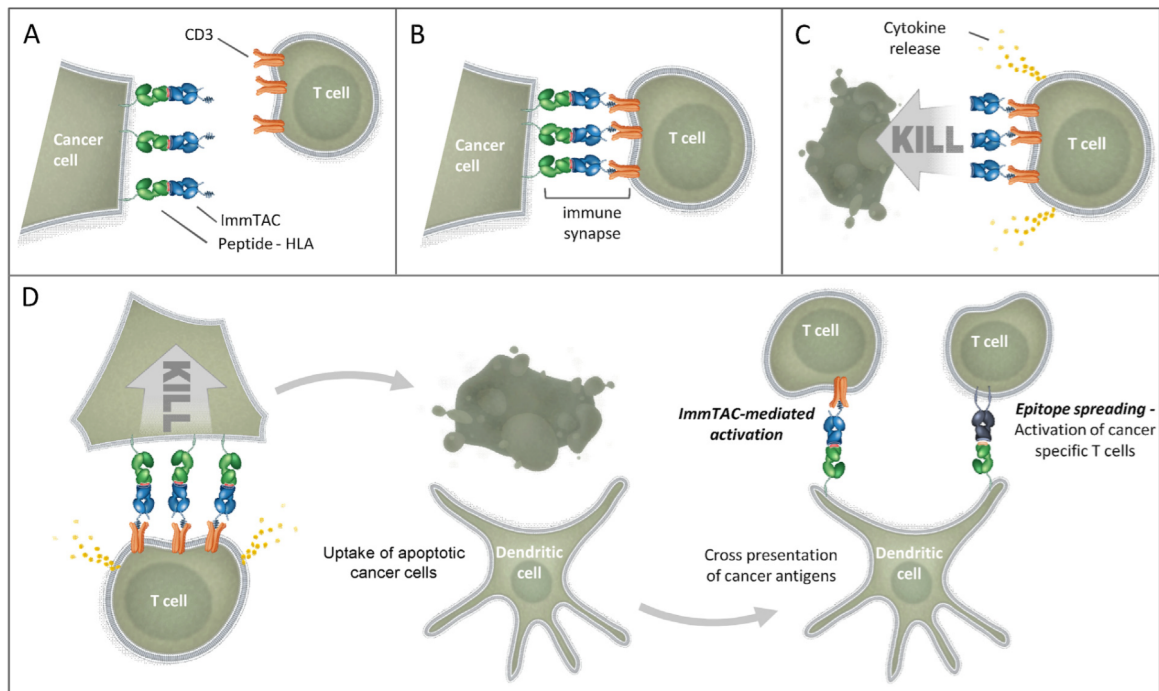


Figure 1-2. ImmTAC mechanism of action. A) ImmTACs bind to a specific peptide-HLA complex on the surface of a cancer cell through a high affinity TCR. B) Polyclonal T cells are redirected to the ImmTAC-bearing cancer cell through the anti-CD3 antibody fragment, forming an immune synapse. C) Redirected T cells are activated and mediate cancer cell death through release of cytokines and lytic molecules. D) ImmTAC-mediated killing leads to antigen uptake by dendritic cells and epitope spreading. Reprinted from Oates *et al.*⁹⁶ Copyright (2015), with permission from Elsevier.

In the first-in-man Phase I/IIa clinical trial, encouraging signs of efficacy were seen with three partial responses (PR) and one complete response (CR) observed in 16 pre-treated patients with metastatic melanoma⁹⁷. The most commonly observed side effect, seen in 82% of patients treated was a transient pruritic rash with an onset within the first 24 hours of treatment⁹⁸. This was accompanied by profound lymphocyte trafficking to the skin on immunohistochemical analysis of skin biopsies⁹⁹.

Whilst ImmTACs have been shown to re-direct T cells into tumour, these cells will potentially be subject to the immunosuppressive effects of the tumour microenvironment. Combining IMCgp100 with checkpoint inhibitors therefore represents a potential strategy to improve upon the efficacy of IMCgp100 alone and overcome resistance in PD-1 non-responders and is the subject of an ongoing clinical trial (IMCgp100-201).

1.2.3 Combination strategies

Given the wealth of potential approaches for augmenting the anti-tumour response, and multiple mechanisms for cancer immune evasion and escape, combining different strategies is likely to prove most effective. For example, immune checkpoint blockade (ICB) alone is unlikely to have efficacy in tumours where defects in antigen processing and presentation prevent T cell recognition, but combinations with strategies which boost tumour-specific immunity such as vaccines and ImmTACs might prove successful. For immune excluded tumours, treatments capable of modulating the tumour microenvironment may be required. However, which particular strategies to combine and in which patients has yet to be fully elucidated.

1.3 Biomarkers of response and resistance to checkpoint inhibitor therapy

In order to try and inform rational combination approaches and to guide the selection of the most appropriate treatment for different patients, much effort has been directed at trying to identify determinants of response and resistance to various therapies, in particular checkpoint inhibitors. These can broadly be divided into three categories: tumour cell intrinsic, microenvironment and systemic¹⁰⁰ (Figure 1-3).

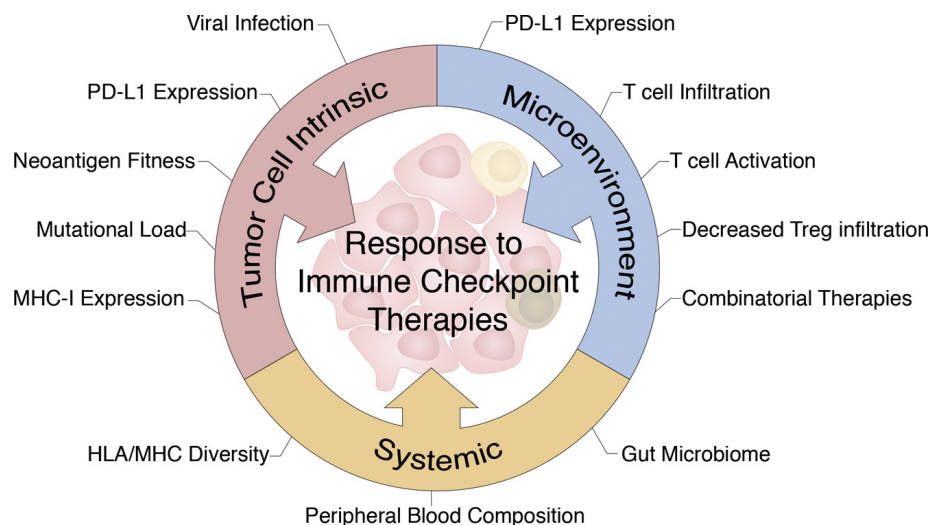


Figure 1-3. Determinants of response and resistance to immune checkpoint blockade. Reprinted from Borchering *et al.*¹⁰⁰, Copyright (2018), with permission from Elsevier.

Although the greatest clinical activity of checkpoint inhibitors is seen in cancers with high levels of somatic mutation such as melanoma^{27,101} and non-small-cell lung cancer (NSCLC)¹⁰², responses are still seen in some patients with low somatic mutation rates, and not all patients with a high mutation burden benefit. There is some suggestion that the heterogeneity of neoantigens within a tumour may be important in this regard, with responders to ICB in NSCLC and melanoma typically having tumours characterised by clonal neoantigens, whilst subclonal neoantigens were seen in non-responders¹⁰³. Limited responses in the presence of sufficient antigenic load may also occur in some patients because they do not have sufficient intra-tumoural CD8⁺ infiltration⁷⁴, however the presence of pre-existing T cell responses at the site of the tumour does not guarantee an anti-tumour response.

It was initially thought that tumour PD-L1 expression might prove a useful biomarker for predicting benefit to checkpoint inhibitor treatment, with higher expression being indicative of a pre-existing anti-tumour response. Whilst in general tumours over-expressing PD-L1 have improved clinical outcomes with PD-1 directed therapy^{104,105}, responses can still be seen in some patients with low expression¹⁰⁶. Furthermore, the use of different antibodies for immunohistochemical analysis, differing thresholds for defining positivity and the fact that PD-L1 expression is upregulated in response to IFN- γ produced by activated T cells and is therefore dynamic over time, has limited the usefulness of this as a biomarker, at least in melanoma¹⁰⁷.

Although reflecting the primary site of the anti-tumour response, biomarkers based on intra-tumoural features are limited in their clinical usefulness due to the difficulties inherent in obtaining tumour biopsies, and in view of both spatial and temporal intra-tumoural heterogeneity. Systemic immune monitoring of peripheral blood therefore provides an attractive alternative for studying the anti-tumour immune response due to its ease of access and greater homogeneity. However, the large number of different immune cell subsets and fact that the anti-tumour immune response represents just a small component

of the total peripheral response, pose challenges. When considering the effect of checkpoint inhibitor treatment upon the systemic immune response, changes in peripheral blood composition can be thought about in terms of phenotypic changes in different immune cell subsets, antigen-specific responses, T cell clonality and also functionality. A summary of some of the extensive literature in this regard relevant to themes explored further in this work will be discussed below.

1.3.1 Immune cell subsets

As the longest established of the checkpoint inhibitor therapies, the effects of anti-CTLA-4 treatment have been the most extensively studied. Increases in absolute lymphocyte counts were observed in response to treatment and positively associated with outcome^{108,109} although no change in CD4:CD8 proportions were observed¹⁰⁸, whilst natural killer (NK) cell¹⁰⁸ and MDSC frequencies were unchanged^{108,110}. Higher frequencies of MDSC at baseline however were a predictor of poor outcome^{108,110}, whilst higher frequencies of non-classical monocytes were associated with positive treatment outcomes¹¹¹.

Consistent with the role of anti-CTLA-4 in T cell priming, reductions in naïve CD4+ and CD8+ T cells and increased frequencies of effector T cells^{108,112}, and effector memory CD45RO+ CD4+ but not CD8+¹¹³⁻¹¹⁶ were observed following treatment. Lower frequencies of CD45RO+ CD8+ T cells were found at baseline in non-responders^{116,117}. Increased expression of the proliferation marker Ki67^{112,118} and activation marker HLA-DR were also observed^{108,113-115,119}. Whilst some studies found positive associations between activation and outcome^{108,115}, others reported no statistically significant association¹¹⁴.

In preclinical models, whilst impacts upon effector T cells were observed, effects upon Tregs were also thought to be important¹²⁰ due to their constitutive expression of CTLA-4, with antibody engagement with activating Fcγ receptors (FcγR) on innate cells causing Treg depletion and altering the intra-tumour immune balance¹²¹⁻¹²³. However, the clinical data on the effect of anti-CLTA-4 treatment upon Tregs has been and remains contradictory, with some studies reporting reductions in Treg frequencies^{108,109,124,125} during CTLA-4 treatment,

and others reporting no change^{113,115,119} or indeed increases intra-tumourally¹²⁶. This may be explained by differences in timing of samples, immunoglobulin (Ig) class of anti-CTLA-4 agent being studied and differences between patients in response status, presence of Fc polymorphisms, and whether the tumours were inflamed¹²⁷.

Unlike anti-CTLA-4 treatment, no changes in absolute lymphocyte counts were observed with anti-PD-1 therapy^{128,129}. Higher baseline frequencies of myeloid and NKT cells¹³⁰, lymphocytes and eosinophils¹³¹ and lower frequencies of gamma delta T cells¹³⁰ have all been reported to associate positively with response to anti-PD-1 therapy, whilst data on whether the frequency of CD5RO+ cells pre-treatment relates to outcome has been conflicting^{116,130}. Upregulation of HLA-DR on both CD4+ and CD8+ cells was observed and positively correlated with response¹³⁰. Proliferation of CD8+ and CD4+ cells (as defined by Ki67 positivity) has been reported in both melanoma and lung cancer^{129,132} but not by all groups¹¹⁸. The proliferating cells appeared to be predominantly PD-1+, were CD45RA^{lo} CD27^{hi} and displayed expression of activation markers CD38 and HLA-DR^{129,132}. Furthermore, increases were typically observed after one or two cycles of treatment but not sustained^{129,132}. Correlations between the number of proliferating PD-1+CD8+ T cells on treatment and positive clinical outcomes have been made^{129,132}, although in the case of Huang *et al.*¹³² this was only when the tumour burden was also taken into consideration. Consistent with this, higher numbers of pre-existing CD8+PD-1+ cells within the tumour, and proliferation of these cells during treatment were found upon serial biopsies from patients responding to anti-PD-1 treatment¹³³.

Relatively little data is published in relation to combination anti-CTLA-4 and anti-PD-1 checkpoint inhibitor therapy. Das *et al.*¹¹⁸ studied a cohort of 9 patients being treated with concurrent combination therapy and compared these to 24 patients treated with anti-PD-1 monotherapy and 9 treated with anti-CTLA-4 monotherapy using a combination of immunophenotyping by flow and mass cytometry together with gene expression analysis of PBMCs. They saw a much greater number of differentially expressed genes with

combination therapy than either agent given alone, with only minor overlap in genes upregulated between the different cohorts, the only one shared being IFN- γ . Proliferation was observed in response to anti-CTLA-4 and combination therapy treatment, however unlike other studies not anti-PD-1 treatment, whilst cytolytic functions indicated by granzyme A and B were induced with anti-PD-1 and combination therapy but not anti-CTLA-4 therapy. Increased expression of HLA-DR was observed for combination therapy. More recent data however¹³⁴, whilst confirming the greater magnitude of effect of combination therapy, suggests greater commonality in gene expression changes between single agent and combination PD-1 based treatments than reported in this previous study. In other work, presence of higher frequencies of intra-tumoural EOMES+CD69+CD45RO+PD-1+ effector memory cells expressing CD103 pre-treatment were observed in responders than non-responders to combination therapy but not anti-PD-1 monotherapy¹³⁵.

1.3.2 Antigen-specific responses

As well as looking at peripheral immune subsets, efforts have been made to study the effect of ICB treatment upon the antigen-specific response. Relatively little however is known about the type of tumour antigens that are the targets of T cells influenced by checkpoint inhibitor therapy. In mice, tumour-specific mutant proteins have been shown to be an important target of checkpoint blockade¹³⁶. Challenges of monitoring patient-specific, neoantigen responses however mean that much of the data we have with regards to antigen specificity in treated patients comes from tumour-associated antigens. As described by Kvistborg *et al.*¹³⁷ effects upon the antigen-specific can be considered in terms of whether treatment produces an increase in the magnitude of the pre-existing anti-tumour response, consistent with primary activity at the level of tumour-T cell interaction, or broadens the melanoma-reactive T cell response, consistent with its primary effect being mediated by T cell priming.

Whilst priming of NY-ESO-1 specific T cell responses has been demonstrated in response to anti-CTLA-4 therapy¹³⁸ and associated with clinical response¹³⁹, other studies have shown no definite trend for changes over time in the frequency of tumour-specific (MART-1, gp100 and tyrosinase) or microbial-specific (Cytomegalovirus (CMV) and EBV) T cells^{115,116}. Transient changes at certain timepoints in some individuals were observed, however the expansion of circulating melanoma antigen-specific T cells did not correlate with clinical benefit from treatment. Accumulation of gp100-specific T cells within a regressing tumour lesion were observed, but discordant with the timing of accumulation in the peripheral blood, raising the possibility that transient peaks observed might be due to accumulation of cells within the tumour¹¹⁵. Although most of these studies were limited to a small selection of tumour-specific antigens, Kvistborg *et al.*¹³⁷ provided strong evidence for anti-CTLA-4 broadening the melanoma-specific CD8 T cell response using tetramers for a much larger selection of tumour-associated antigens. Whilst pre-existing and virus-specific CD8 T cell responses were largely unchanged following anti-CTLA-4 treatment, an increase in the number of melanoma-specific T cell responses (new specificities detectable by tetramer staining) was observed in nearly half of patients studied. For most patients this was only a single novel melanoma-specific reactivity, however for some patients up to 6 new specificities were detectable. It is not known however whether these changes were associated with treatment response.

For anti-PD-1 therapy, high pre-treatment NY-ESO-1 and MART-1-specific CD8+ T cells and a decrease in antigen-specific T cells after 12 weeks on therapy were associated with progression of disease¹⁰⁶. Further recent work looking at neoantigen-specific T cells in patients being treated with the anti-PD-L1 inhibitor Atezolizumab, showed a trend towards greater abundance at baseline and enrichment post-treatment in patients responding to treatment¹⁴⁰. They also observed that these antigen-specific cells displayed diverse phenotypes and that among responders, the majority of antigen-specific cells had a late-differentiated effector phenotype (expressing KLRG-1, CD57, 2B4, CD161, TIGIT and

CD25) similar to CMV and EBV specific cells, compared with non-responders which had higher expression of CD127, CD28, CD27 and CCR7, consistent with a more memory-like phenotype. Amongst the responders, they also saw in one individual high HLA-DR and CD38 expression as well as high PD-1 and CD39.

As previously discussed, proliferation of PD-1+ positive cells in response to treatment has been observed by a number of investigators^{129,141}. Evidence that these proliferating cells might be tumour-specific comes from observations that proliferation of EBV specific cells is not observed in response to PD-1 therapy¹²⁹, and that when these cells were sorted from 6 patients and their TCR sequence analysed, clones that are shared between tumour and blood display an activated phenotype whilst those found only in blood and not tumour have a mixed phenotype¹³². However, evidence directly demonstrating tumour-reactivity of these proliferating cells is lacking.

1.3.3 T cell clonality

A number of studies have also investigated the association of T cell clonality in response to ICB. A diverse TCR repertoire pre-treatment has been associated with clinical benefit to anti-CTLA-4 treatment^{128,142}, whilst more clonal, less diverse repertoires were predictive of responses to anti-PD-1 blockade¹⁴². In patients responding to anti-PD-1 treatment, 10 fold more clones expanded after treatment compared to baseline in responders than non-responders¹³³. In vitro re-stimulation studies on PBMCs isolated from 9 patients undergoing anti-PD-1 therapy have shown changes in the TCR β repertoire of MART-1-specific T cells upon treatment, with both expansion of existing clonotypes and emergence of new clonotypes observed. Predominant expansion of emerging clonotypes was only observed in patients exhibiting objective responses¹⁴³. PD-1 expression was higher on clonotypes amplified upon treatment (regardless of whether they were pre-existing or emerging) than on clonotypes that did not expand on treatment. Furthermore, emerging clonotypes were found to display the highest functional avidities and both emerging and pre-existing

clonotypes which expanded on treatment displayed a higher and less diverse functional avidity than those that did not amplify on treatment.

As discussed previously, the proliferation of PD-1+ T cells in response to anti-PD-1 therapy has generally been interpreted as anti-PD-1 treatment causing reinvigoration of exhausted tumour-specific cells. However, more recent data studying in greater detail TCR clonality within tumours of patients with basal or squamous cell carcinoma receiving anti-PD-1 therapy, demonstrated that it was not pre-existing T cell clones expanding within the tumour, but that novel clonotypes not previously observed were replacing pre-existing exhausted clonotypes²⁰. Furthermore, whilst T cells of the same clonotype could be found in phenotypically distinct clusters, suggesting transitions between activation states, clonotypes displaying an exhausted phenotype were not found amongst effector cells and did not revert to non-exhausted phenotypes post-treatment. This suggests that exhausted pre-existing TILs have limited potential for re-invigoration and that response to treatment is driven by a novel repertoire of tumour-specific clones. Bulk TCR sequencing of the blood showed that 41% of clonotypes found in TILs could also be detected in the blood, and that 12% of novel TIL clonotypes could be detected in the periphery, but not the tumour, pre-treatment suggesting that these novel TCR clones might be recruited from the periphery.

1.3.4 Synopsis

Whilst some features predictive of response to ICB have been identified, to date no definitive or clinically useful markers to determine which patients will benefit from treatment have been established. The dynamic nature of the cancer-immune system response and the rapid mutation rate of cancer makes this particularly challenging, resulting in numerous evolving antigen-specific responses, each associated with distinct HLA-restricted epitopes and TCR repertoires, which may or may not be successful in containing the growth of some or all of the various clones and subclones contained within a tumour. A lack of useful biomarkers may also be because previous studies have typically addressed these individual aspects of the immune response in isolation. For example, many of the studies describing

phenotypic or TCR clonality changes do so without a knowledge of their antigen-specificity, or infer this indirectly through TCR sharing between the tumour and periphery without directly demonstrating that these cells are tumour-specific and not simply viral-specific tumour resident bystander cells¹⁴⁴. Other studies taking an antigen directed approach have not always considered the TCR repertoire or phenotype of the cells in vivo. Integrated approaches are therefore likely to be more informative of the anti-tumour immune response.

1.4 Summary and aims

There is no doubt that strategies such as immune checkpoint blockade have revolutionised treatment for patients with cancers such as metastatic melanoma however, despite this success, responses are still only seen in a subset of patients. Whilst treatment with combination anti-CTLA-4 and anti-PD-1 antibody therapy has been shown to produce numerically higher response rates, progression free survival and overall survival than anti-PD-1 monotherapy or anti-CTLA-4 monotherapy in metastatic melanoma, this comes at the expense of greater toxicity in the form of immune-related adverse events^{107,145,146}. In addition, there are substantial healthcare costs associated with both the treatment itself, which can be ongoing for up to 2 years, as well as the management of checkpoint inhibitor toxicity.

There is therefore a need to identify biomarkers of response and resistance which might predict which patients are most likely to benefit from which treatments, to provide early assessments of response to help inform the balance of benefits vs risks of ongoing treatment, and to predict effective combination strategies to overcome both primary and adaptive resistance. A deeper understanding of T cell behaviour during immunotherapy treatment and how this relates to response or failure of treatment is integral to this. Furthermore, despite the success of immune-checkpoint inhibitor therapies, more targeted approaches might be needed for those patients who still progress after treatment. Among the different personalised therapies currently under development, adoptive T cell therapy requires the identification of TCRs and/or of autologous T cell responses capable of

recognising tumour cells. Current methodologies to identify tumour-specific T cells rely on the availability of tumour tissues, and either on the in vitro expansion of tumour infiltrating lymphocytes, or on the identification of paired TCR sequences of TILs by single-cell sequencing technologies and their in vitro reconstruction. However, tumour tissues are not always available, and individual biopsies might provide only a partial representation of the repertoire of tumour-specific T cells. Approaches for the identification of tumour-specific T cells within the periphery might therefore provide a more accessible alternative for obtaining T cells for therapeutic use.

In an attempt to address this, we set out to assess the effects of immune checkpoint inhibitor therapy upon the peripheral immune response and more specifically, to try and establish the effects upon the antigen-specific response and TCR repertoire. Given the relatively limited data available on the current standard of care treatment for metastatic melanoma of combination anti-CTLA-4 and anti-PD-1 therapy, we focused our attention on this treatment. To allow us to fully elucidate the specific effects of the combination treatment, we also considered the respective monotherapy treatments, as well as the ImmTAC IMCgp100 as a non-ICB based treatment. We employed an integrated immune monitoring approach as shown in Figure 1-4 utilising a combination of high dimensional single-cell phenotypic characterisation by mass cytometry, and fluorescence-activated cell sorting (FACS) to allow us to interrogate the specificity, functionality and TCR repertoire both by single cell cloning and single-cell RNA-sequencing (scRNA-seq).

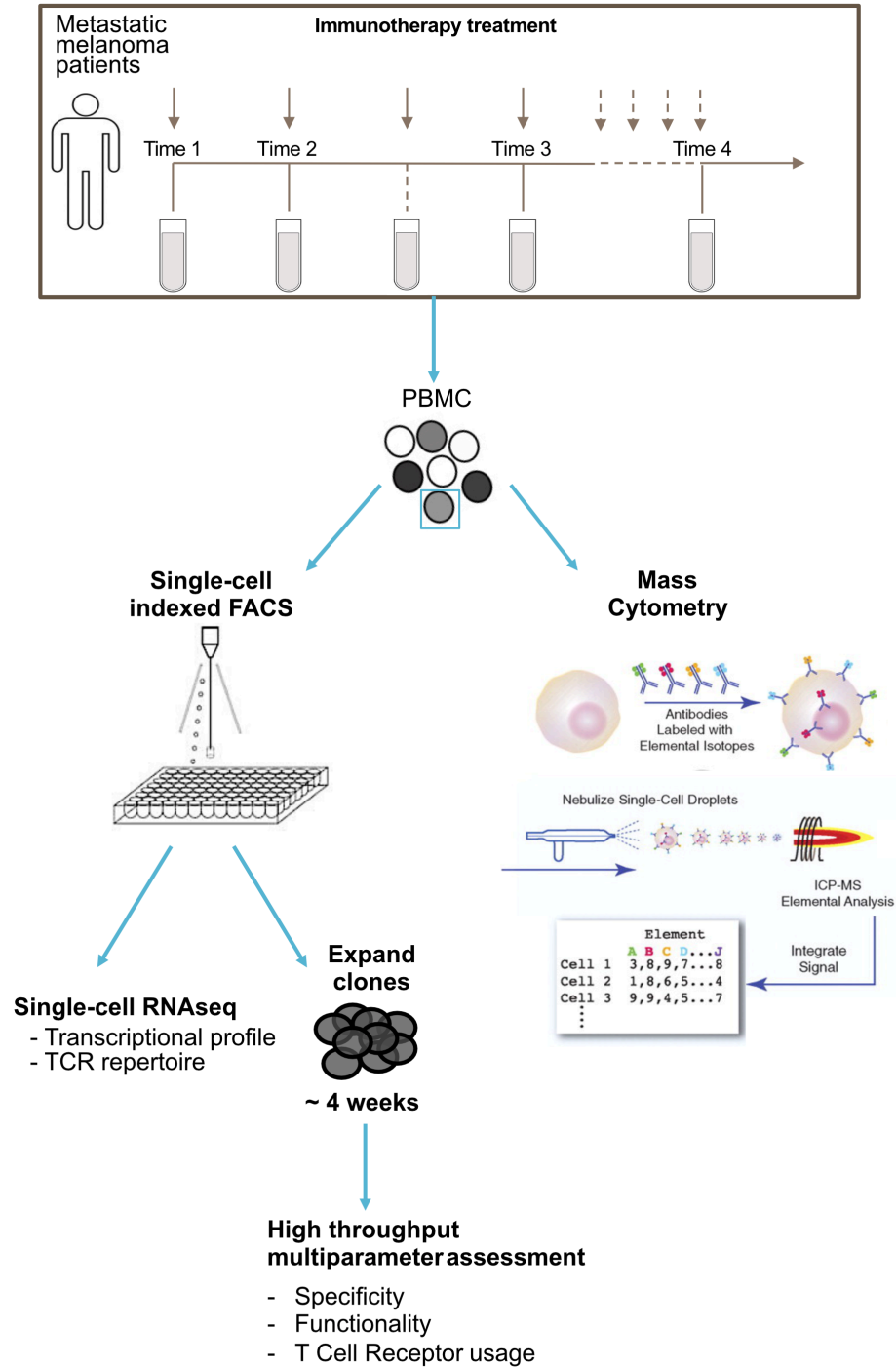


Figure 1-4. Integrated peripheral immune monitoring approach. Mass cytometry illustration adapted from Bendall *et al.*¹⁴⁷. Reprinted with permission from AAAS.

Chapter 2. Materials and methods

2.1 Common reagents

A list of reagents used across various different experimental protocols in this thesis are listed in Table 2-1.

Table 2-1. Common reagents

Reagent	Source	Catalogue Number
Roswell Park Memorial Institute media (RPMI)-1640	Sigma-Aldrich	R8748
Hepes Buffer Solution 1M	Gibco	15630-056
Lymphoprep	Axis-Shield	LYS3773
Trypan Blue Stain 0.4%	Gibco	15250-061
Minimal Essential Medium Non-Essential Amino Acids 10mM	Gibco	11140-035
Sodium Pyruvate 100mM	Gibco	11360-039
L-Glutamine 200mM	Sigma	G7513
Penicillin-Streptomycin (10,000units penicillin and 10mg streptomycin/ml)	Sigma	P0781
2-Mercaptoethanol 50mM	Gibco	31350-010
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich	D5879
Foetal bovine serum (FBS)	Gibco	10500-064
Human serum (HS) pooled from 15 donors	NHS Blood & Transplant	NA
Recombinant human IL-2	In house as per Traunecker ¹⁴⁸	NA
Benzonase nuclease ≥ 250 units/ μ l, $\geq 90\%$ purity	Sigma-Aldrich	E1014
Phosphate-buffered saline (PBS)	Alfa Aesar	J61196
Cell Stimulation Cocktail 500X	eBioscience	00-4970-93
Protein Transport Inhibitor Cocktail 500X	eBioscience	00-4980-93
Intracellular fixation buffer	eBioscience	00-8222-49
Permeabilization Buffer (10X)	eBioscience	00-8333
Ultracomp eBeads	Invitrogen	01-2222-42
Purified Phytohemagglutinin (PHA)	Thermo Scientific	10082333
Purified anti-human HLA-A,B,C (W632)	Biolegend	311412
Purified Mouse IgG2a, κ isotype control (MOPC-173)	Biolegend	400224

2.2 Common media and buffers

The composition of common media and buffers used are detailed in Table 2-2. All additives were filtered with a 22µM filter.

Table 2-2. Common culture media and buffers

Media/Buffer	Base media	Additive	Final concentration
RPMI + Hepes	RPMI-1640	Hepes	10mM
Complete RPMI	RPMI-1640	Hepes Sodium Pyruvate L-Glutamine Penicillin Streptomycin Non-essential amino acids 2-mercaptoethanol	10mM 1mM 2mM 100U/ml Penicillin 100µg/ml Streptomycin 10µM 50µM
R10-FBS	Complete RPMI	FBS	10% (v/v)
R5HS-IL-2	Complete RPMI	Human serum IL-2	5% (v/v) 1000U/ml
FBS Buffer	PBS	FBS	2% (v/v)
Freezing media	FBS	DMSO	10% (v/v)

2.3 Patient samples

Blood and tumour samples were collected from patients with metastatic melanoma under ethical approval from the Oxford Radcliffe Biobank (Reference No. 09/H0606/5+5 16A019). Serial blood samples were collected from patients receiving immunotherapy treatment either as standard-of-care treatment or within clinical trials at the Churchill Hospital, Oxford. Blood samples were taken before commencing treatment as a baseline and then at subsequent timepoints during therapy, guided by the scheduling and administration of the relevant immunotherapy treatment. In some cases where treatment was ongoing after the initial sample collection schedule, an opportunistic 'late on-treatment' blood sample was also collected.

2.4 Isolation of peripheral blood mononuclear cells

Patient blood samples were collected in either sodium heparin or EDTA anticoagulant containing blood collection tubes and processed within 4 hours of sampling. Blood was diluted 1:1 with room-temperature RPMI-1640 + Hepes media and layered over Lymphoprep density gradient medium prior to centrifugation at 800 x *g* for 30 minutes at room temperature. After centrifugation, mononuclear cells were removed from the sample/medium interface using a Pasteur pipette and washed once with RPMI + Hepes with centrifugation at 500 x *g* followed by a further wash with centrifugation at 250 x *g* to remove platelets. The pelleted PBMCs were then resuspended in R10-FBS and counted using 0.4% Trypan Blue. Following centrifugation at 500 x *g* cells were resuspended at 10×10^6 cells/ml in FBS supplemented with 10% DMSO and frozen in 1ml aliquots in cryovials at -80C using a controlled rate cell freezing container. After 24 hours the aliquots were transferred to liquid nitrogen for long term storage.

2.5 Peptides

A panel of HLA-A*02:01 restricted peptides (Sigma-Aldrich) for 25 common melanoma tumour-associated antigens (Table 2-3) as well as 16 common microbial antigens (Table 2-4) were used for tetramer synthesis as well as profiling of CD8 T cell clones. Melanoma-associated antigens were chosen from a list of 145 epitopes previously described by Kvistborg *et al.*¹³⁷ to cover a range of different types of antigen and where detectable responses had been reported in melanoma patients in their study or in the literature previously.

Table 2-3. Peptides for melanoma tumour-associated antigens

Antigen	Peptide sequence	Type of antigen
Melan-A/MART-1*	ELAGIGILTV	Melanocyte differentiation antigen
TRP-2	SVYDFFVWL	Melanocyte differentiation antigen
gp100*	ITDQVPFSV	Melanocyte differentiation antigen
gp100*	KTWGQYWQV	Melanocyte differentiation antigen
gp100*	VLYRYGSFSV	Melanocyte differentiation antigen
gp100*	YLEPGPVTA	Melanocyte differentiation antigen
gp100*	IMDQVPFSV	Melanocyte differentiation antigen
NY-ESO-1/LAGE-2*	SLLMWITQA	Cancer testis antigen
LAGE-1*	MLMAQEALAFI	Cancer testis antigen
LAGE-1	SLLMWITQC	Cancer testis antigen
MAGE-A1	KVLEYVIKV	Cancer testis antigen
MAGE-A10*	GLYDGMEHL	Cancer testis antigen
MAGE-A2*	YLQLVFGIEV	Cancer testis antigen
MAGE-A3	FLWGPRALV	Cancer testis antigen
MAGE-A3*	KVAELVHFL	Cancer testis antigen
MAGE-C2*	ALKDVEERV	Cancer testis antigen
MAGE-C2	KVLEFLAKL	Cancer testis antigen
MAGE-C2*	LLFGLALIEV	Cancer testis antigen
RAGE-1*	LKLSGVVRL	Cancer testis antigen
SSX-2*	KASEKIFYV	Cancer testis antigen
GnT-V (MGAT5)*	VLPDVFIRCV	Over-expressed antigen
BING-4 (WDR46)*	CQWGRLWQL	Over-expressed antigen
Meloe-1*	TLNDECWPA	Over-expressed antigen
PRDX5 (OMT3-12)*	AMAPIKVRL	Over-expressed antigen
Tyrosinase	YMDGTMSQV	Over-expressed antigen

* - used for tetramers in mass cytometry experiments

Table 2-4. Peptides for microbial antigens

Organism	Antigen	Peptide sequence
Flu	M1*	GILGFVFTL
Varicella-Zoster virus	IE62	ALWALPHAA
Adenovirus	Ad11 Hexon	LLFEVFDVV
Adenovirus	AdV Hexon	YLLFEVFDV
Cytomegalovirus	pp65*	NLVPMVATV
Cytomegalovirus	IE1	VLEETSVML
Epstein-Barr virus	LMP2*	TVCGGIMFL
Epstein-Barr virus	LMP1	YLQQNWWTL
Human papillomavirus	E6	KLPQLCTEL
Human papillomavirus	E7	YMLDLQPET
Mycobacterium tuberculosis	16kDa	GILTVSVAV
Mycobacterium tuberculosis	Ag85A	GLPVEYLQV
Mycobacterium tuberculosis	Ag85B	KLVANNTRL
House dust mite	Der p 1A	MMIEEYPYV
House dust mite	Der p 1B	SLDLAEQEL
House dust mite	Der p 1C	YLAYRNQSL

* - used for tetramers in mass cytometry experiments

A number of predicted neoantigen peptides for one individual were also ordered from Pepscan (not listed). Crude peptides were supplied lyophilised and were resuspended in DMSO to a concentration of 30mM

2.6 Tetramers

Recombinant peptide-MHC (pMHC) multimer technology as previously described^{149,150} was used for the detection and quantification of antigen-specific T cell responses. Reagents used are listed in Table 2-5.

Table 2-5. Reagents for tetramer synthesis

Reagent	Source	Catalogue Number
LB Broth – Ezmix powder microbial growth medium	Sigma	L7658
Isopropyl β - d-1-thiogalactopyranoside (IPTG) 99%	Flurochem	M02726
Ampicillin	Roche Diagnostics	10835243001
Tris 1M pH 8	Santa Cruz Biotechnology	Sc-296649
Sodium Chloride (NaCl)	Sigma-Aldrich	7647-14-5
Triton X-100	Acros Organics	9002-93-1
DTT (DL-Dithiothreitol) 0.5M	Sigma-Aldrich	D97798-6G
EDTA 0.5M	Corning	46-034-cl
Sodium azide 10%	Sigma-Aldrich	08591
Urea 10M	Sigma-Aldrich	U5378
Sodium phosphate monobasic (NaH ₂ PO ₄) 1M	Sigma-Aldrich	S8282
Pierce BCA protein assay kit	ThermoScientific	23225
L-arginine hydrochloride	Acros Organics	105000025
L-Glutathione reduced	Sigma-Aldrich	G4251
L-Glutathione oxidized	Sigma-Aldrich	G4376
d-Biotin 100mM	Sigma-Aldrich	B4501
Magnesium chloride (MgCl ₂) 1M	Sigma-Aldrich	M8266
Adenosine triphosphate (ATP)	Sigma-Aldrich	A2383
E. coli biotin ligase (BirA) enzyme	In house	NA
Leupeptin	Santa Cruz Biotechnology	Sc-295358A
Pepstatin A $\geq$ 100,000 U/mg	Sigma-Aldrich	77170
Ultraviolet (UV) cleavable peptide	Pepscan	Custom
D-biotin 50mM	Invitrogen	B20656

2.6.1 Synthesis of HLA-A*02:01 UV monomer

HLA-A*02:01 MHC class I complexes occupied with a conditional UV ligand were produced by Ji-Li Chen (Cerundolo lab, University of Oxford) according to the following method.

2.6.1.1 Protein expression in E coli and purification

BL21 E coli expressing recombinant HLA-A*02:01 class I heavy chains containing a COOH-terminal biotinylation sequence and $\beta 2$ microglobulin ($\beta 2M$) light chain were cultured separately in LB broth containing 100 μ g/ml ampicillin until $OD_{600\text{ nm}} = 0.6$. They were then induced with 0.5ml/L of 1M IPTG and the cells isolated after 4 hours by centrifugation, resuspended in minimum volumes of cold PBS and frozen.

Inclusion bodies were isolated from thawed bacterial pellets by bursts of sonication followed by centrifugation at 13000rpm for 30 minutes at 4°C and then removal of the supernatant. To remove bacterial debris, pellets were resuspended using a glass homogeniser in 25ml of cold Triton wash buffer (consisting of water containing 50mM Tris, 100mM NaCl, 0.5% Triton, 1mM DTT, 1mM EDTA and 0.1% Azide) followed by centrifugation at 15000rpm for 10 minutes and discarding of the supernatant, repeated three times. The detergent was then washed out by resuspending pellets in 25ml of cold resuspension buffer (consisting of 50mM Tris, 100mM NaCl, 1mM DTT and 1mM EDTA) and centrifugation at 15000rpm for 10 minutes. Pellets were then resuspended in 25ml cold fresh urea solution (consisting of 8M Urea, 10mM Tris, 100mM NaH_2PO_4 , 0.1mM EDTA and 0.1mM DTT) and left on a rotator in a cold room overnight to denature. Urea extracts were centrifuged the following day at 15000rpm for 30 minutes and supernatants combined on ice. The protein content was then quantified by BCA Protein Assay and stored at -80°C in aliquots of 32mg for the A2 heavy chain and 26mg for $\beta 2M$.

2.6.1.2 Refolding

For refolding, the conditional UV ligand (H-KILGVFJV-OH where J = the UV sensitive amino acid 2-NO₂- β -Phe) was dissolved in DMSO to a concentration of 10mg/ml and added

dropwise to 1L of chilled refolding buffer (consisting of 100mM Tris, 400mM L-arginine hydrochloride, 2mM EDTA, 5mM reduced glutathione, 0.5mM oxidised glutathione in water). Once the peptide was completely dissolved 26mg of β 2M was added dropwise followed by 32mg of A2 heavy chain and the refolding mix stirred in a cold room for 48 hours. After this time the mixture was concentrated over a 10kDa ultrafiltration membrane (Millipore) using a nitrogen pressurised stir cell (Amicon) and then purified by fast protein liquid chromatography FPLC using a Superdex-75 column with Tris-buffered saline as the running buffer. Fractions consistent with monomeric complexes were collected and then further concentrated using a 10kDa centrifugal filter unit. After de-salting by buffer exchange using PD10 columns (GE Healthcare) the purified monomers were then biotinylated by the addition of d-Biotin and BirA enzyme at final concentrations of 400 μ M and 50 μ M respectively in the presence of 5mM MgCl₂, 1 μ M of the protease inhibitors leupeptin and pepstatin and 5mM of ATP and incubated overnight at room temperature. Biotinylated complexes were then separated from residual free biotin by fast protein liquid chromatography (FPLC) using a Superdex-75 column, further protease inhibitors added and concentrated to 1mg/ml using 10KDda centrifugal filter units. Successful biotinylation was confirmed by enzyme-linked immunosorbent assay (ELISA) and aliquots of monomer frozen at -80°C for future use.

2.6.2 UV induced peptide exchange and tetramerization

Individual HLA-A*02:01 restricted peptides of interest and HLA-A*02:01 monomer loaded with UV conditional ligand were diluted in PBS to concentrations of 400 μ M and 200 μ g/ml (~4 μ M) respectively. Equal volumes (typically 10 μ l) were mixed in 96-well polypropylene plates to give a final concentration of 100 fold molar excess of peptide over MHC (200 μ M peptide and 100 μ g/ml (~2 μ M) monomer). The plate was then placed under a UV lamp (366nm) for 1 hour at a distance of approximately 5cm and temperature of 4°C. After 1 hour the plate was centrifuged at 2,250 x g for 10 minutes and the supernatant collected (15 μ l in the case of a 20 μ l exchange corresponding to 1.5 μ g of monomer) avoiding the pellet

containing aggregates and transferred to a fresh plate. Streptavidins for tetramerisation (Table 2-6) were diluted in PBS to a concentration of 37.5µg/ml in PBS and 0.1µg (typically 4µl) was added per 1µg (10µl) of monomer retrieved. In the case of tetramers dual-labelled with different fluorophores, both streptavidins were combined for tetramerisation. 10% milk power in PBS (w/v) was added at a final volume of 1% (v/v) to prevent aggregation and background staining and incubated for 30 minutes on ice in the dark (final streptavidin concentration of ~7.18 µg/ml). An equal volume of 50µM D-biotin and 0.04% sodium azide was then added to block any free binding sites and incubated overnight at 4°C. The following day, tetramers were centrifuged at 3300 x *g* for 5 minutes and the supernatant collecting avoiding the pellet and used for subsequent staining or transferred to Eppendorf tubes for storage.

Table 2-6. Streptavidins used for MHC tetramers

Fluorophore	Concentration	Source	Catalogue Number
PE	0.2mg/ml	eBioscience	12-4317-87
APC	0.2mg/ml	eBioscience	17-4317-82
BV421	0.1mg/ml	Biolegend	405231
PerCP-eFluor710	0.2mg/ml	eBioscience	46-4317-80

2.6.3 Tetramer staining

In most cases tetramers were freshly synthesised as above 24 hours in advance of staining, but in cases where they were stored for up to a week in the dark at 4°C, tetramers were centrifuged for 3300 x *g* for 5 minutes before use. Cells were washed and stained with 2µl of each tetramer being used in 100µl final staining volume per 3 x 10⁶ cells to be stained for single fluorophore labelled tetramers, or 4µl in the case of dual-labelling. In the case of tetramer staining for flow cytometry FBS buffer was used, whilst for mass cytometry staining this was performed in cell staining buffer (CSB). Staining was performed at room temperature for 30 minutes and cells were then washed before further antibody staining. In order to minimise TCR internalisation following tetramer binding subsequent steps were performed on ice. In addition, where streptavidins conjugated to PE or APC were used,

unconjugated antibodies against these fluorophores were added during antibody staining (0.4µl/20µl stain) for flow cytometry experiments to boost the fluorophore signal and reduce the off-rate of the pMHC tetramer during staining and washing¹⁵¹.

2.7 Mass cytometry

2.7.1 Reagents

A list of reagents used in mass cytometry experiments is listed in Table 2-7.

Table 2-7. Mass cytometry reagents

Reagent	Source	Catalogue Number
Cell-ID 20-Plex Pd Barcoding Kit - Cell-ID Barcodes - Maxpar Fix 1 Buffer (5X) - Maxpar Barcode Perm Buffer (10X)	Fluidigm	201060
Maxpar Cell Staining Buffer (CSB)	Fluidigm	201068
Maxpar Fix and Perm Buffer	Fluidigm	201067
Maxpar Nuclear Antigen Staining Buffer Set - Maxpar Nuclear Antigen Staining Buffer Concentrate (4X) - Maxpar Nuclear Antigen Staining Buffer Diluent - Maxpar Nuclear Antigen Staining Perm (1X)	Fluidigm	201063
Maxpar Water	Fluidigm	201069
Maxpar PBS	Fluidigm	201058
Cell-ID Cisplatin	Fluidigm	201064
Cell-ID Intercalator-Ir 125µM	Fluidigm	201192
16% Formaldehyde Methanol Free	Life Technologies	28906
EQ Four Element Calibration (EQ) Beads	Fluidigm	201078
OneComp eBeads	Invitrogen	01-1111-42

2.7.1.1 Antibodies

Antibodies used in mass cytometry staining experiments are listed in Table 2-8. Whilst the exact antibodies used in each staining varied slightly between experiments, with the exception of CD14 and CD19 which were in some cases used together on the same metal as a 'dump' channel, only one antibody per metal was used in a given staining.

Table 2-8. Mass cytometry antibodies

Metal	Target	Clone	Source	Catalogue Number	Purity	Cocktail
89Y	CD45	HI30	Fluidigm	3089003	-	B
Qdot-114Cd	CD14	TuK4	Invitrogen	Q10056	-	A
Qdot-114Cd	CD19	SJ25-C1	Invitrogen	Q10179	-	B
115In	CD57	HCD57	Biolegend	322325	M	B
141Pr	CCR6	GO34E3	Fluidigm	3141003	-	A
142Nd	Bcl2	100	Biolegend	658702	P	C
143Nd	CD45RA	HI100	Fluidigm	3143006	-	B
144Nd	Granzyme B	GB11	Abcam			C
145Nd	CD4	RPA-T4	Fluidigm	3145001	-	B
146Nd	TCR $\nu\delta 2$	B6	Biolegend	331402	P	B
146Nd	CD39	A1	Biolegend	328221	M	B
147Sm	CD127	A019D5	Biolegend	351337	M	A
147Sm	CD20	2H7	Fluidigm	3147001	-	B
148Nd	ICOS	C398.4A	Fluidigm	3148019	-	B
149Sm	CXCR5	RF8B2	BD Pharmingen	552032	P	A
150Nd	CD95	DX2	Biolegend	305631	M	B
151Eu	CD103	Ber-ACT8	Fluidigm	3151011	-	B
152Sm	TCR $\gamma\delta$	11F2	Fluidigm	3152008	-	B
153Eu	TCR $V\alpha 7.2$	3C10	Fluidigm	3153024	-	B
154Sm	Anti-APC	APC003	Biolegend	408005	M	A
154Sm	Anti-PE	PE001	Biolegend	408105	M	C
155Gd	PD-1	EH12.2H7	Fluidigm	3155009	-	B
156Gd	CXCR3	G025H7	Fluidigm	3156004	-	A
158Gd	CD27	L128	Fluidigm	3158010	-	B
159Tb	FoxP3	259DC7	Fluidigm	3159028	-	C
160Gd	CTLA-4	14D3	eBioscience	14-1529-82	P	C
161Dy	Anti-PE	PE001	Biolegend	408105	M	A
161Dy	Anti-APC	APC003	Biolegend	408005	M	C
162Dy	Integrin- $\beta 7$	FIB504	Fluidigm	3162026	-	B
163Dy	CD49a	Ts27	Fluidigm	3163015	-	B
164Dy	Ki-67	Ki-67	Biolegend	350523	M	C
165Ho	CD45RO	UCHL1	Fluidigm	3165011	-	A
166Er	CD56	NCAM16.2	BD Pharmingen	559043	P	A
166Er	CD56	HCD56	Biolegend	318345	M	A
167Er	CCR7	150503	R&D Systems	MAB197	P	A
168Er	CD161	HP-3G10	Biolegend	339919	M	B
169Tm	CD38	HIT2	Biolegend	303502	P	B
170Er	CD3	UCHT1	Biolegend	300443	M	B
171Yb	CD49d	9F10	Biolegend	304319	M	B

172Yb	CD25	M-A251	Biolegend	356102	P	B
173Yb	CCR10	6588-5	Biolegend	341502	P	A
173Yb	HLA-DR	L243	Fluidigm	3173005	-	B
174Yb	HLA-DR	L243	Biolegend	307651	M	B
174Yb	CD127	A019D5	Biolegend	351337	M	A
175Lu	CCR4	205410	R&D Systems	MAB1567	P	A
176Yb	CLA	HECA-452	Fluidigm	3176018	-	B
198Pt	CD8a	RPA-T8	Biolegend	301053	M	B
209Bi	CD16	3G8	Fluidigm	3209002	-	B
APC	CCR10	1B5	BD Pharmingen	564771	-	A
PE	LAG-3	T47-530	BD Pharmingen	565617	-	B
APC	Microbial Tetramers	NA	NA	NA	-	NA
PE	Tumour tetramers	NA	NA	NA	-	NA
Purity: P = purified, M = MaxPar ready, L = LEAF purified. Antibody cocktail: A = pre-barcoding, B = post-barcoding, C = intracellular. Cocktails A and B were diluted in CSB, cocktail C in Nuclear Antigen Staining Perm.						

The antibody panel was designed to try and optimise signal and minimise background for each target taking into account the abundance of the target, detection efficiency for the various metal isotopes and signal overlap into other detector channels, and likelihood of co-expression of targets in overlapping channels. Antibodies not sourced already metal-conjugated from Fluidigm were conjugated to the metals indicated according to the Fluidigm Maxpar Antibody Labelling Kit Protocol V6 and diluted to 0.5mg/ml using Candor antibody stabilizer supplemented with 0.05% sodium azide. Conjugated antibodies were tested at different titrations by staining healthy donor PBMCs to find the minimum concentration required to provide good discrimination between positive and negative populations whilst minimising the amount of spillover into other detector channels.

For experiments where > 5 different samples were being stained simultaneously, barcoding of individual samples using a unique combination of three palladium isotopes was used. This enabled samples to be pooled and stained together with the same antibody cocktail to try and ensure uniformity of staining and reduce reagent usage. As this process required a light fixation of the cells, antibodies were also tested for their sensitivity to the buffers used

for barcoding fixation. Based on this, together with the cellular location of the target being stained, antibodies were combined in different antibody cocktails (pre-barcoding, post-barcoding and intracellular) which were added at different steps of the staining process. Antibody cocktails for staining were freshly assembled on the day of staining according to the volumes determined during antibody titration and filtered before use using a 0.1µm centrifugal unit to remove any antibody aggregates.

2.7.2 Sample preparation

Frozen PBMCs were thawed in a water bath at 37°C and washed twice with warm R10-FBS supplemented with Benzonase nuclease ≥ 25 U/ml to prevent cell clumping, followed by a further wash with R10-FBS without Benzonase. Cells were resuspended in R10-FBS without Benzonase, counted with 0.4% Trypan Blue and rested for one hour in an incubator at 37°C. Cells were then washed with CSB and resuspended at a concentration of up to 3×10^6 total cells per 50µl of CSB for staining.

2.7.2.1 Staining

Staining was typically performed using a modified and miniaturised version of the standard Fluidigm staining protocols as described below. All staining steps were performed at room temperature. 50µl of cells were stained with an equal volume of pre-barcoding antibody cocktail and incubated for 30 minutes before the addition of cisplatin (freshly diluted in PBS) as a viability marker at a final concentration of 5µM for 5 minutes. Cells were then washed by addition of 200µl cell staining buffer, centrifugation at $300 \times g$ for 5 minutes and discarding of the supernatant, repeated three times. 200µl of Fix I buffer (diluted to 1X with PBS) was then added and incubated for 10 minutes before 3 washes by addition of 200µl of Barcode Perm Buffer (diluted to 1X with PBS). Centrifugation for these and all subsequent wash steps following this initial fixation were performed at $800 \times g$. Cells were then resuspended in 200µl of Barcode Perm Buffer and 50µl of Cell-ID barcode consisting of a unique combination of 3 palladium isotopes added and incubated for 30 minutes. Cells

were then washed three times with CSB before pooling individual samples together and counting using Trypan Blue to determine the effective number of samples n (total number of pooled cells divided by 3) for subsequent staining steps. Pooled cells were resuspended in a volume of $50\mu\text{l} \times n$ of CSB and an equal volume of post-barcoding antibody cocktail added for 30 minutes. Following 2 washes with CSB, $1\text{ml} \times n$ of Nuclear Antigen Staining Buffer working solution was added for 30 minutes before 2 washes with Nuclear Antigen Staining Perm. Cells were then resuspended in a volume of $50\mu\text{l} \times n$ of Nuclear Antigen Staining Perm and an equal volume of intracellular antibody cocktail added and incubated for 30 minutes. Two washes with CSB were then performed before addition of 10ml of freshly prepared 1.6% formaldehyde (diluted in PBS) for 10 minutes. Cells were then centrifuged, the supernatant discarded and resuspended in $1\text{ml} \times n$ of intercalator solution containing Iridium diluted to a concentration of 125nM in Fix and Perm Buffer and kept at 4°C overnight. For experiments where barcoding was not used, antibodies used in the pre- and post-barcoding cocktails were combined together into a single surface antibody cocktail which was added to cells before cisplatin staining as described above. After the CSB washes the subsequent barcoding steps were then omitted, instead proceeding directly to the addition of nuclear antigen staining buffer working solution.

In experiments where tetramer staining was included in the CyTOF panel for HLA-A*02:01 positive patients, tetramers labelled with either APC or PE fluorophores were synthesised as described in Section 2.6.2. Following thawing and resting, $50\mu\text{l}$ of cell suspension was stained for 30 minutes at room temperature with an equal volume of tetramer cocktail consisting of a reduced pool of tetramers from the peptides in Table 2-3 and Table 2-4 (peptides used indicated by *). Cells were then washed once with CSB and before following the standard staining protocol as described above with the exception of the addition of metal conjugated anti-PE and anti-APC antibodies during the first incubation as part of the pre-barcoding antibody cocktail.

2.7.2.1 Preparing cells for acquisition

Following overnight incubation with intercalator, cells were washed twice with CSB before counting and dividing into aliquots of no more than 2×10^6 cells which were kept pelleted at 4°C until acquisition. Immediately prior to acquisition, individual aliquots of cells were washed twice with Maxpar water and resuspended at a concentration of 1×10^6 cells/ml. A volume of 0.1X EQ beads was added and the cell/bead suspension filtered into cell strainer cap tubes.

2.7.3 Acquisition

All samples were acquired on a single Helios mass cytometer (Fluidigm). Prior to running samples a full machine tuning protocol and EQ bead sensitivity test was performed. This was repeated after ~4 hours of acquisition time to correct for any drift in detector sensitivity. Samples were acquired at a flow rate of 30 μ l/min and an event rate of up to 750 events/second. Where required, samples were further diluted to try and ensure this event rate was not exceeded to avoid excessive doublets. Between samples, de-ionized water was run for at least 2 minutes to remove residual sample, minimise potential cross-contamination and prevent clogging.

2.7.4 Data pre-processing and analysis

Due to computational challenges posed by the size of the dataset, pre-processing and clustering analysis was performed with assistance from Christina Bligaard Pederson and Lars Ronn Olsen (Technical University of Denmark). Event data was processed to .FCS files with randomisation of non-zero ion count values by uniform negative distribution using CyTOF software version 6.7.

2.7.4.1 Concatenation and normalisation

Files from individual aliquots/acquisitions from each sample were concatenated using CATALYST¹⁵² and then normalised using EQ beads to correct for instrument performance over time using the MATLAB implemented normalisation algorithm¹⁵³ in CATALYST. This

approach identifies all beads in all files (across runs and days) and then calculates a global median to use in normalisation, therefore making files from different days comparable. Examples of before and after normalisation plots for individual samples across a single run and across different samples run on different days are shown in Figure 2-1 and Figure 2-2 respectively.

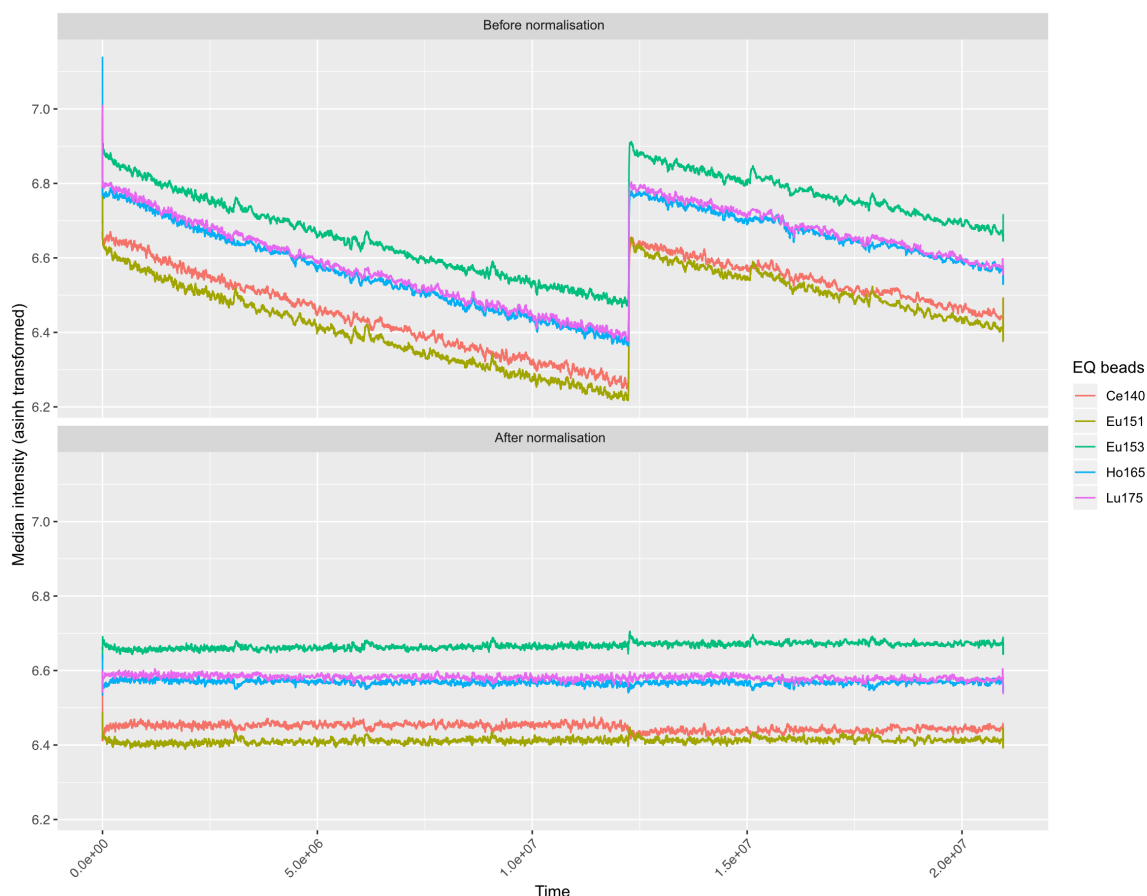


Figure 2-1. Median EQ bead intensity over time for a single concatenated sample before (top) and after (bottom) normalisation. EQ beads consist of 5 different metal isotopes, indicated by different colours. A gradual reduction in median intensity of the bead signal recorded is observed during the course of the acquisition and corrected for by normalisation. The increase in signal intensity mid-way through the acquisition in the before-normalisation data is due to re-tuning of the machine, typically performed after 4 hours of acquisition.

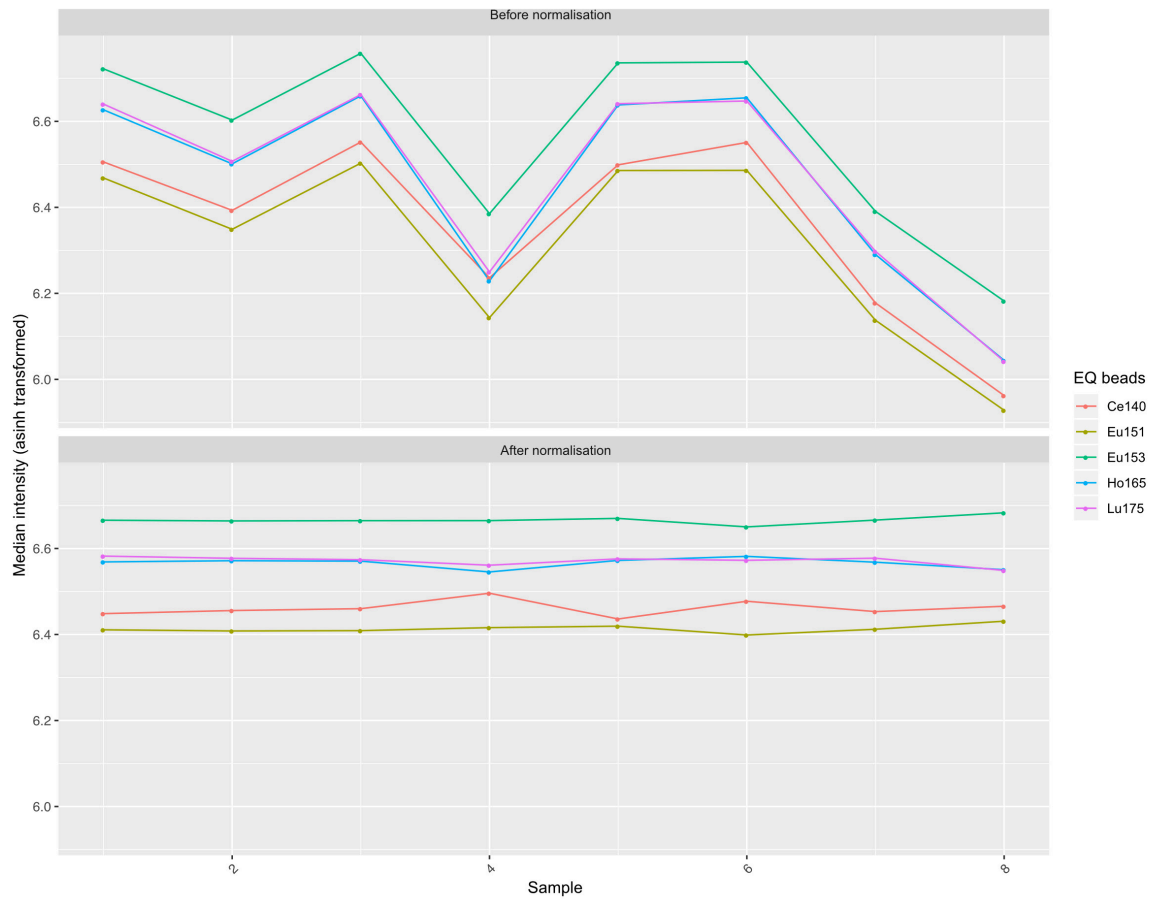


Figure 2-2. Median EQ bead intensity for different samples acquired on different days before (top) and after normalisation (bottom) across samples.

2.7.4.2 Pre-gating

Pre-gating of the data to obtain only live, intact, single cells was performed using the biaxial gating strategies shown in Figure 2-3.

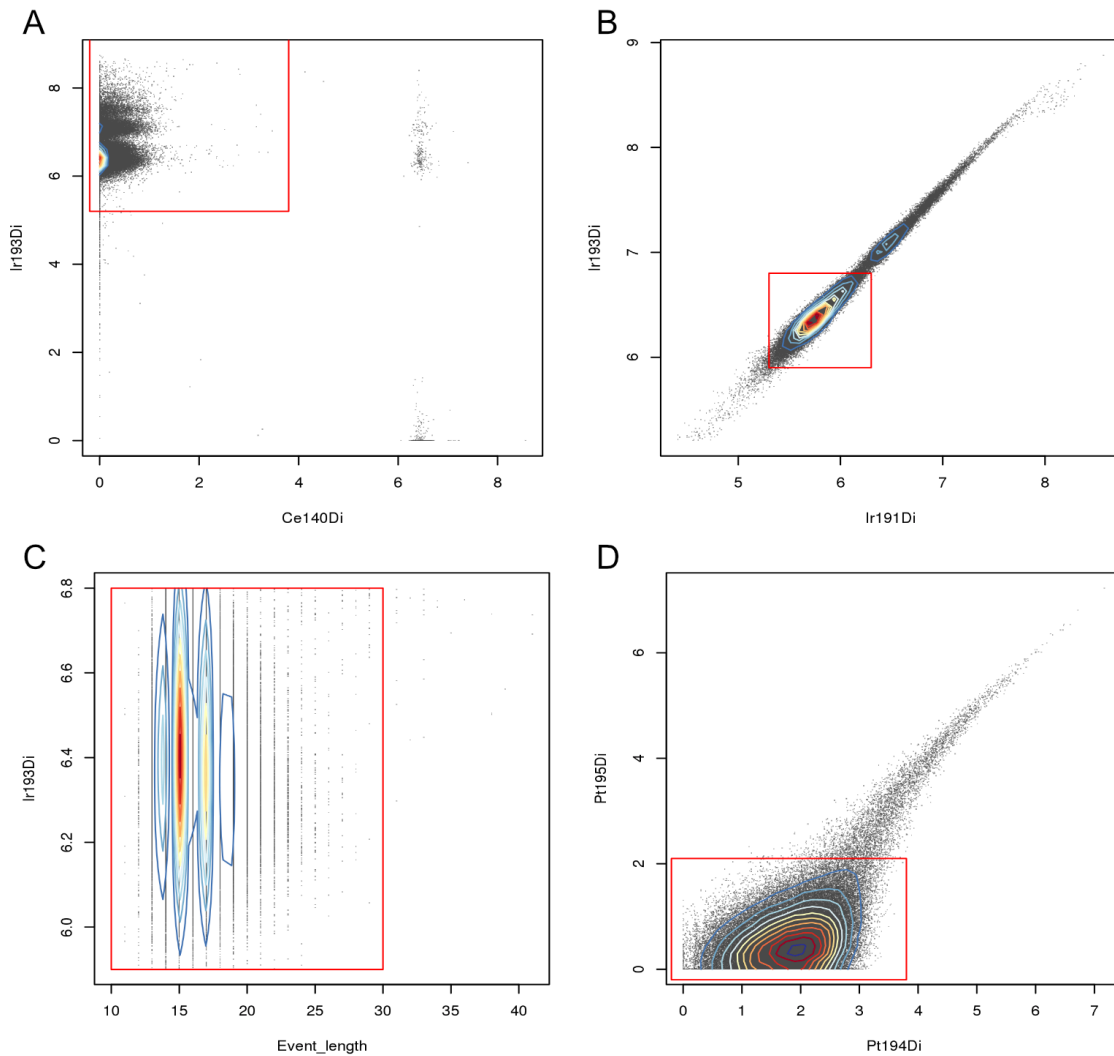


Figure 2-3. Example pre-gating plots. A) Cells gate – DNA channel (Ir193) vs bead-only isotope (Ce140) used to remove beads (Ce140 positive) and debris (Ir193 and Ce140 negative). B) Intact singlets gate – DNA channel Ir193 vs DNA channel Ir191 used to exclude debris (DNA low) and doublets (DNA high). C) Secondary singlets gate – DNA channel Ir193 vs Event length to exclude any residual doublets (event length >30). D) Live cells gate – Pt195 vs Pt194 viability markers (high in dead/dying cells due to disrupted cellular membranes). Typical recovery of live, intact, single cells using this method was 70-75%.

2.7.4.3 Compensation

Whilst spillover between different channels is less problematic with mass cytometry than flow cytometry, this can still lead to unwanted noise so compensation was performed using the compensation tool in CATALYST¹⁵⁴. A compensation matrix was determined by calculating the spillover from each isotope into different channels from a sample of single-

stained OneComp eBeads labelled with the various antibodies used in the staining panel and the data was then corrected according to this.

2.7.4.4 Debarcoding

Where cells were barcoded and pooled for staining and acquisition, debarcoding was performed to separate the data back into individual samples on the basis of the combinations of palladium isotopes used for each barcode. This was again performed using CATALYST but with manual adjustment of barcode separation cut-offs to ensure the differences in signal between the various palladium channels were sufficient to be confident of correct sample assignment (see Figure 2-4 for an example event plot).

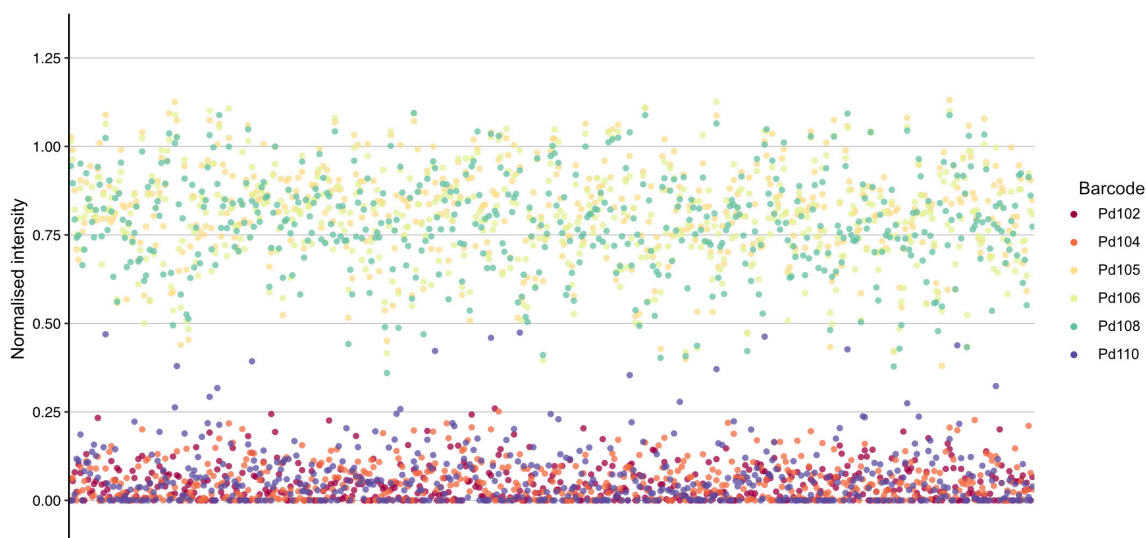


Figure 2-4. Example event plot of discrimination between palladium isotopes for a single barcode. Each palladium isotope is indicated by a different colour and each cell is labelled with a barcode containing a unique combination of three of the six isotopes. Cells belonging to different barcodes can be identified by separation of the signal intensity for the positive and negative isotopes of that barcode.

2.7.4.5 Secondary clean-up and manual gating

A secondary clean-up was performed on individual de-barcoded files based on Gaussian discrimination parameters of the .fcs files (width, residual, centre and offset) to remove any residual low quality data points resulting from ion fusion events or debris. Manual gating

was then performed based on bivariate plots to obtain major populations of interest for subsequent clustering analyses.

2.7.4.6 Data analysis/clustering

Analysis and clustering of CyTOF data was conducted broadly in line with the workflow described by Nowicka *et al.*¹⁵⁵. For clustering, FlowSOM¹⁵⁶, a type of artificial neural network, was chosen on the basis of its performance, speed and ability to operate on large datasets. Briefly, this works by assigning individual cells based on the similarities of the markers expressed to nodes on a self-organising map (SOM) consisting of 100 (10x10) grid points. Hierarchical consensus metaclustering is then performed using ConsensusClusterPlus¹⁵⁷ with similar nodes grouped together into varying numbers of clusters (we typically used between 10 and 40 metaclusters to avoid the risk of under-clustering and missing rare populations). The number of metaclusters which best described the relevant populations in the data was determined by interpretation of delta area plots (Figure 2-5), clustering hierarchy plots and based on domain knowledge of biologically meaningful populations. Manual merging of metaclusters was then performed where required.

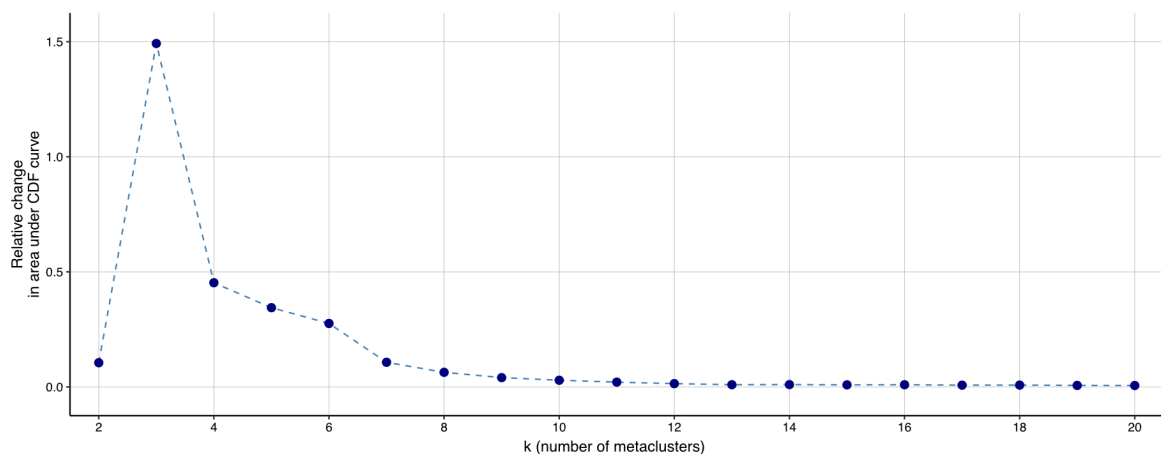


Figure 2-5. Example delta area plot for number of metaclusters. Relative change in area under cumulative distribution function (CDF) curve indicates the amount of extra cluster stability gained when clustering into k metaclusters compared to $k-1$ metaclusters. The likely number of ‘natural’ clusters in the data is typically found where the curve plateaus and there is no longer a considerable increase in stability with increasing numbers of metaclusters.

All data were transformed using the hyperbolic arcsine (arcsinh) function with a co-factor of 5 to accommodate negative values and allow data to be displayed in a log-like manner. Unscaled data was used to generate the hierarchical structure of the metaclusters and for visualisation in Uniform Manifold Approximation and Projection (UMAP)¹⁵⁸, whilst channel scales were normalised between 0 and 1 for visualisation in heatmaps to account for the difference in expression level between markers. For visualisation of clusters and corresponding marker expression, UMAPs were used with data downsampled to 5000 cells per sample to provide acceptable computational demand/run-times. For clustering analyses, channels that had already been used to gate the population on which the clustering was performed were typically excluded. Channels with little range in signal across the population of interest were not included in the clustering. For reproducibility the random seed was defined for the starting point of each analysis. Statistical testing for differential abundance of clusters over time and associations with outcome was performed using negative-binomial generalised linear models through the Diffcyt package¹⁵⁹ implementation of edge-R¹⁶⁰.

2.8 Fluorescence-activated single cell sorting and cloning

2.8.1 Sample preparation and staining

Frozen PBMCs were thawed in a water bath at 37°C and washed twice with warm R10-FBS supplemented with Benzonase nuclease ≥ 25 U/ml to prevent cell clumping, followed by a further wash with R10-FBS without Benzonase. Cells were resuspended in R10-FBS without Benzonase, counted with 0.4% Trypan Blue and rested for one hour in an incubator at 37°C. After resting, up to 9×10^6 cells were stained in a 96-well round-bottom plate. If applicable, tetramer staining was performed as detailed in Section 2.6.3. Cells were then washed once with PBS before incubation with $20\mu\text{l}/3 \times 10^6$ cells of Aqua Live/Dead stain (diluted 1:200 in PBS) for 10 minutes at room temperature. Following this cells were washed once with FBS buffer and incubated with antibody cocktail (made up to a final volume of

20µl/3 x 10⁶ cells stained) for 30 minutes at 4°C. Cells were then washed twice with FBS buffer and resuspended in 500µl FBS buffer and put through a 35µm nylon mesh cell strainer and kept on ice whilst proceeding directly to cell sorting.

2.8.2 FACS sorting

Samples were acquired using a FACSAria III or FACSAria Fusion cell sorter (BD Biosciences) with compensation calculated and applied from single stained control beads. Live, CD8⁺ single cells were sorted based on either tetramer positivity or activation marker expression. For tetramer staining, gates were set using PBMCs from a healthy donor with and without T cell clones spiked in of known specificity for the tetramers being tested. Single cells were sorted with indexing into 96-well round-bottom plates containing 125,000 feeder cells which consisted of a mixture of irradiated PBMCs isolated from two healthy donors resuspended in R5HS-IL2 media.

2.8.3 Single cell cloning

After sorting, PHA was added to each well to give a final concentration of 1µg/ml in a final volume of 150µl and plates were placed in an incubator at 37°C and 5% CO₂. A further 100µl of R5HS-IL2 media without PHA was added to each well after 5 days. After between 10 and 21 days, depending on the rate of growth, single cell clones which were found to have expanded on visual inspection were transferred to 48-well flat-bottom plates. Once confluent in 48-well plates, typically between 3-7 days later, clones were further transferred to 24-well flat-bottom plates. Downstream analyses were typically performed on expanded clones around 6 weeks after sorting. In some cases where prolonged maintenance of clones in culture was desirable, re-stimulation was performed with addition of further feeder cells and PHA after 6-8 weeks. In this case, any downstream assays were performed at least 2 weeks after re-stimulation.

2.9 Flow cytometry

2.9.1 Antibodies used for flow cytometry

Antibodies used in flow cytometry staining are detailed in Table 2-9.

Table 2-9. Antibodies used for flow cytometry

Target	Fluorophore (abbreviation)	Clone	Supplier	Catalogue Number
CCR10	APC	1B5	BD Biosciences	564771
CCR4	BV605	L291H4	Biolegend	359417
CCR7	PE/Cy7	G043H7	Biolegend	353225
CD127	BB515	HIL-7R-M21	BD Biosciences	564423
CD14	BV510	M5E2	Biolegend	301841
CD19	BV510	H1B19	Biolegend	302241
CD3	BV650	OKT3	Biolegend	317323
CD3	PE/Cy7	HIT3a	Biolegend	300316
CD38	BV786	HIT2	BD Biosciences	563964
CD39	BV711	A1	Biolegend	328227
CD4	Alexa Fluor 700 (AF700)	OKT4	Biolegend	317425
CD45RA	BV711	HI100	Biolegend	304137
CD8	BV605	RPA-T8	Biolegend	301039
CD8	PerCP/Cy5.5	HIT8a	Biolegend	300924
CD8	PerCP/Cy5.5	SK1	Biolegend	344709
CD8	FITC	HIT8a	BD Biosciences	555634
CD8	Alexa Fluor 700 (AF700)	HIT8a	Biolegend	300919
CLA	FITC	HECA-452	Biolegend	321305
CXCR3	Alexa Fluor 647 (AF647)	G025H7	Biolegend	353712
CXCR3	FITC	G025H7	Biolegend	353703
HLA-DR	APC/Cy7	L243	Biolegend	307617
PD-1	PE-eFluor610 (PEe610)	eBioJ105	eBioscience	61-2799-41
Live/Dead Stain	Aqua	NA	Invitrogen Life Technologies	L34965
Anti-PE	NA	PE001	Biolegend	408101
Anti-APC	NA	APC003	Biolegend	408001
IFN- γ	PE/Cy7	4S.B3	Biolegend	502527
IFN- γ	APC-eFluor780 (APCe780)	4S.B3	eBioscience	47-7319-42
TNF- α	PerCP/Cy5.5	MAb11	Biolegend	502925
TNF- α	PE/Cy7	MAb11	Biolegend	502929
CD170a (LAMP-1)	BV605	H4A3	Biolegend	328633
HLA-A2	PE	BB7.2	Biolegend	343305

2.9.2 Combination dye labelling

In order to allowing multiplexing and increase throughout, fluorescent barcoding was used to uniquely label individual T cell clones prior to testing against stimuli of interest. Four different fluorescent dyes were used (Table 2-10) and diluted to different concentrations in PBS (Table 2-11).

Table 2-10. Dyes used for fluorescent barcoding

Dye	Colour	Supplier	Cat No
CellTracker Orange CMDA	Orange	Invitrogen	C34551
Vybrant CFDA SE Cell Tracer	CFDA	Invitrogen	V12883
CellTrace Far Red	Far Red	Invitrogen	C34572
CellTrace Violet	Violet	Invitrogen	C34571

Table 2-11. Dilutions of fluorescent dyes

Violet	Dilution	Orange	Dilution	Far Red	Dilution	CFDA	Dilution
V1	1:2500	O1	1:2500	FR1	1:2500	C1	1:2500
V2	1:12500	O2	1:25000	FR2	1:16666	C2	1:10000
V3	PBS only	O3	PBS only	FR3	PBS only	C3	1:40000
						C0	PBS only

Dyes were then combined in equal volumes (18.75µl) to give 54 unique combinations of dyes, each in a volume of 75µl (Figure 2-6). Dyes mixes were freshly prepared immediately prior to staining and protected from light throughout the staining and subsequent incubations.

V1	V1	V1	V1	V1	V1	V1	V1	V1
O1	O2	O3	O1	O2	O3	O1	O2	O3
FR1	FR1	FR1	FR2	FR2	FR2	FR3	FR3	FR3
C1	C2	C1	C2	C1	C2	C1	C2	C1
V1	V1	V1	V1	V1	V1	V1	V1	V1
O1	O2	O3	O1	O2	O3	O1	O2	O3
FR1	FR1	FR1	FR2	FR2	FR2	FR3	FR3	FR3
C3	C0	C3	C0	C3	C0	C3	C0	C3
V2	V2	V2	V2	V2	V2	V2	V2	V2
O1	O2	O3	O1	O2	O3	O1	O2	O3
FR1	FR1	FR1	FR2	FR2	FR2	FR3	FR3	FR3
C1	C2	C1	C2	C1	C2	C1	C2	C1
V2	V2	V2	V2	V2	V2	V2	V2	V2
O1	O2	O3	O1	O2	O3	O1	O2	O3
FR1	FR1	FR1	FR2	FR2	FR2	FR3	FR3	FR3
C3	C0	C3	C0	C3	C0	C3	C0	C3
V3	V3	V3	V3	V3	V3	V3	V3	V3
O1	O2	O3	O1	O2	O3	O1	O2	O3
FR1	FR1	FR1	FR2	FR2	FR2	FR3	FR3	FR3
C1	C2	C1	C2	C1	C2	C1	C2	C1
V3	V3	V3	V3	V3	V3	V3	V3	V3
O1	O2	O3	O1	O2	O3	O1	O2	O3
FR1	FR1	FR1	FR2	FR2	FR2	FR3	FR3	FR3
C3	C0	C3	C0	C3	C0	C3	C0	C3

Figure 2-6. Fluorescent combinatorial barcoding scheme. All 54 combinations of dyes at different dilutions used are shown. Each well/clone was stained with the combination of 4 dyes indicated within a single square.

T cell clones were harvested from cell culture (approximately 0.5 million cells per clone) and transferred to 96-well u-bottom plates. Up to 54 different clones were placed into a single plate in adjacent wells in a 9x6 well configuration. Cells were washed twice in PBS, resuspended in 75µl of PBS and combined with the dyes, noting which clone was assigned to each dye barcode. After 10 minutes incubation at room temperature 150µl of FBS was added to quench the staining. Cells were then centrifuged and washed three times with R10-FBS before transferring to a fresh 96-well plate and resting for 1 hour at 37°C. After resting, individual clones were pooled and counted and resuspended at the desired concentration for subsequent assays.

2.9.3 Testing antigen specificity of CD8+ T cell clones

In order to determine the antigen specificity of T cell clones, pooled dye labelled clones were incubated with either tumour cell lines or peptides and their resultant cytokine response assayed by flow cytometry.

For cell lines, tumour cells were harvested from culture, counted and resuspended in R10-FBS media at a concentration of 4×10^6 cells/ml. 100µl of each tumour cell suspension was then added to individual wells of a 96-well round-bottom plate in triplicate. MHC Class I blocking antibody (W6/32 IgG2a, Biolegend) or isotype control (IgG2a, Biolegend) were then added to a concentration of 20µg/ml and incubated with the cell lines for 60 minutes at 37°C. 100µl of pooled clone suspension was then added to give a tumour:clone ratio of 2:1 (400,000 tumour cells: 200,000 T cell clones). For peptides, individual peptides or pools of peptides were diluted in R10-FBS and combined with pooled T cell clones in R10-FBS at a final concentration of 10µg/ml. In all experiments a negative control well of T cell clones in R10-FBS media alone and a positive control well of T cell clones combined with cell stimulation cocktail, containing phorbol myristate acetate (PMA) and Ionomycin, were also included.

After 1 hour of incubation at 37°C, protein transport inhibitor cocktail consisting of Brefeldin A and Monensin was added to block the secretion of cytokines and prevent degranulation, resulting in accumulation within the endoplasmic reticulum and Golgi apparatus of the targets of interest for further assessment by intracellular staining. In cases where CD107a was being used as a readout this antibody was added into the stimuli/clone mix from the outset of the incubation. Co-culture of the clones with the stimuli was continued for a further 4 hours at 37°C to a total of 5 hours, previously shown to be an optimal duration of incubation for the measuring IFN-γ production¹⁶¹, before washing and performing viability staining with aqua live/dead stain. Cells were then washed and fixed with intracellular fixation buffer (eBioscience) at 4°C overnight. The following day cells were washed twice with permeabilization buffer (eBioscience) and then stained with 20µl/well of antibody

cocktail prepared in permeabilization buffer for 30 minutes at 4°C. They were then washed with FBS buffer before being acquired directly from the plate using an autosampler on an Attune NxT flow cytometer (Invitrogen) with compensation applied from single-stained beads.

2.9.4 Testing functional avidity of sorted CD8⁺ T cells

Testing of the functional avidity of expanded CD8⁺ T cell clones was performed by incubating pooled, dye labelled clones with reducing concentrations of a pool of TAA peptides over a 5 log Molar range (final peptide concentration range 10 µg/mL to 0.0001 µg/mL). Pooled T cell clones were either cultured directly with peptides or with peptide-pulsed T2 cells. This human cell line is deficient in the TAP peptide transporter involved in antigen processing, meaning that they do not display endogenous peptides and can be used to study antigen recognition in cytotoxic lymphocytes of exogenous peptides presented on class I MHC in a non-competitive manner. Where T2 cells were used, these were incubated with a pool of TAA peptides at the concentrations previously specified for 90 minutes at 37°C, before washing twice in R10-FBS media, and co-culturing with T cell clones at an effector:target ratio of 1:2. T cell clones were co-cultured with peptides or peptide-pulsed T2 cells in the presence of CD107a antibody for a total of 5 hours, with the addition after 1 hour of protein transport inhibitor cocktail. Viability staining and overnight fixation was then performed, followed by permeabilization, intracellular cytokine staining and acquisition the following day as described in Section 2.9.3. Controls including T cell clones stimulated with PMA/Ionomycin and unstimulated T cell clones (either no peptide or non-pulsed T2 cells) were included in each experiment and titrations performed in duplicate or triplicate.

2.10 TCR sequencing

TCR sequencing was performed as previously described¹⁶² with assistance from Margarida Rei and Prathiba Kurupati (Cerundolo Lab, University of Oxford). Briefly, RNA was

extracted from individual clones or sorted bulk cell populations using RNeasy Mini Kit (Qiagen), following the manufacturer's instructions. cDNA was generated by template switch reverse transcription using SMARTScribe Reverse Transcriptase (Clontech), using a template-switch oligo (TSO) with a 6bp unique molecular identifier (TSO-UMI) and primers designed for the constant regions of the T cell receptor constant alpha (TRAC) and beta (TRBC) genes. TCR amplification was achieved by performing two rounds of nested polymerase chain reaction (PCR) using Phusion High-Fidelity PCR Master Mix (New England Biolabs). During the first PCR priming, indexes were included to identify each sample. A last PCR was performed to add the Illumina adaptors. Primer sequences are described in Table 2-12. TCR libraries were sequenced on Illumina Miseq using Miseq Reagent Kit V2 300-cycle (Illumina). FASTQ files were demultiplexed for each clone or cell population. TCR sequences from clones were determined by Margarida Rei or Mike Shugay (Skolkovo Institute of Science and Technology, Russia) using MiXCR¹⁶³. Sequences from cell populations were analysed using MIGEC¹⁶⁴. Post analysis was performed using VDJtools¹⁶⁵.

Table 2-12. Primers used for TCR sequencing

Primer	Usage	Sequence 5'-3'
TraRv0	Reverse transcription	TCA GCT GGA CCA CAG CCG CAG
TrbRv0		CAG TAT CTG GAG TCA TTG A
TSO-UMI		CAG TGG TAT CAA CGC AGA GTA C NNN NNN rGrGrG *
1stPCRFw-index	1st nested PCR	CAC GAC GCT CTT CCG ATC TXX XXX XXX CAG
1stPCRTraRv		TGG TAT CAA CGC AGA GTA C **
1stPCRTrbRv		TAC ACG GCA GGG TCA GGG T
2ndPCRFw	2nd nested PCR	TGC TTC TGA TGG CTC AAA CAC
2ndPCRTraRv		CAC TCT TTC CCT ACA CGA CGC TCT TCC GAT C
2ndPCRTrbRv		TGG AGT TCA GAC GTG TGC TCT TCC GAT CTG GGT CAG GGT TCT GGA TAT
3rdPCRi7	Illumina adaptors PCR	TGG AGT TCA GAC GTG TGC TCT TCC GAT CTA CAC STT KTT CAG GTC CTC
3rdPCRi5		CAA GCA GAA GAC GGC ATA CGA GAT TCG CCT TAG TGA CTG GAG TTC AGA CGT G AAT GAT ACG GCG ACC ACC GAG ATC TAC ACC TCT CTA TAC ACT CTT TCC CTA CAC GAC

* NNN NNN - unique molecular identifier, ** XXX XXX - sample index

2.11 Single-cell RNA-sequencing

A modified version of the SmartSeq2 protocol by¹⁶⁶ was used for single-cell RNA-sequencing (scRNA-seq). Optimisation experiments and library preparation were performed with the assistance of Prathiba Kurupati. Reagents used not listed elsewhere and sequences for primers used are detailed in Table 2-13 and Table 2-14.

Table 2-13. Reagents for single-cell RNA-sequencing

Reagent	Supplier	Catalogue Number
DNAse I solution 1mg/ml	STEMCELL Technologies	07900
EDTA 0.5M	Amresco	E522
EasySep™ Human CD8+ T Cell Enrichment Kit	STEMCELL Technologies	19053
EasyEights Magnet	STEMCELL Technologies	18103
Recombinant RNAse inhibitor 40U/μl	Takara	2313A
Deoxyribonucleotide triphosphate (dNTP) mix 10mM	Thermo Scientific	R0192
External RNA Control Consortium (ERCC) Spike In	Thermo Fisher	4456740
SMARTScribe Reverse Transcriptase	Takara	639538
SeqAmp DNA Polymerase	Takara	638509
RT-PCR Grade Water	Invitrogen	AM9935
Ethanol absolute (Molecular Biology Grade)	Fisher Scientific	10644795
Agencourt AMPure XP beads	Beckman Coulter	A63881
Buffer EB	Qiagen	19086
Qubit dsDNA High Sensitivity Assay Kit	Invitrogen	Q32851
High Sensitivity NGS Fragment Kit	Advanced Analytical	DNF-474-0500
High sensitivity DNA Kit	Agilent	5067-4626
Quant-iT PicoGreen dsDNA Kit	Invitrogen	P11496
Nextera XT DNA Library Prep Kit	Illumina	FC-131-1096
Nextera XT Index Kit v2 Set A	Illumina	FC-131-2001
Nextera XT Index Kit v2 Set D	Illumina	FC-131-2004
NextSeq 500/550 High Output Kit v2.5	Illumina	20024907

Table 2-14. Primers for single-cell RNA-sequencing

Primer	Sequence	Source
Oligo-dT30VN	5'-AAGCAGTGGTATCAACGCAGAGTACT30VN-3'	IDT
ISPCR Oligo	5'BiosgAAGCAGTGGTATCAACGCAGAGT 3'	IDT
5' Bio-LNA-TSO	5'BiosgAAGCAGTGGTATCAACGCAGAGTACATrGrG+G	Qiagen

ISPCR oligonucleotide and template switching oligonucleotides were biotinylated to help overcome problems with concatemer formation seen during optimisation experiments.

2.11.1 Experimental design

Samples for three patients were chosen based on analysis of previous antigen-specific T cell frequency and specificity. As the primary comparison of interest was the effect of treatment over time within each patient, all the samples for each patient were thawed, isolated and stained in parallel and sorted on the same day. Samples from different patients were thawed, stained and sorted on consecutive days.

2.11.2 Isolation of CD8+ T cells

In order to preserve RNA integrity and minimise the time taken for sorting, enrichment for CD8+ T cells was performed by immunomagnetic negative selection. PBMC samples were thawed and rested in R10 media containing 0.1mg/ml DNase I for 15 minutes at room temperature before washing with separation buffer consisting of PBS supplemented with 2% FCS and 1mM EDTA. Cells were resuspended in 150µl of separation buffer in polystyrene FACS tubes and 50µl/ml of EasySep Human CD8+ T cell enrichment cocktail was added. Cells were incubated for 10 minutes at room temperature before addition of 150µl/ml of EasySep magnetic particles and further incubation for 5 minutes. Separation buffer was added to a final volume of 2.5ml and tubes were inserted into an EasyEight magnet for 5 minutes. The enriched cell suspension was then aspirated using a serological pipette and transferred to a 15ml Falcon and centrifuged for 5 mins at 1500rpm to pellet the cells and the supernatant discarded.

2.11.2.1 Tetramer and antibody staining for FACS sorting

CD8+ enriched cells were stained with 4µl of each dual-labelled tetramer synthesised as described in Section 2.6.2. in a final volume of 100µl of separation buffer for 30 minutes at room temperature. Cells were then washed with PBS and stained with 20µl of PBS/antibody cocktail combining live/dead stain, FACS antibodies and anti-tetramer antibodies for 15

minutes on ice. Cells were washed twice with FBS buffer and resuspended in 500µl FBS buffer and kept on ice before proceeding directly to sorting.

2.11.2.2 FACS Sorting

Cells were acquired on a FACSAria III (BD Biosciences) using a 100 micron nozzle. CD8+ T cells isolated from an HLA-A*02:01 negative donor, with and without T cell clones of known specificity for the tetramers spiked-in, were used to determine the level of background staining and set tetramer positive sorting gates. In the case of patient 1 and 2, OR gating of cells that were positive for either specificity 1 or 2 was performed with adjustment to gating just the lower frequency population once the higher frequency population had reached 50% of the planned total cells to be collected for that timepoint. Live tetramer positive CD8+ single cells were sorted into chilled breakable 96-well plates containing 4µl of lysis buffer (0.4% Triton, 2U/µl RNase inhibitor, 2.5µM oligo dT, and 2.5mM dNTP and 1:16,000,000 ERCC spike-in). To prevent RNA degradation, sorting into each 3x8 well segment was restricted to 10 minutes of acquisition time before the segments were sealed and the cells were vortexed, spun down and frozen on dry ice before being stored at -80°C.

To try and minimise batch effects caused by duration of time between processing and sorting of serial samples from different timepoints, the order in which samples were sorted was varied between patients as per Table 2-15.

Table 2-15. Order of sorting for samples from different timepoints by patient

Patient	Timepoint sorted			
	1st	2nd	3rd	4th
1	1	2	3	4
2	4	3	1	2
3	2	4	1	3

A number of wells across plates were also left intentionally blank without a cell sorted into them to allow assessment for cross contamination between wells.

2.11.3 Library preparation and sequencing

Reverse transcription and PCR preamplification was performed in a stepwise manner with cells from different patients processed on consecutive days. To avoid confounding per-patient timepoint analysis by potential sources of technical variation such as processing plate or thermocycler used, cells from the same timepoint were distributed by segment across different processing plates. Samples were thawed on ice and then heated for 3 minutes at 72°C to hybridise the oligo-dT primer to the poly(A) tail of the mRNA molecules before addition of 6µl of first strand synthesis reverse transcriptase reaction buffer consisting of:

Reagent	Volume (µl/reaction)	Final concentration (in 10µl reaction)
5X SmartScribe First-Strand Buffer	2	1X
RNAse inhibitor 40U/µl	0.25	1U/µl
TSO (100µM)	0.1	1µM
Water (RT-PCR grade)	3.15	-
SMARTScribe RT (100U/µl)	0.5	5U/µl
Total	6	

Cells were incubated in a thermocycler as follows:

Temperature	Duration	Cycles (n)
42°C	90 mins	1
50°C	2 minutes	9
42°C	2 minutes	
70°C	15 minutes	1
4°C	Hold	

Following the first-strand reaction, PCR preamplification of cDNA was performed by adding 15µl of PCR mix was added to each well consisting of:

Reagent	Volume (µl/reaction)	Final concentration (in 25µl reaction)
SeqAmp PCR Buffer (2X)	12.5	1X
ISPCR primers (10µM)	0.125	0.05µM
Water	1.875	-
SeqAmp DNA Polymerase	0.5	-
Total	15	

PCR was performed as follows and samples were then frozen at -20°C.

Temperature	Duration	Cycles (n)
98°C	3 mins	1
98°C	20 seconds	24
67°C	15 seconds	
72°C	6 minutes	
72°C	5 minutes	1
4°C	Hold until frozen	

Thawed PCR products were bead cleaned by addition of a ratio of 0.6X AMPure beads (15µl) to PCR product (25µl), mixing and then incubating for 8 minutes at room temperature before placing on a magnet for 5 minutes. After removing the supernatant, the beads were washed twice with 200µl of 80% ethanol, dried and then removed from the magnet and re-suspended in 17.5µl of buffer EB. After 2 minutes the samples were placed back on a magnet and 15µl of supernatant aspirated and transferred to a new plate and frozen at -20°C. Prior to freezing, a 5µl aliquot of cleaned cDNA was transferred to a separate plate for quality control assessments. These included quantification of 48 randomly selected cells per patient taken from across different processing plates for molecular size and weight by Fragment Analyzer (Agilent) and quantification of cDNA for all samples using a fluorescent nucleic acid stain PicoGreen (Promega). Example fragment analyser profiles are shown in Figure 2-7.

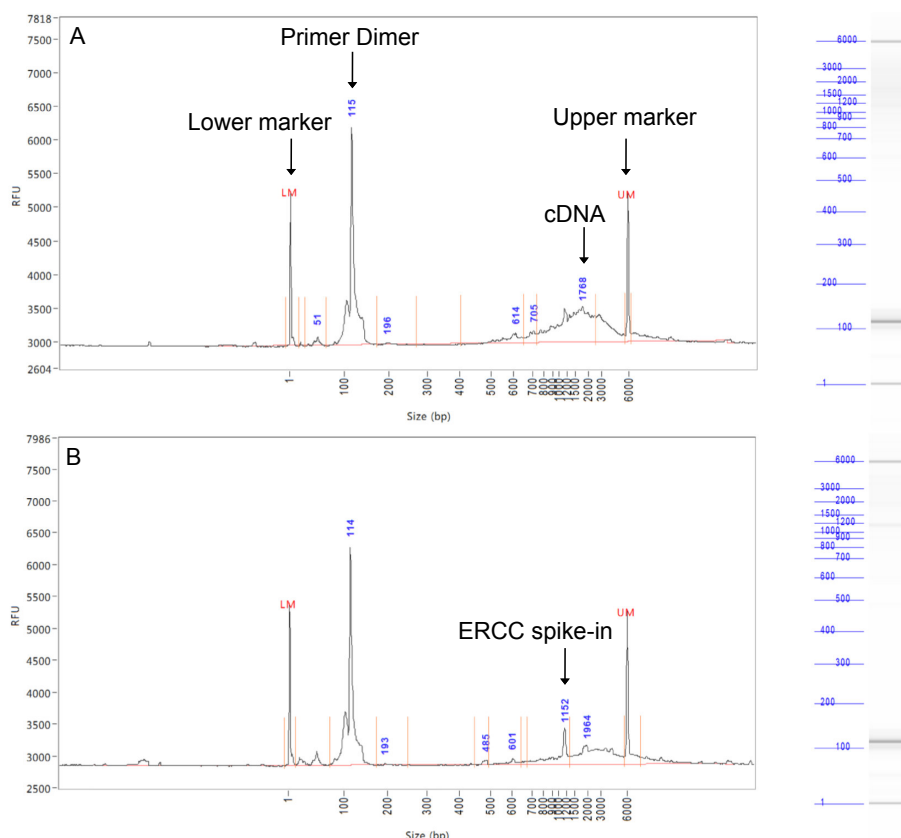


Figure 2-7. Representative Fragment Analyser electropherogram and digital gel images. A) Single cell profile with lower (LM) and upper (UM) ladder markers, cDNA with average size 1500-2000 base pairs (bp) and primer dimer. B) Blank well profile with ERCC spike-in cDNA indicated.

Tagmentation to construct sequencing libraries from the amplified cDNA was performed using the Illumina Nextera XT DNA sample preparation kit. For single cell libraries, miniaturisation of the standard Illumina protocol down to a final reaction volume of 4µl was performed¹⁶⁷ using a Mosquito HTS nanolitre liquid handler (TTP LabTech Ltd) in 384-well plate format (one plate per patient). 800nl Tagment DNA (TD) buffer, 400nl Amplicon Tagment Mix (ATM) and 400nl cDNA were combined and tagmentation performed for 6 minutes at 55°C. 400nl of Neutralise Tagment Buffer (NT) buffer was then added and incubated at room temperature for 5 minutes to terminate the tagmentation reaction. 1.2µl of Nextera PCR Master Mix (NPM) and 400nl each of Index 1 adapters (S5XX) and Index 2 adapters (N7XX) were added and 12 cycles of PCR performed as follows:

Temperature	Duration	Cycles (n)
72°C	3 mins	1
95°C	30 seconds	1
95°C	10 seconds	12
55°C	30 seconds	
72°C	30 seconds	
72°C	5 minutes	1
4°C	Hold until frozen	

1.2µl of each single cell library was pooled and then pooled libraries for each patient cleaned by bead purification using a 0.6X ratio of beads as previously described with samples eluted in 125µl EB buffer.

Following cleaning, the size distribution of pooled single cell libraries were assessed by Bioanalyzer using the Agilent High Sensitivity DNA Kit and the concentration quantified using Qubit dsDNA high sensitivity assay kit using a Qubit 3 fluorometer (Invitrogen). As expected, libraries displayed a broad distribution from around 400bp to 1500bp with an average size of ~800-1000bp in length. The molarity of each library was then calculated using the concentration obtained by Qubit and average size by Bioanalyzer and libraries for each patient were pooled proportionally by volume and diluted to produce a final library of 2nM concentration confirmed by Qubit. Libraries for each patient were denatured and diluted to 1.8pM and 2 x 75bp paired-end sequencing was performed on separate days with the same NextSeq500 machine using NextSeq v2.5 high output 150bp reagent kit.

2.11.4 Data processing and analysis

Single-cell RNA-seq data pre-processing and analysis was performed with the help of Hashem Koohy (Human Immunology Unit, University of Oxford) and for TCR analysis, Mike Shugay.

2.11.4.1 Read mapping and expression quantification

Raw reads from each sequencing run were deconvoluted within Illumina BaseSpace into paired-end fastq files for each individual library based on the Nextera indexes used. Read

mapping and expression quantification were performed by Hashem Koohy. Reads were trimmed using Trim Galore with initial quality assessment performed on the resulting output files using FastQC. Trimmed reads were then mapped to the hg38 human genome using STAR aligner. Mapped reads were then assigned and counted to each gene against the human genome annotation hg38 with ERCC RNA spike-in features using featureCounts (v1.5.0-p2) from the subread package.

2.11.4.2 Quality control

In order to allow us to be confident in the data resulting from analysis of individual single cells, a number of measures were built into the experimental design to enable us to check for evidence of cross-contamination between wells during the initial sorting and library preparation steps and to control for variation in read depth between cells in different plates and sequencing runs. This included the inclusion of a mix of control external polyadenylated RNA transcripts (ERCC spike-in) to all wells, including a number of 'blank wells' into which no cells were sorted. As such it was expected that in the absence of any cross-contamination any resultant reads after sequencing in these blank wells should map only to ERCC spike-in. Cell level quality control (QC) was performed using Scrn¹⁶⁸ and Seruat v3^{169,170} based on the number of features (genes) and percentage of reads mapping to ERCC spike-in, with cells with few genes expressed or a high percentage of ERCC indicating likely low quality cells which were removed from further analysis. Filters set at a minimum of 200 features per cell and maximum of 20% of reads from ERCC spike-in were set as cut-offs based upon inspection of the data across the three sequencing runs. Gene level QC was performed to remove any genes which were expressed in less than 20 cells across the whole experiment.

2.11.4.3 Normalisation, highly variable gene identification and scaling

Data were normalised by dividing the feature counts for each cell by the total counts for that cell, multiplying by a scaling factor of 10,000 and then natural-log transformed. To focus our analysis on the genes contributing most to cellular heterogeneity, the top 1500 highly

variable genes per patient were identified using Seurat v3 method *vst*, which calculates feature variance from a local polynomial regression line fitted to the data based on the relationship between $\log(\text{variance})$ and $\log(\text{mean})$. Data were then scaled and centred to give equal weight to individual genes in downstream analyses, to avoid this being dominated by highly-expressed genes.

2.11.4.4 Dimensionality reduction, clustering and visualisation

Principal component analysis was performed on the scaled data using the highly variable genes identified as described above. To reduce noise inherent in single cell data, the first 5 principal components were selected for further analysis as these corresponded to the greatest proportion of the variance in the dataset. Clustering was performed using a *k*-nearest neighbour graph-based clustering approach in Seurat v3 which partitions cells based on similar feature expression patterns and then applies Louvain algorithms to group cells together. A resolution of 0.8 was used to ensure sufficient granularity in the clustering given the relatively heterogeneous nature of the cells being studied (all antigen-specific CD8⁺ T cells). Non-linear dimensionality reduction by UMAP was used to visualise the clustering in low-dimensional space, using the same 5 principal components.

2.11.4.5 Differential expression analysis

Testing of differential expression of genes between clusters or groups of cells at different timepoints was performed using non-parametric Wilcoxon rank-sum test within Seurat with *p*-values and log fold change thresholds used specified in the relevant analyses. Gene ontology annotation was performed using the package *clusterProfiler*¹⁷¹.

2.11.4.6 TCR calling

TCR calling was performed by Mike Shugay using MiXCR¹⁶³ with modifications for scRNA-seq. Briefly, transcripts for TCR alpha (TRA) and beta (TRB) genes were pre-filtered based on a minimum of 5 reads and >30% frequency for a given cell and aggregated in the following order to identify T cell clones:

TRA-TRA-TRB
 ← TRA-TRB
 ← TRA-TRA-TRB-TRB
 ← TRA-TRB-TRB
 ← TRB-TRB
 ← TRA-TRA
 ← TRB
 ← TRA

The resultant list of T cell clones was then combined with the expression data for cells which had passed QC based on gene expression for downstream analysis.

2.12 DNA/RNA extraction from PBMCs and tumours

DNA extraction from PBMCs and tumour cell lines or single cell suspension was performed using Qiagen DNAeasy blood/tissue kit (Cat 69504) according to the manufacturer's instructions, with the resultant DNA eluted in AE buffer and quantified by Nanodrop. RNA extraction was performed using the Qiagen RNeasy mini kit (Cat 74104) according to the manufacturer's instructions, with RNA eluted in RNase free water and quantified by Nanodrop.

In the case of the tumour biopsy from patient NPB_03284, snap frozen tissue stored at -80°C was divided into two. Half was minced into small pieces and DNA extracted as per the DNAeasy (Qiagen) blood and tissue protocol. The other half was added immediately to chilled RNAlater-ICE (Qiagen) and transferred to -20°C for 7 days. This was subsequently cut into small fragments, added to RLT buffer containing beta-mercaptoethanol and dissociated and homogenised using a Qiagen TissueRuptor probe. The supernatant was then aspirated and RNA extracted using the Qiagen RNeasy kit.

2.13 HLA typing

2.13.1 MHC class I haplotyping

DNA for haplotyping was obtained from PBMCs or tumour cell lines as described in Section 2.12. MHC class I HLA typing was performed by the MRC WIMM Sequencing Facility, University of Oxford, using single specific primer polymerase chain reaction (SSP-PCR) for high-resolution typing of alleles at HLA-A, HLA-B and HLA-C loci.

2.13.2 HLA-A*02 testing

Where not otherwise known, the HLA-A*02 status of patient samples was tested to determine suitability for tetramer staining. This was performed by staining approximately 50,000 PBMCs with a PE conjugated anti-HLA-A*02 antibody and subsequent assessment by flow cytometry in comparison with a known HLA-A*02 positive and negative control. An example staining is shown in Figure 2-8.

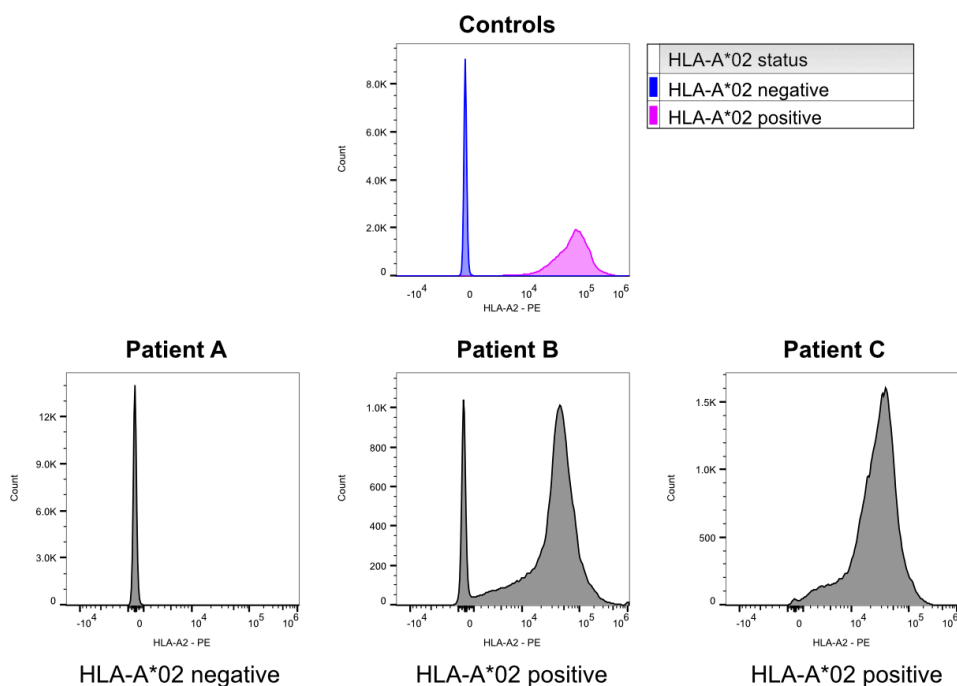


Figure 2-8. HLA-A*02 staining by flow cytometry. Top panel shows HLA-A*02 staining for known HLA-A*02 positive and negative patient confirmed by haplotyping. Bottom panels show example staining for 3 patients displaying positive and negative HLA-A*02 staining.

2.14 Tumour cell lines

2.14.1 Allogeneic cell lines

A panel of established melanoma cell lines was assembled from frozen stocks held by the Cerundolo lab, University of Oxford. A glioblastoma (GBM) cell line generated by Rui Ma (Cerundolo lab, University of Oxford) was also included in the panel as a non-melanoma control. MHC class I HLA-typing of these tumour lines was performed as detailed in Section 2.13.1. Expression of HLA-A*02 was confirmed by FACS staining using the method described in Section 2.13.2.

Known tumour-associated antigen expression as documented in the literature, where available, for these tumour cell lines is shown in Table 2-16.

Table 2-16. Tumour-associated antigen expression of allogeneic cell lines

Cell line	Antigen							
	gp100	Tyrosinase	MART-1	MAGE-A3	NY-ESO-1	LAGE-1	MAGE-A1	Trp1
Sk-MEL23	+	+	+		-			
Sk-MEL29		+	+	+			+	
MZ2	-	-	-	+	-			
Na8	-	-	-	-	-			
D10	+	+	+	+	-			
ME275	+	+	+	+	+	+		
Sk-MEL28	+	+	+		+		-	+
Mel526	+	+	+	+				

Expression (+) or absence of expression (-) of known tumour-associated antigens on tumour cell lines as documented in the literature¹⁷²⁻¹⁸³ where available.

All cells were cultured as adherent monolayers in R10-FBS media in cell culture-treated polystyrene flasks. When approaching confluency, cells were detached and either split or harvested for downstream experiments using Accutase cell detachment solution (STEMCELL technologies Cat:07922).

2.14.2 Autologous cell lines

For one individual (P20c) an autologous cell line was generated from a single cell suspension previously prepared using the Milteyni human tumour dissociation kit (Cat 130-

095-929) by Jane Maraka (Cerundolo Lab, University of Oxford). Cells were thawed and counted then plated at 2×10^6 cells in 2ml of R10-FBS media in a 24-well plate (10 wells of 2×10^6 cells). Media was replaced every 3-5 days and once the adherent cells became confluent, cells were detached using Accutase cell detachment solution and transferred to a 12-well plate initially and subsequently a T25 flask. Once expanded to T75 flasks, individual aliquots of each cell line were frozen and the remainder pooled in equal number to provide a single culture used for subsequent experiments.

2.14.3 Characterising tumour-associated antigen expression on tumour cell lines

TAA expression on melanoma cell lines was tested by antibody staining and flow cytometry. Tumour cells were harvested from culture, fixed, permeabilised and then stained with primary antibody for 30 minutes followed by washing and staining with secondary antibody for 15 minutes. Antibodies used are listed in Table 2-17.

Table 2-17. Antibodies for characterisation of tumour antigen expression

Target	Supplier	Conjugate	Catalogue Number	Note
Melan A	Santa Cruz	Mouse IgG1	Sc-20032	Primary
HMB45 (anti-gp100)	Santa Cruz	Mouse IgG1	Sc-59305	Primary
Donkey anti-mouse IgG	Life Technologies	AF647	A31571	Secondary
Anti-melanoma MSCP	Mileyni Biotec	PE	130-099-413	Surface

2.15 Exome and RNA-sequencing

DNA and RNA for whole exome sequencing (WES) and RNA-sequencing (RNA-seq) was obtained as described in Section 2.12. Library preparation, quality control and sequencing was performed by Novogene (HK) Company Limited. Illumina 150bp paired-end sequencing was performed to a depth of 50X for germline samples, 100X for tumour samples, with the exception of P20 tumour line DNA from a single cell suspension which was sequenced to 150X in view of reduced purity, and 50 million paired-end raw reads for RNA-seq.

2.16 Neoantigen prediction

Whole exome sequencing and RNA-seq raw data was provided by external collaborators (Christian Ottensmeier, University of Southampton) or by Novogene. Neoantigen prediction was performed by Isaac Woodhouse (Koohy group, Human Immunology Unit, University of Oxford) according to the following methodology.

WES reads were trimmed using Trim Galore v0.4.1^[1] (utilising Cutadapt version¹⁸⁴ 1.2.1 and FastQC v0.10.1^[2]) and mapped against Homo sapiens genome version hg38 using Bowtie2¹⁸⁵ v2.2.5. Duplicate reads in mapped files were detected and removed using Picard MarkDuplicates^[3] v2.3.0 and resulting mapped sequences were sorted and indexed using Samtools^{186,187} v1.6. Somatic variants were called using Strelka¹⁸⁸ v2.9.2 in WES mode, with small indel candidates provided by Manta¹⁸⁹ v1.3.2, and then filtered by “PASS” in the variant call format filter field. RNA-seq reads were processed using the same approach including Trim Galore, Picard MarkDuplicates and Samtools but were mapped against Homo sapiens genome version hg38 using STAR¹⁹⁰ v2.5.3a. The featureCounts tool¹⁹¹ from the Subread package v1.6.2 was used to quantify reads to generate counts per gene which were then transformed into transcripts per million (TPM) values using an in-house python script.

Neoantigen prediction was performed by an in-house re-developed version of MuPeXI¹⁹² codenamed TUNAPASTA v0.5, which follows much of the same rationale. Patient mutations, HLA-types and TPM expression data are input and processed to determine a ranked list of neoantigens. TUNAPASTA relies on the Ensembl Variant Effect Predictor (VEP)¹⁹³ v92.1, NetMHCpan¹⁹⁴ v4.0 and Python v3.

[1] Krueger, F. (2015). Trim galore. A wrapper tool around Cutadapt and FastQC to consistently apply quality and adapter trimming to FastQ files. Available online at: http://www.bioinformatics.babraham.ac.uk/projects/trim_galore

[2] Andrews S. (2010). FastQC: a quality control tool for high throughput sequence data. Available online at: <http://www.bioinformatics.babraham.ac.uk/projects/fastqc>

[3] “Picard Toolkit.” 2019. Broad Institute, GitHub Repository. Available online at: <http://broadinstitute.github.io/picard/>

Chapter 3. Characterising the effect of checkpoint inhibitor treatment upon the antigen-specific immune response

3.1 Introduction

As discussed in Chapter 1, although treatment with combination anti-CTLA-4 and anti-PD-1 checkpoint inhibitor treatment has dramatically improved outcomes for patients with metastatic melanoma, limited data exists on the effect of combination treatment upon the peripheral immune response. Furthermore, data on the effects of combination checkpoint inhibitor therapy upon tumour-specific T cell responses are lacking and it is not known to what extent cellular subsets responding to combination ICB are tumour-specific or whether they represent a subset of a wider immunotherapy induced antigen-independent response.

3.2 Patient samples and clinical characteristics

To provide an initial assessment of the effect of combination ICB upon the peripheral immune response, we performed longitudinal immune profiling on serial blood samples collected from 6 metastatic melanoma patients receiving combination Ipilimumab and Nivolumab immunotherapy treatment at the Churchill Hospital, Oxford. As combination checkpoint inhibitor therapy was not standard of care treatment at the beginning of this study, samples were collected from patients enrolled in two distinct clinical trials: CheckMate-511 and CheckMate-401. The CheckMate-511 trial was a double-blind study comparing different doses of Ipilimumab plus Nivolumab in combination. Patients in this study were randomised to either four doses of Ipilimumab 3mg/kg + Nivolumab 1mg/kg given in 3 weekly cycles (q3w) for 4 doses followed by Nivolumab 480mg 4 weekly (q4w), or four doses of Ipilimumab 1mg/kg + Nivolumab 3mg/kg q3w followed by Nivolumab 480mg q4w. In the CheckMate-401 trial, patients received Ipilimumab 3mg/kg + Nivolumab 1mg/kg q3w for four doses followed Nivolumab 3mg/kg q2w, a regime that has subsequently been approved as standard of care. The clinical characteristics of the patients in this cohort and the treatment they received are detailed in Table 3-1.

Table 3-1. Clinical characteristics of initial cohort of melanoma patients

Patient ID	Sex	Age	Prior systemic treatment	Treatment received
NPB_03284	M	61	Nil	Ipi + Nivo [†]
NPB_03663	M	66	Nil	Ipi3 + Nivo1
NPB_03642	F	42	Nil	Ipi + Nivo [†]
NPB_03661	M	76	Nil	Ipi3 + Nivo1
NPB_03659	M	58	Nil	Ipi3 + Nivo1
NPB_03617	F	48	Nil	Ipi + Nivo [†]
Ipi3 + Nivo1 (CheckMate-401 trial) = Ipilimumab 3mg/kg + Nivolumab 1mg/kg q3w for 4 doses then Nivolumab 3mg/kg q2w. Ipi + Nivo [†] (CheckMate-511 trial) = Ipilimumab 3mg/kg + Nivolumab 1mg/kg q3w for 4 doses then Nivolumab 480mg q4w OR Ipilimumab 1mg/kg + Nivolumab 3mg/kg q3w for 4 doses then Nivolumab 480mg q4w.				

Blood samples were taken pre-treatment as a baseline and then at two subsequent timepoints during therapy to coincide with the administration of subsequent doses of the treatment at cycle 2 (C2, 3 weeks) and cycle 4 (C4, 9 weeks). In some cases, where treatment was ongoing after the initial sample collection schedule, an opportunistic 'late on-treatment' blood sample was also collected (Figure 3-1). All on-treatment blood samples were taken prior to administration of the drug on that day, and can therefore be considered to reflect the effect of the treatment upon the immune response in the interval between the dose preceding it and the timing of the sample. PBMCs were isolated and frozen as described in Chapter 2.4 and then subsequently used for downstream analyses.

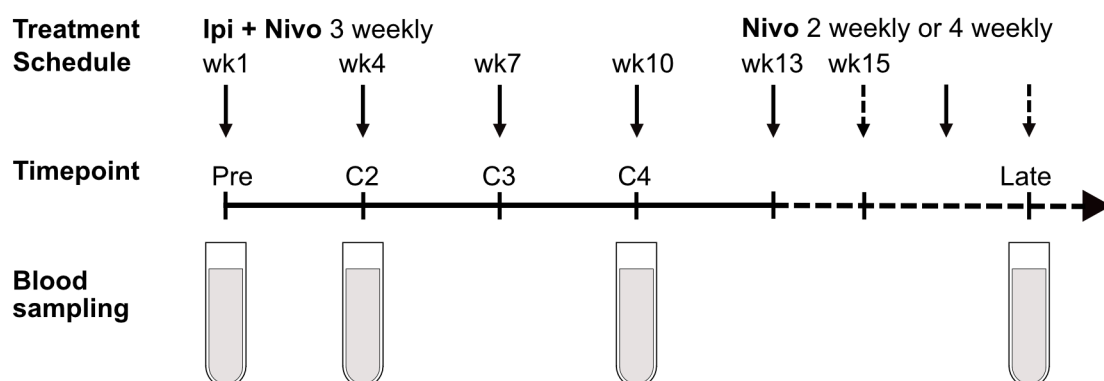


Figure 3-1. Treatment and sampling schedule for patients receiving combination Ipilimumab and Nivolumab checkpoint inhibitory therapy

3.3 Characterisation by mass cytometry of the peripheral immune response to combination Ipilimumab and Nivolumab checkpoint inhibitor therapy

In order to characterise the cellular composition of PBMCs during therapy, we decided to use mass cytometry, also known as CyTOF (Cytometry by time-of-flight). This is a relatively new technology, similar to flow cytometry, but which uses antibodies labelled with heavy metal ion tags instead of fluorophores to stain cells, which are then measured by time-of-flight mass spectrometry. The use of metal isotopes instead of fluorophores has the advantage of significantly reducing the issues of spectral overlap and autofluorescence associated with flow cytometry, allowing the simultaneous assessment of up to 40 phenotypic and functional markers at a single-cell resolution. This allowed us to obtain both a broad overview of the major different immune cell populations as well as to characterise in-depth individual sub-populations. Previous studies suggested that T cell responses are particularly important in the response to checkpoint inhibitor treatment so a number of markers in our panel were allocated to defining this population in detail including a number of T cell homing markers. Sample preparation and staining was performed as described in Chapter 2.7.2.

3.3.1 Unsupervised clustering of PBMCs identifies the main peripheral immune cell subsets and reveals substantial inter-patient heterogeneity

Traditional analysis approaches used for multicolour flow cytometry, such as manual gating, are sub-optimal for the analysis of CyTOF data, as the number of possible combinations of markers that can be defined by inspection of biaxial plots increases exponentially in line with the number of single cell parameters measured. They also fail to adequately explore the multidimensional nature of the data. For this reason, we used an unsupervised clustering approach (FlowSOM¹⁵⁶) to identify cell populations based on similarities and differences in the expression of phenotypic markers across different groups of cells across

patients, and dimensionality tools such as UMAP¹⁵⁸ to visualise cellular heterogeneity in bi-dimensional space.

To ensure we did not overlook changes in non-T cell populations in response to therapy and to validate this analysis approach, we performed initial unsupervised clustering on the total CD45+ leucocyte populations using 11 lineage markers capable of discriminating the major leucocyte sub-populations including B cells, monocytes, dendritic cells, NK cells and T cells. Granulocytes such as neutrophils were not expected to be present in significant numbers as these typically separate on top of the erythrocyte layer during density-based separation, and are thus absent in the PBMC fraction. In order to identify low frequency non-lymphocyte sub-populations, 100 FlowSOM clusters were generated, and grouped into 20 metaclusters using ConsensusClusterPlus¹⁵⁷ (Appendix A, Figure A-1). We then used UMAP plots and heatmaps to identify markers expressed in the different clusters to manually merge and annotate the populations (Figure 3-2 A-C). By doing this we were able to identify all the expected major leucocyte sub-populations within the PBMCs. Heterogeneity was observed between patients in the proportions and compositions of these different cellular populations at baseline before they commenced treatment (Figure 3-2 D). Furthermore, variation between individuals appeared to be greater than within individuals over time as demonstrated by the observation that samples from the same patients at different timepoints were typically more closely associated when we performed dimension reduction by multidimensional scaling (Figure 3-2 E). Inter-individual variation might depend on underlying features such as increasing age, which has been associated with fewer CD8+ T cells and more NK cells¹⁹⁵, and prior infection history, but also on differences in the pre-existing anti-cancer immune response. In view of this, when studying the effect of treatment upon the immune response, we focused on changes in frequency of distinct populations from baseline during therapy for each individual rather than absolute frequencies.

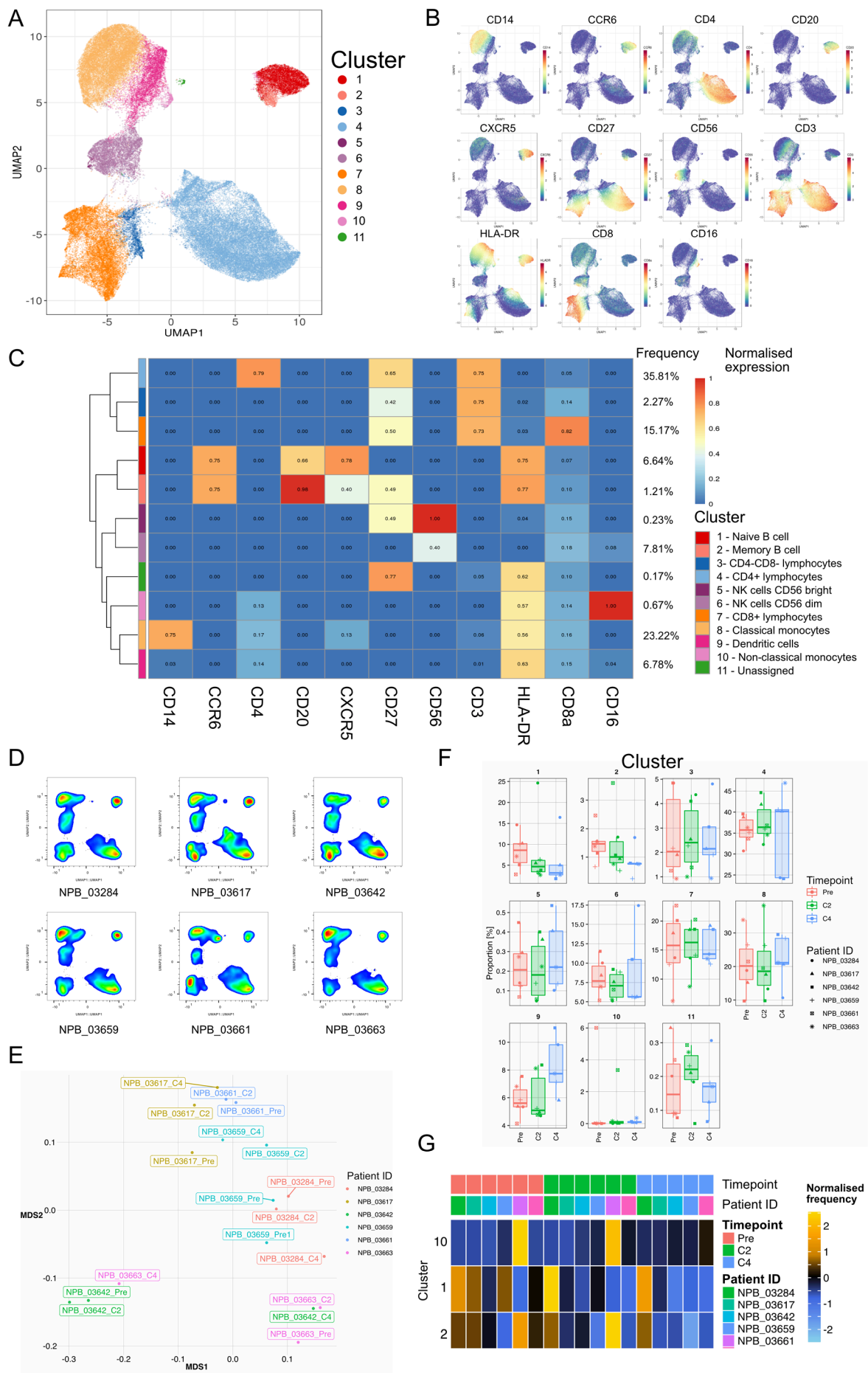


Figure 3-2. Clustering of CD45+ cells. A) UMAP based on expression of 11 lineage markers in CD45+ cells for all patients (5000 cells randomly selected per patient). Cells coloured according to manually merged PBMC populations obtained from FlowSOM clustering. B) UMAP plots with cells coloured according to arcsinh-transformed expression of lineage markers. C) Heatmap of median arcsinh transformed and 0-1 normalised marker intensities for the manually merged and annotated PBMC populations obtained from FlowSOM clustering. D) Per-patient UMAPs at baseline coloured by relative population density. Colouring corresponds to cell density from low density (blue and green) through mid-range (yellow) to high cell density (orange and red). E) Multi-dimensional scaling plot showing similarity of individual samples based on expression of all markers. Greater distances between points indicate less similarity. F) Proportion of cells in each cluster at different timepoints on therapy (indicated by colours) with individual patients indicated by different symbols. G) Heatmap of arcsine-square-root transformed cell frequencies normalised per cluster (row) to mean of zero and standard deviation of 1 for significantly differentially abundant clusters at C4 (FDR adjusted p -value <0.05). Each column represents one patient sample from one timepoint. The colour indicates the extent of relative under-representation (blue) to over-representation (orange).

Having identified the major PBMC populations, we looked at how the frequency of these different populations changed with treatment at each timepoint and in each of the six individuals assessed (Figure 3-2 F). Differential abundance testing was performed using negative-binomial generalised linear models (diffcyt implementation of edgeR¹⁵⁹). No significant changes were observed in the frequency of cells in any of the clusters at cycle 2 after 3 weeks on treatment. After 4 cycles (9 weeks on therapy) we observed a significant reduction in both B cell clusters (clusters 1 and 2) as well as an increase in CD16+ non-classical monocytes (cluster 10) compared with baseline (Figure 3-2 G). This decrease in circulating B cells is consistent with previous observations¹⁹⁶ of reduced peripheral B cell frequencies in a cohort of melanoma patients treated with combination checkpoint blockade, although the mechanisms underlying this are unknown. Although we could not detect any overall changes in lymphocyte frequencies, the limited resolution at this level of clustering was not sufficient to discriminate between different lymphocyte subpopulations. We therefore proceeded to perform higher resolution clustering on CD8+ and CD4+ T cell populations using the T cell specific markers included in our panel.

3.3.2 Clustering of lymphocyte subpopulations reveals activation of both CD8+ and CD4+ T cells in response to treatment

CD4+ and CD8+ T cell populations were identified by manual gating of CD3+ lymphocytes into CD4+ and CD4- lymphocyte populations, followed by subsequent exclusion of CD8- and non-conventional CD8+ T cells from the CD4- population by clustering. FlowSOM clustering was then performed using 24 functional and phenotypic markers in the respective subsets. Meta-clustering to 15 metaclusters was performed based upon inspection of delta area plots for different numbers of metaclusters as described in Chapter 2.7.4.6. The expression of the various markers in the different clusters is shown in Figure 3-3 A & B. Changes in the differential abundance of the clusters over time was then evaluated (Figure 3-3 C-F).

For the CD8+ population, statistical testing of the differential abundance of the clusters revealed six clusters differentially expressed at C2 and 11 clusters differentially expressed at C4, when compared to baseline (Figure 3-3 E). Inspection of the markers defining these clusters (Figure 3-3 A) reveals that six of them (11,13, 5, 9,10 and 12) shared features of activation as indicated by expression of CD38 and HLA-DR. These clusters of activated cells differed in the presence of homing markers, and degrees of expression of activation markers and proliferation markers such as Ki67 (highly expressed in cluster 12). Whilst all of these clusters remained elevated at C4 compared to baseline, the frequency of clusters 13 and 10 decreased at C4 compared to C2. These clusters appeared to express higher levels of the memory marker CD45RO, the activation markers HLA-DR and CD38, and the proliferation marker Ki67 compared to the other activated clusters. It is now known whether their reduction at C4 represents a change in differentiation in these cells causing them to shift from one cluster to another, or is due to disappearance of these cells from the circulation.

Figure 3-3. Clustering of CD8+ and CD4+ cells. A & B) Heatmap of median marker intensities of CD8+ (A) and CD4+ (B) metaclusters (arcsinh, 0-1 transformed marker expression calculated using cells from all the samples). C & D) Proportion of cells found in each cluster at different timepoints on treatment for CD8+ (C) and CD4+ (D) clusters. E & F) Differential abundance of CD8+ (E) and CD4+ (F) clusters at different timepoints on treatment. Clusters significantly differently abundant (FDR <0.05) at C2 and C4 are indicated in green in the bar to the right. Clusters are ordered by significance at C2. G) PD-1 expression of different CD8+ clusters. H) Biaxial plots of CD8+ T cells showing expression of CD38 vs HLA-DR over time for 4 individuals.

At C4 changes in the small population of circulating memory cells expressing the tissue resident markers CD103 and integrin β 7 (cluster 15 and 14) were observed with an increase in cells expressing CCR4 (cluster 15) and a reduction in those expressing CXCR3 (cluster 14). As well as the increases across these various low frequency clusters, an increase in cells with an effector memory (cluster 2) and CD57- T_{EMRA} phenotype (cluster 7) were seen accompanied by a reduction in the abundance of naïve cells (cluster 8).

For the CD4+ population, once again variation between patients was evident at baseline, with one cluster of cells (cluster 1) characterised by Granzyme B and CD57 expression consistent with 'cytotoxic' type CD4 cells being present in 2 individuals but almost entirely absent in the others. Seven clusters were found to be differentially abundant at C2 and 12 at C4 compared to baseline (Figure 3-3 D & F). The changes were similar to those observed in the CD8 compartment with increases in abundance of a large number of small frequency activated populations (clusters 15, 6, 9, 5, 12, 7, 4, 14) and a reduction in naïve CD4 (cluster 8). An increase in frequency of Treg cells (cluster 10) was also observed at C4.

Given that PD-1 blockade is expected to modulate the activity of cells expressing PD-1, we investigated whether the treatment affected the frequency of CD8+ cells expressing PD-1 in peripheral blood. Since drug occupancy of the antibody target is known to cause interference with the antibody staining for PD-1^{89,118} at the on-treatment timepoints, we excluded PD-1 from the FlowSOM clustering and assessed PD-1 expression in each cluster based on the baseline timepoint. As expected, all clusters that displayed PD-1 expression at baseline showed a reduction in median PD-1 expression on treatment consistent with

receptor occupancy (Figure 3-3 G). At baseline, clusters 12 and 13 displayed uniformly relatively high PD-1 expression with lower and more variable PD-1 expression seen in clusters 11, 9, 10 and 2. Accordingly, clusters 12 and 13 accounted for 2 out of the 3 clusters with the greatest log fold-change upon treatment (3.19 and 2.81 respectively) with only cluster 10 showing a greater change (3.48).

To validate the observation of CD8+ T cell activation based on unsupervised clustering, we went back to the biaxial plots for each patient and looked at the expression of the activation markers CD38 and HLA-DR over time. This confirmed the pronounced activation of CD8+ T cells, and also revealed different activation kinetics between patients. This could be categorised as transient where activation was seen at three weeks but not nine weeks, sustained where activation was seen both at three weeks and nine weeks, delayed where activation was not observed until nine weeks, and pre-activated where an elevated frequency of activated cells was present at baseline and did not change during treatment (Figure 3-3 H).

In summary, these CyTOF analyses demonstrated that despite substantial heterogeneity between individuals, treatment with combination immune checkpoint inhibitor therapy lead to the accumulation of both CD4+ and CD8+ activated cells.

3.4 CD8+ T cells activated by combination checkpoint inhibitor therapy contain cells capable of recognising melanoma tumour-associated antigens or tumour cell lines

Having established that combination checkpoint inhibitor therapy leads to an increase in the frequency of activated CD8+ T cells, we set out to establish the relevance of this to the anti-tumour response by determining whether the cells activated during treatment were tumour-specific. Whilst other studies have described shared TCRs between TILs and cells activated in the periphery in response to PD-1 monotherapy¹³², direct evidence that cells

expressing these shared TCRs are actually tumour-specific and not simply tumour resident bystander cells¹⁴⁴ has been lacking.

In order to test whether circulating cells activated upon immune checkpoint inhibitor treatment were tumour-specific, we isolated activated T cells at the peak of the immune response and investigated whether these could recognise tumour antigens. As we did not have fresh tumour samples from these patients to generate autologous tumour cell lines, we performed haplotyping on a panel of allogeneic melanoma tumour cell lines expressing known tumour-associated antigens (Table 3-2). Those tumour lines which partially matched to the patient's haplotypes, were then used to test for antigen recognition of the isolated T cells. A glioblastoma (GBM) cell line was also included in the panel for use as a non-melanoma control.

Table 3-2. Class I haplotypes of allogeneic tumour cell lines

Cell line	Tumour type	Class I HLA locus					
		A1	A2	B1	B2	C1	C2
Sk-MEL23	Melanoma	01:01/04N	02:01	07:02	08:01	07:01	07:01
Sk-MEL29	Melanoma	02:01	68:01	44:02	45:01	05:01/03	06:02
MZ2	Melanoma	01:01/04N	01:01/04N	37:01	37:01	06:02	06:02
Na8	Melanoma	01:01/04N	02:01	08:01	40:01	03:04	07:01
D10	Melanoma	02:01	30:02	07:02	51:01	07:02	15:02
ME275	Melanoma	02:01	02:01	07:02	07:02	07:02	07:02
Trombelli	Melanoma	01:01/04N	02:01	27:05	38:01	02:02	12:03
Sk-MEL28	Melanoma	11:01	11:01	40:01	40:01	03:04	03:04
MelJuso	Melanoma	01:01/4N	01:01/4N	08:01	08:01	07:01	07:01
DX3	Melanoma	02:05	31:01	35:02	50:01	04:01	06:02
Mel526	Melanoma	02:01	03:01	15:01	15:01	03:03	03:04
MM25	Melanoma	01:01/4N	25:01	08:01	15:01	04:01	07:01
DAG1	Melanoma	02:01	25:01	18:01	44:02	05:01/03	05:01/03
U251	GBM	02:01	02:01	18:01	18:01	05:01/03	05:01/03

We began by testing activated cells from 3 HLA-A*02:01 patients (Table 3-3) receiving combination checkpoint inhibitor treatment, for their capacity to recognise partially HLA matched melanoma tumour cell lines or a pool of peptides of commonly expressed HLA-A*02:01 restricted immunodominant epitopes from melanoma tumour-associated antigens

Table 3-3. Patient HLA-Class I haplotypes

Patient ID	HLA-Class I haplotype
NPB_03284	A*02:01/29:02 B*44:03/51:01 C*02:02/16:01
NPB_03663	A*02:01/03:01 B*15:01/35:01 C*03:03/04:01
NPB_03659	A*02:01/24:02 B*15:01/51:01 C*02:02/03:03

To isolate activated T cells, we performed single cell sorting of T cells expressing HLA-DR and CD38 after 3 weeks of checkpoint inhibitor treatment (Figure 3-4) and expanded these to generate T cell clones as described in Chapter 2.8.3.

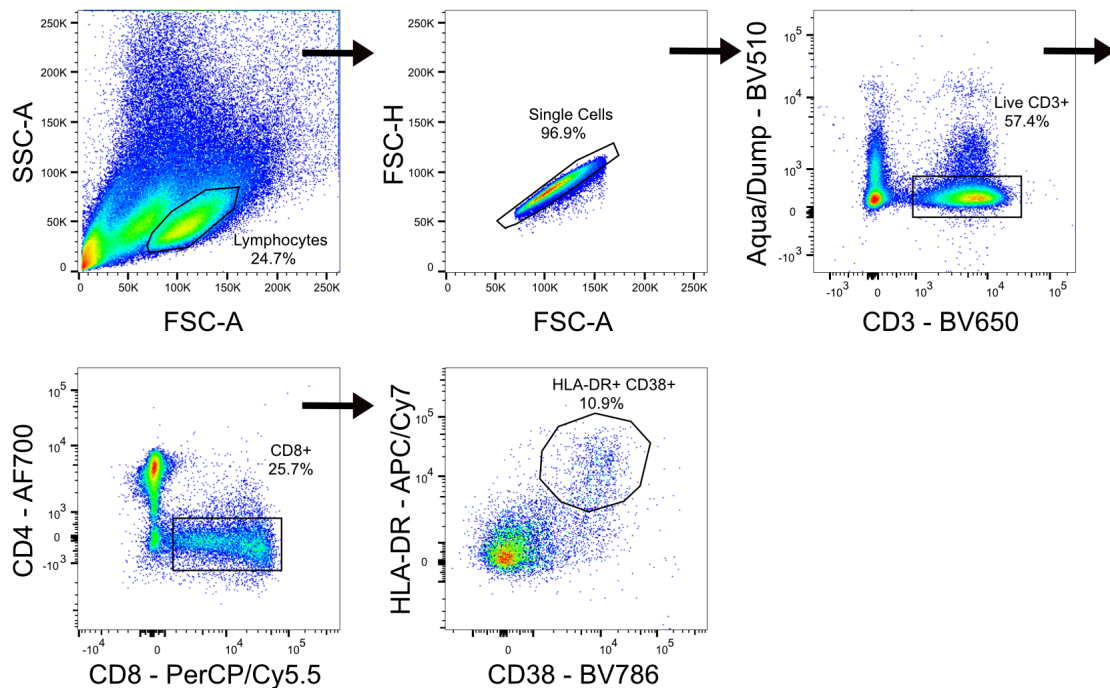


Figure 3-4. FACS gating strategy for sorting of activated HLA-DR+ CD38+ CD8+ cells from PBMCs. Sequential gating was performed as follows: identification of lymphocytes based on size and granularity using forward scatter (FSC) and side scatter (SSC), exclusion of doublets, exclusion of dead or CD14 or CD19 positive cells (dump channel) and selection of CD3+ cells, selection of CD8+ cells and finally gating and sorting of activated cells positive for HLA-DR and CD38.

In order to allow each individual clone generated to be tested against multiple tumour lines and peptides in a high-throughput manner, a combinatorial barcoding approach was used

whereby individual clones were labelled with a unique combination of fluorescent dyes at different concentrations (Chapter 2.9.2). Labelled clones were then pooled and incubated with tumour cell lines or peptides and the resultant cytokine response assayed by multicolour flow cytometry. The response of individual clones was then deconvoluted after acquisition based on the dye labelling. Clones were considered to be reactive when more than 10% of cells were positive for IFN- γ and/or CD107a in response to culture with tumour lines or peptides (based on gates set relative to the unstimulated and stimulated controls). Where sufficient numbers of cells were available, clones found to be reactive against the pool of tumour peptides were then tested against individual peptides using the same approach to determine their specificity.

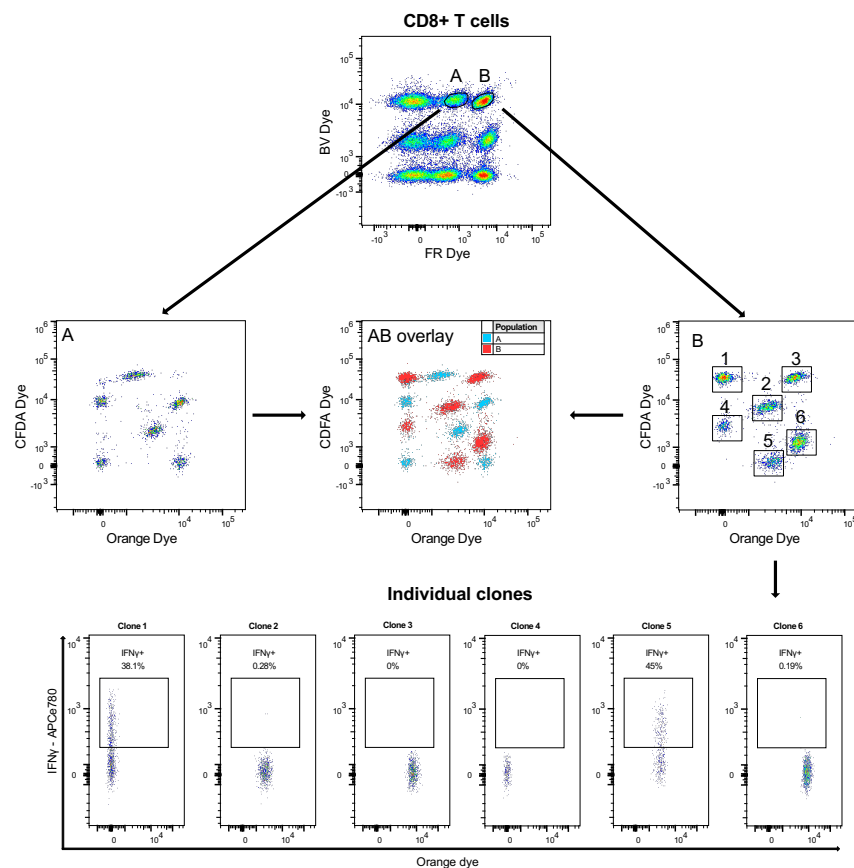


Figure 3-5. Combinatorial dye labelling scheme. Clones can be initially segregated based on the expression of two of the dyes used into 9 populations (top panel). Each population (e.g. A and B) can then be subdivided into a further 6 populations based on the expression of the other two dyes (middle panel). The dye staining for clones with neighbouring dye labelling in the first dimension are offset in the second dimension to avoid overlap (AB overlay). Production of IFN- γ can then be assessed for individual clones.

A total of 155 clones were successfully expanded from CD38+HLA-DR+ cells from the different patients, of which 23 were tumour reactive, representing between 5-25% of the clones tested in each individual (Table 3-4).

Table 3-4. Number of tumour reactive clones identified per patient

Patient	Number of CD38+HLA-DR+ clones tested	Number of tumour-specific clones	Percentage tumour-specific clones
NPB_03284	60	15	25%
NPB_03596	34	2	5.8%
NPB_03663	61	6	9.8%

Example summary data for positive clones identified in individual NPB_03284 are shown in Figure 3-6. In this analysis, we tested expanded clones for their capacity to recognise HLA-A*02:01 positive melanoma tumour cell lines known to express tumour-associated antigens such as MART-1, gp100 and tyrosinase (cell lines D10, ME275 and Sk-MEL23) and TAA peptides. As a negative control we used the HLA-A*02:01 positive cell line Na8, which is reported to not express tumour-associated antigens, and the HLA-A*02:01 negative melanoma cell line MZ2, alongside the unstimulated condition and microbial peptides. In this individual, 9 clones recognised antigens expressed in a number of cell lines as well as the peptide TAA panel (group 2), while 2 recognised tumour peptides alone (group 1) and 2 recognised tumour cell lines but not peptides in the TAA panel (group 3). Testing against individual peptides established that both the clones in group 1 were reactive to the MAGE-C2 peptide (ALDVEERV) which is not known to be expressed on any of the cell lines that were tested. T cell clone responses to the melanoma cell lines showed partial or complete inhibition in the presence of a Class I blocking antibody confirming that these were being mediated through MHC:TCR interactions. Partial blockade of responses to TAA peptides was observed, possibly because the peptide concentration used in this assay (10µM) was too high for the antibody to completely block the MHC:TCR interaction, whilst the TCR independent stimulation by PMA/Ionomycin was unaffected by class I blockade.

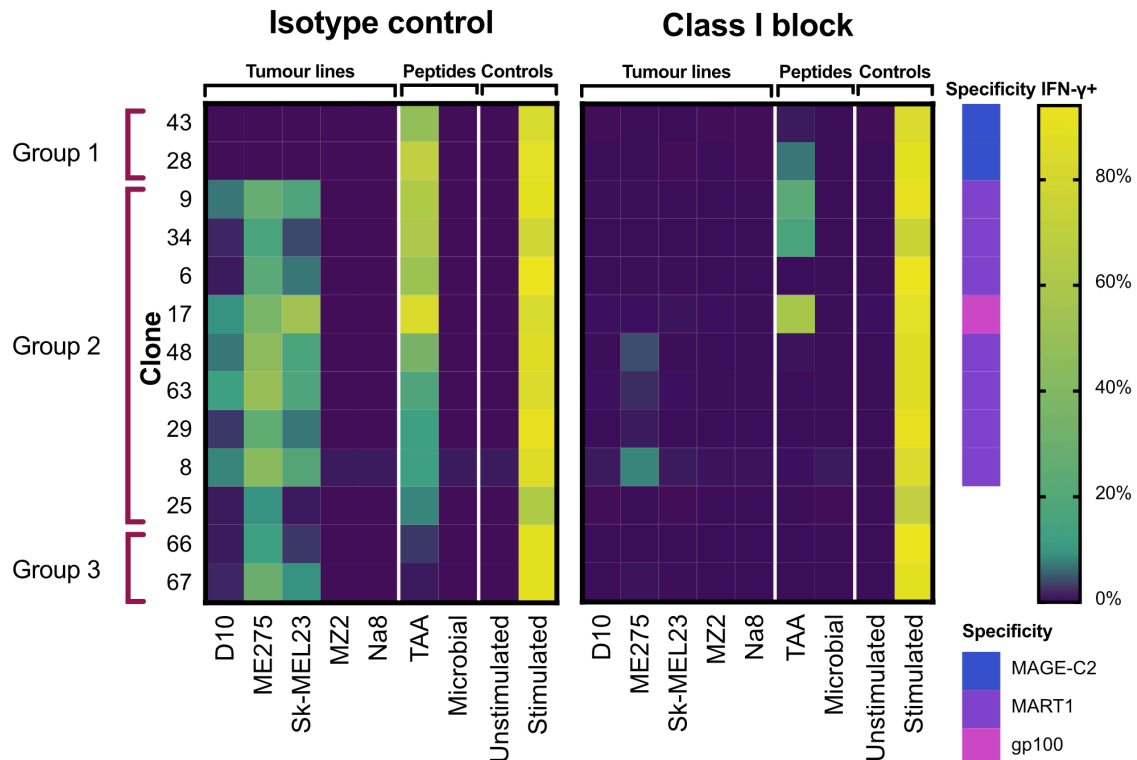


Figure 3-6. Heatmaps displaying reactivity of positive clones for patient NPB_03284. Columns contain the various conditions grouped into tumour lines (D10, ME275,Sk-MEL23, MZ2 and Na8), peptides (TAA or Microbial pools) and controls (unstimulated or stimulated with PMA/Ionomycin cell stimulation cocktail). Rows contain the patient derived T cell clones tested. The left panel shows the percentage of cells producing IFN- γ as measured by intracellular FACS staining in the presence of an IgG2a isotype control antibody, whilst the panel on the right shows the response in the presence of a class I blocking antibody (W6/32 IgG2a). Grouping as indicated to the left is based on reactivity to TAA peptides alone (group 1), tumour lines and peptides (group 2) and tumour lines alone (group 3). Data for 13 out of 15 positive clones identified for this patient shown where testing in the presence of blocking antibody was conducted. Specificity as determined by testing against individual peptides is shown in the coloured side-bar.

Tumour reactive T cells clones were also identified for patients NPB_03663 and NPB_03659, although at a lower frequency within the total expanded clones than for patient NPB_03284. No reactivity was detected for these patients against tumour peptides so we could not determine the specific antigens these cells are recognising.

A wide variation in the nature of the anti-tumour response was observed both between different clones reacting against the same tumour lines and within individual clones in response to different tumour lines. Whilst some clones displayed strong upregulation of

both IFN- γ and CD107a (Figure 3-7), others showed only a CD107a degranulation response (Figure 3-8). We did not find any clones that produced IFN- γ without upregulation of CD107a. In the presence of PMA/Ionomycin stimulation all clones were capable of both IFN- γ and CD107a production suggesting the extent of the response may be indicative of the affinity of each clone for their cognate antigen.

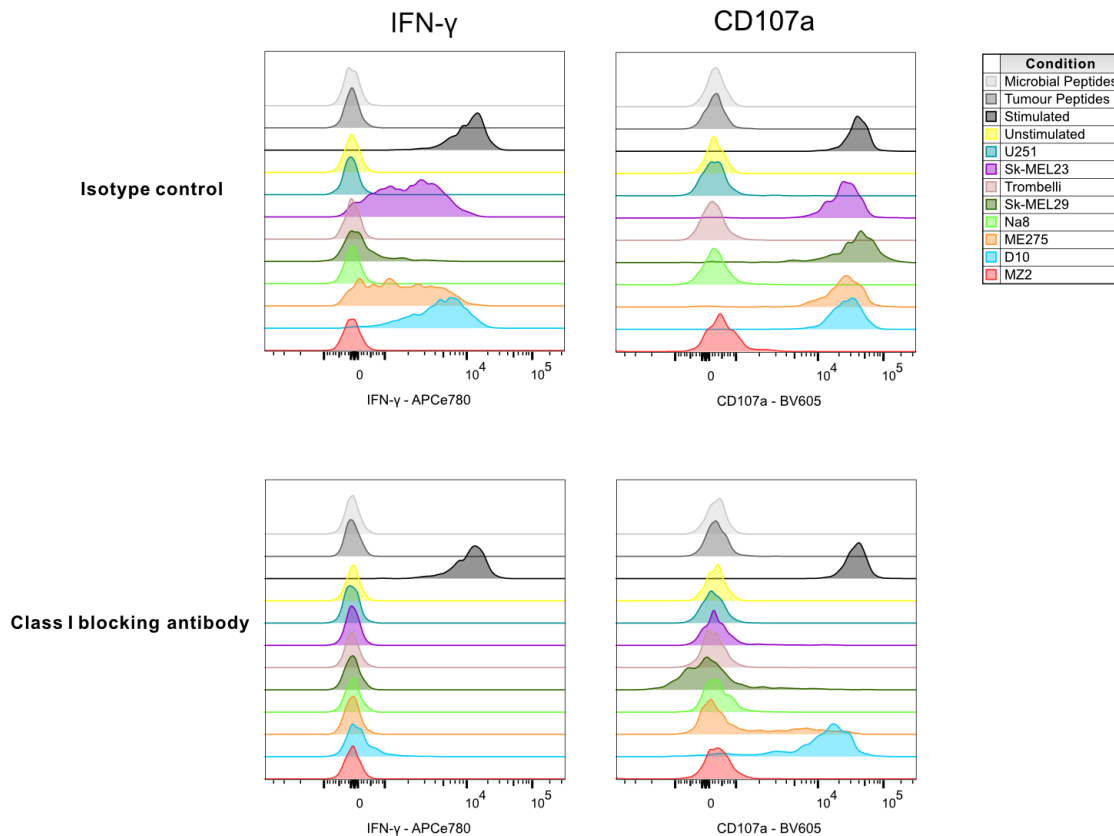


Figure 3-7. Polyfunctional T cell clone response. Production of IFN- γ (left) and CD107a (right) by T cell clone 2 (from patient NPB_03659) in response to stimulation with various tumour cell lines, peptides and controls in the presence of an IgG2a isotype control antibody (top), or class I blocking antibody W6/32 IgG2a (bottom). Increased expression of both IFN- γ and CD107a are seen when this T cell clone is incubated with cell lines D10, ME275 and SK-MEL23, but only CD107a for SK-MEL29.

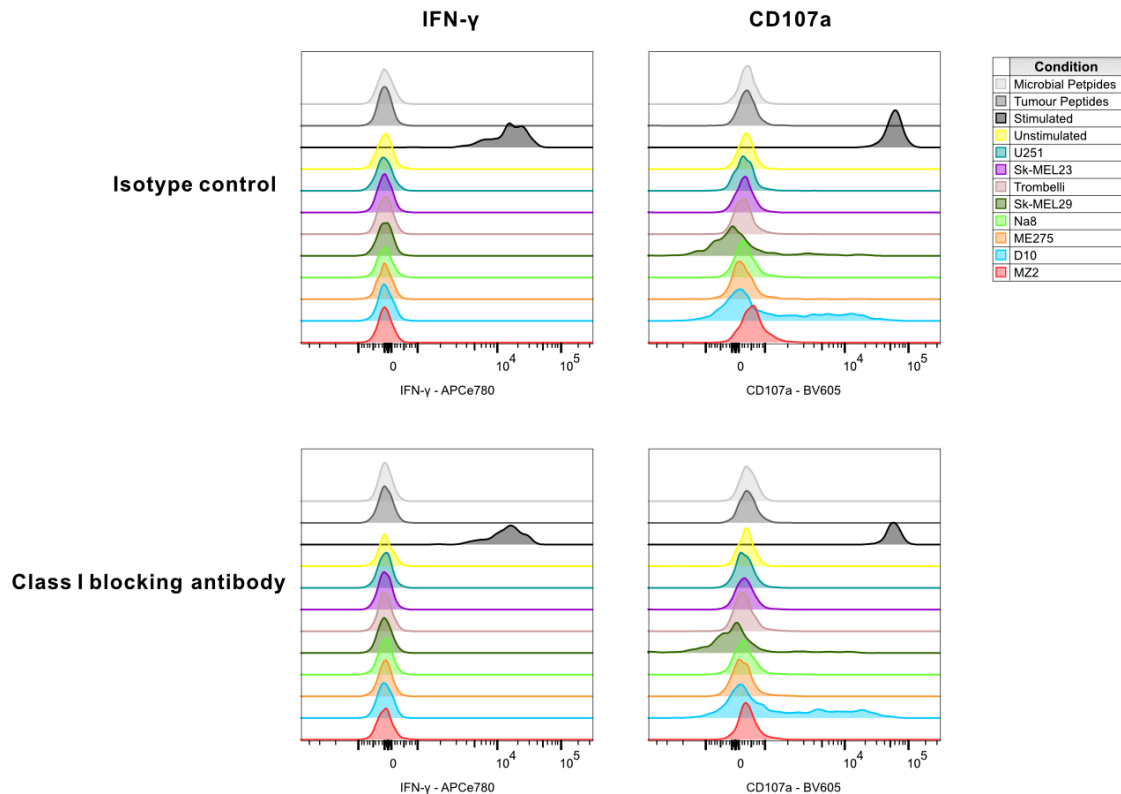


Figure 3-8. CD107a monofunctional T cell clone response. Production of IFN-γ (left) and CD107a (right) by T cell clone 26 (from patient NPB_03659) in response to stimulation with various tumour cell lines, peptides and controls in the presence of an IgG2a isotype control antibody (top), or class I blocking antibody W6/32 IgG2a (bottom). Reactivity for this clone is detected only against the D10 tumour line and only by CD107a, not IFN-γ response.

Whilst the IFN-γ response was consistently blocked in the presence of class I blocking antibodies, CD107a degranulation in some cases showed only a partial downregulation, possibly reflecting a lower threshold of activation for CD107a degranulation than for IFN-γ production. Interestingly, we also observed class I dependent CD107a reactivity of one clone tested against the Na8 tumour cell line which is not known to express any of the common tumour-associated antigens (Figure 3-9). It is possible this could represent a false positive, however the specific and selective response observed suggest that this clone might recognise a previously undescribed tumour-associated antigen expressed on this cell line but not on the other tested lines.

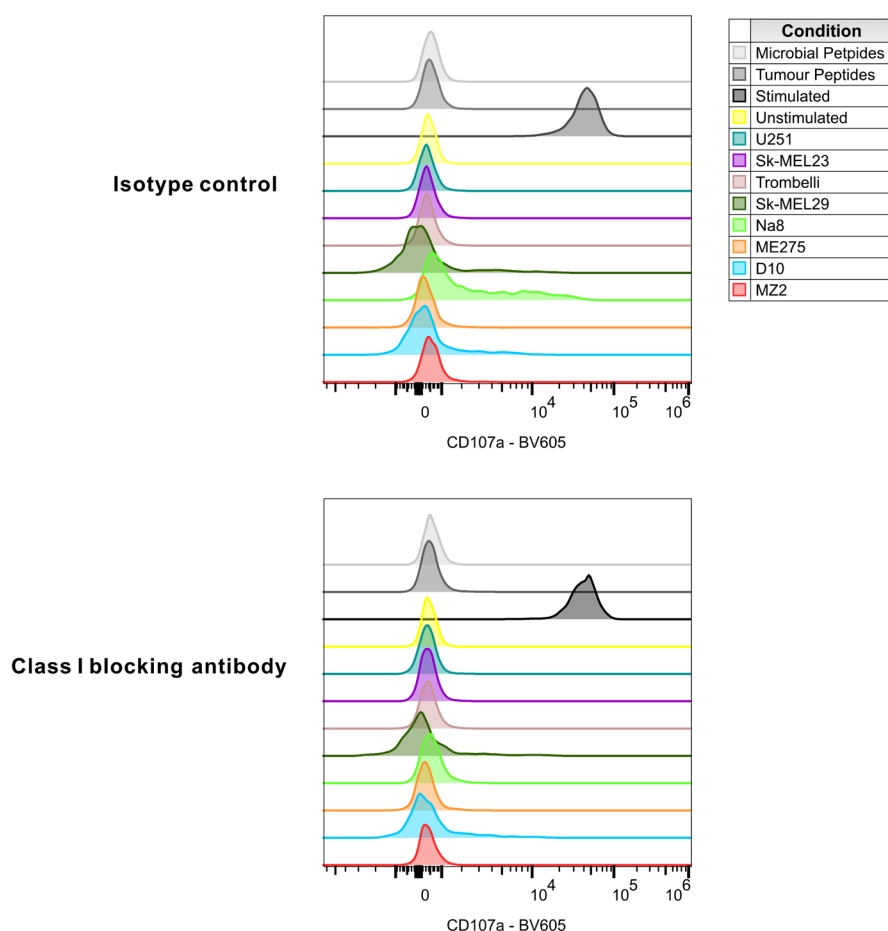


Figure 3-9. CD107a reactivity against tumour line Na8. Production of CD107a by T cell clone 88 (from patient NPB_03663) in response to stimulation with various tumour cell lines, peptides and controls in the presence of an IgG2a isotype control antibody (top), or class I blocking antibody W6/32 IgG2a (bottom). Class I dependent CD107a (but not IFN- γ) reactivity for this clone is seen against the Na8 tumour line.

As the T cell clones generated for these analyses were expanded from individual T cells isolated by FACS using indexed single cell deposition into 96-well plates, this allowed us to retain information on the phenotypic profile of each of the individual cells isolated, including the fluorescence intensity of each antibody included in our staining panel. We therefore investigated associations between the phenotype of the individual sorted cells and the cloning efficiency or tumour specificity (Figure 3-10). The only feature that appeared to be consistently associated with successful expansion of single cells to clones was the level of CD8 expression, with successfully expanded clones being almost universally CD8 bright

rather than CD8 moderate or dim. All cells which were found to be tumour-specific were confined to the CCR7 negative compartment, consistent with them being antigen experienced and of either T_{EMRA} , T_{EM} or effector phenotype, although variation was observed between patients as to whether tumour reactive cells were CD45RA positive or negative. The majority of expanded clones and tumour-specific clones were CXCR3 positive and except for one single clone, all tumour reactive clones were CCR4 negative. Being on-treatment samples, PD-1 expression is masked by binding of the anti-PD-1 antibody to the receptor, thus the expression of PD-1 in these cells could not be determined.

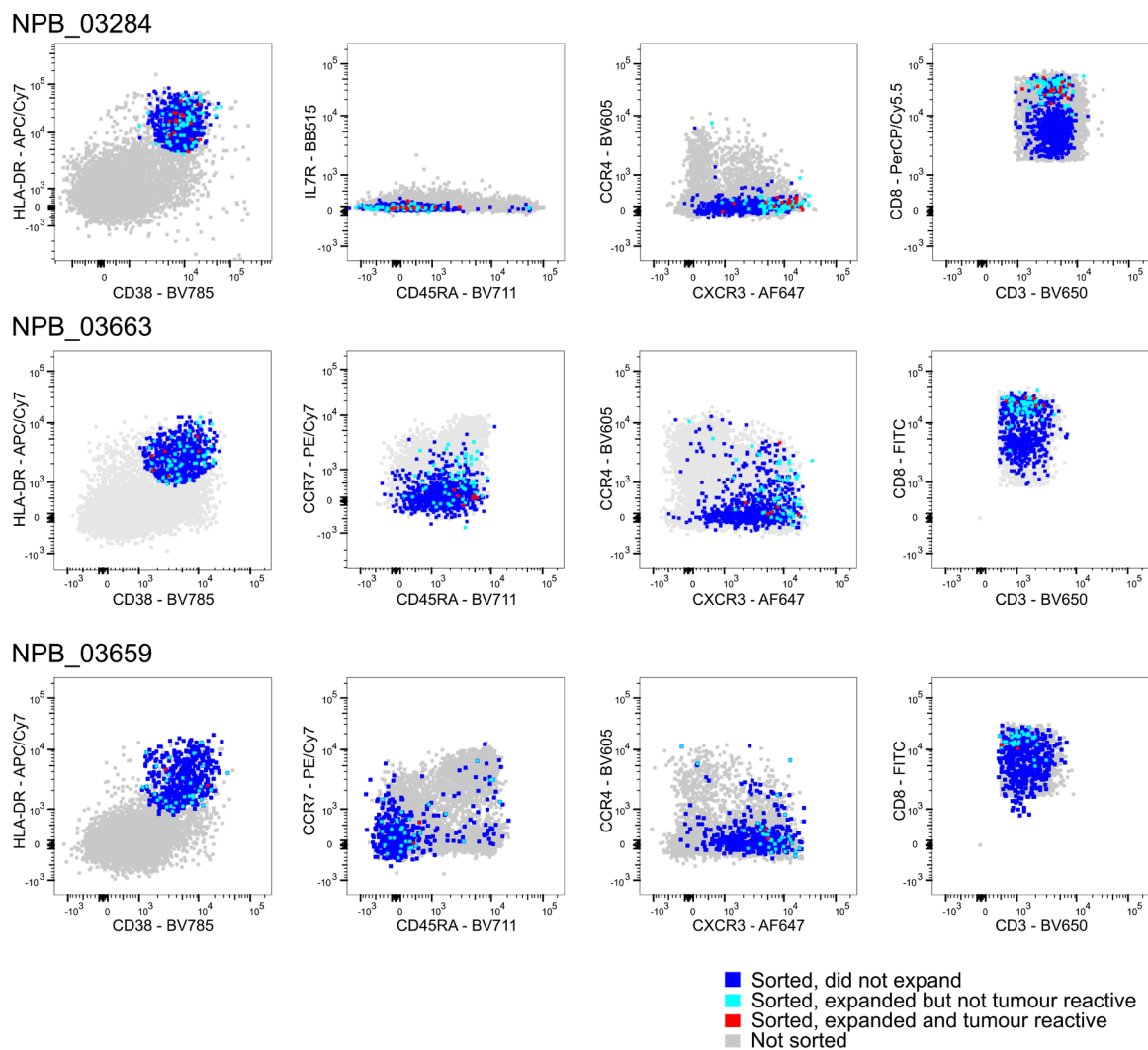


Figure 3-10. Phenotype of sorted activated T cells by expansion status and tumour reactivity. Plots of index sorted activated T cells overlaid upon the total CD8⁺ T cell population for different phenotypic markers in 3 patients. Sorted activated T cells that did not expand upon cloning are coloured blue. T cells that were successfully expanded are coloured by whether they were tumour-reactive (red) or not tumour-reactive (aqua). Non-sorted CD8⁺ T cells are coloured grey.

3.4.1 Investigation of CD39 as a marker of tumour-specific T cells in the periphery

During the course of our experiments, emerging data was published suggesting that expression of the activation marker CD39, in association with CD103 expression, may be a marker of tumour specificity within tumour infiltrating lymphocytes¹⁹⁷. However, these cells were only observed at a low frequency in the peripheral blood of the patients studied (who were not being treated with immunotherapy treatment). Given the enrichment we had observed for tumour-specific T cells in the periphery at the peak of the immune response to immunotherapy treatment amongst activated cells, as defined by CD38 and HLA-DR expression, we investigated whether CD39 could further enrich for tumour-specific cells when used in conjunction with CD38 and/or HLA-DR. In order to clone enough cells to be able to compare the distinct populations, we selected a patient (60yr old male, A015), who had a very pronounced activation response, with around 35% of CD8+ T cells becoming activated at 3 weeks on therapy with combination checkpoint inhibitor treatment (Figure 3-11). Non-naïve single cells were sorted from this patient as shown in Figure 3-12 and expanded to form T cell clones.

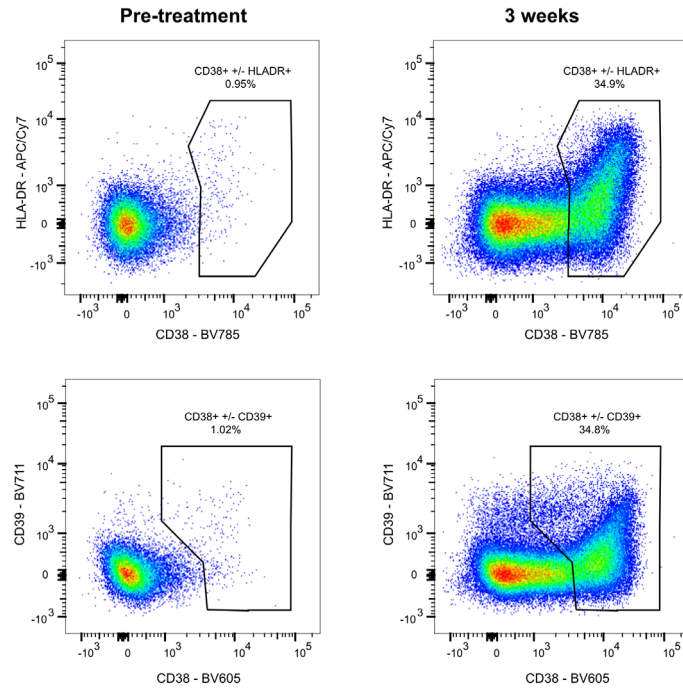


Figure 3-11. Expression of activation markers CD38, HLA-DR and CD39 before and during treatment for patient A015. Biaxial plots of CD38 vs HLA-DR or CD39 expression on CD8+ T cells pre-treatment and at 3 weeks on combination checkpoint inhibitor treatment for patient A015.

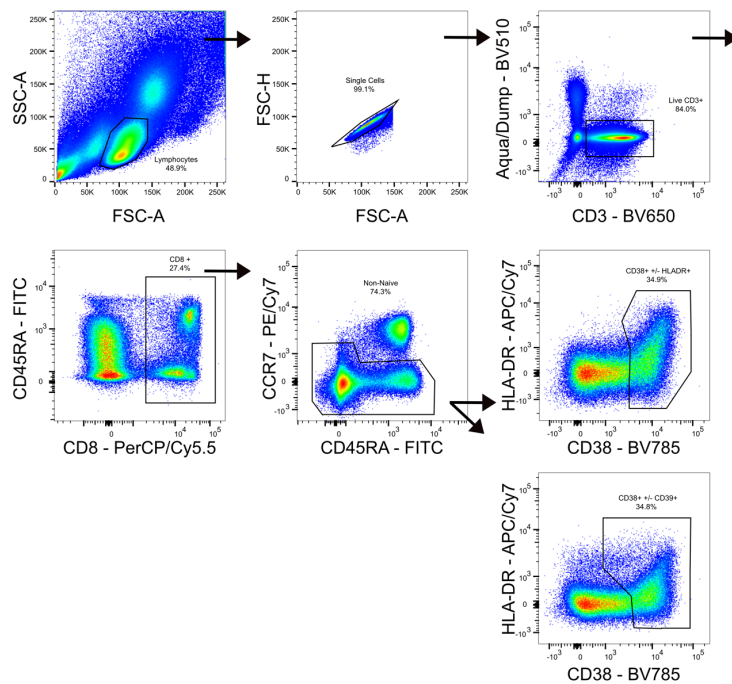


Figure 3-12. Gating strategy for sorting of CD38+ +/- CD39+ +/- HLA-DR positive cells. Sequential gating was performed as follows: identification of lymphocytes based on size and granularity using forward scatter (FSC) and side scatter (SSC), exclusion of doublets, exclusion of dead or CD14 or CD19 positive cells (dump channel) and selection of CD3+ cells, selection of CD8+ cells, exclusion of CCR7+CD45RA+ naïve cells, and finally gating and sorting of activated cells positive for CD38 ± HLA-DR OR ± CD39.

In this individual 189 clones were successfully established from 597 sorted cells giving a cloning efficiency of 31.6%. Class I haplotyping was performed for this patient (HLA-A*01:01/04N/11:01, B*08:01/35:01, C*04:01/07:01) and tumour lines from those listed in Table 3-2 were selected based on matching of Class I alleles, with cell lines DX3, MZ2, Na8 and Sk-MEL28 being matched at one or more loci and cell lines Sk-MEL29 and D10 included as unmatched controls. 46 of the clones tested were found to be reactive to allogeneic HLA Class I matched tumour lines (24%). Expression of IFN- γ and CD107a for the positive clones is shown in Figure 3-13.

The vast majority of positive clones in this patient recognised the two tumour lines Na8 and Sk-MEL28 (35/46 clones recognised both Na8 and Sk-MEL28. A further 3 clones recognised Na8 but not Sk-MEL28 and 2 recognised Sk-MEL28 but not Na8 (<10% CD107a positive). There were also a more limited number of clones reactive to DX3 and MZ2 (3 and 2 clones respectively). In the case of MZ2, both responses were seen against MZ2 only, whilst 2 of the 3 responses to DX3 were in clones that also reacted to Sk-MEL28 or MM25. In most cases (40/46 positive clones) CD107a degranulation was observed without IFN- γ production and again the effect of Class I blocking antibody was more complete for prevention of IFN- γ than CD107a expression. Of note, there was a high level of background CD107a production for all clones tested regardless of their positivity in the presence of the HLA mismatched tumour lines Sk-MEL29 and D10. However, this did not appear to be affected by class I blockade.

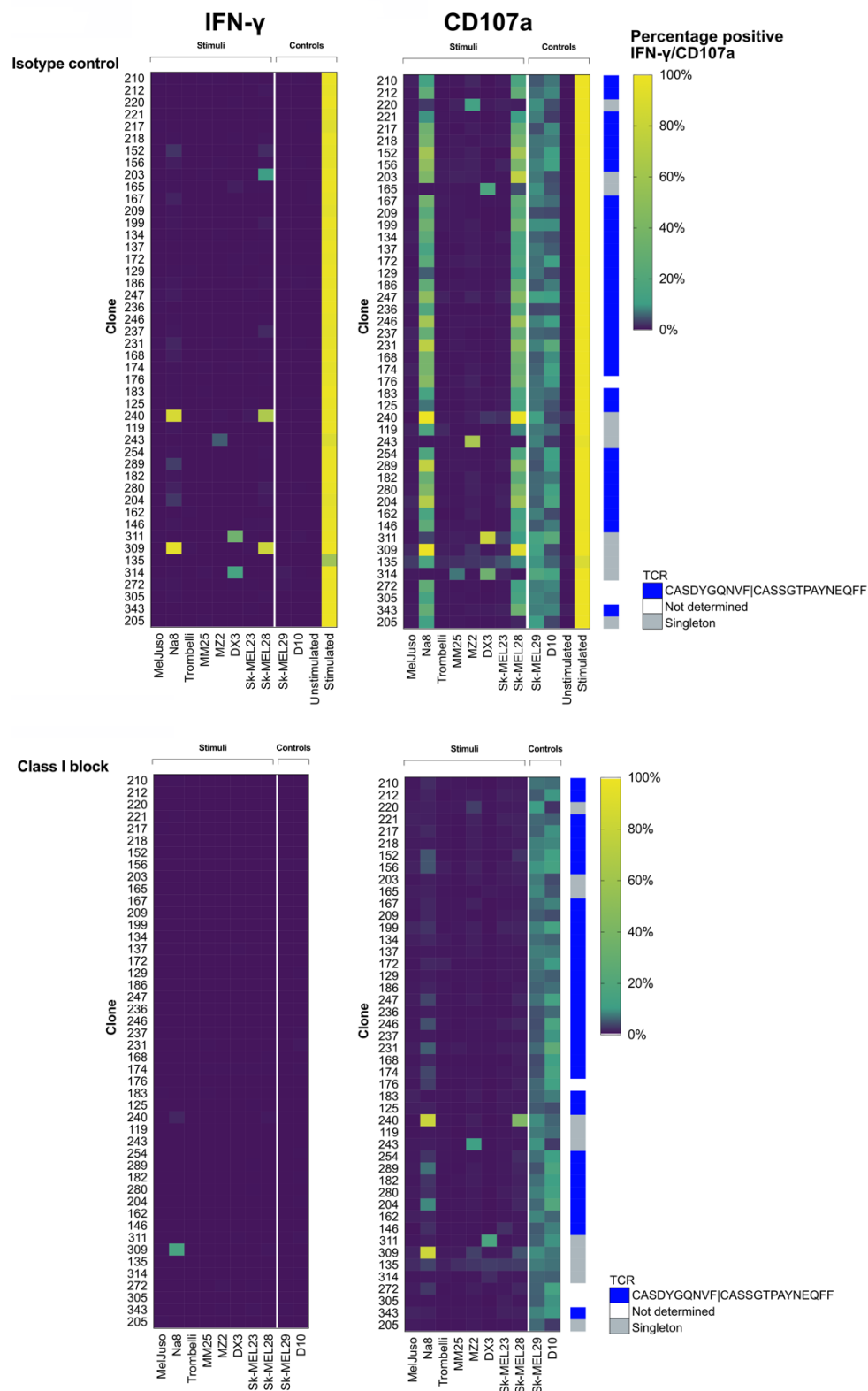


Figure 3-13. Tumour line reactive clones for patient A015. Expression of IFN- γ and CD107a in the presence of isotype control or class I blocking antibody against different stimuli for positive clones from patient A015. Tumour lines MeJuso, MZ2, Na8, Sk-MEL28, DX3 and MM25 were matched by at least one HLA locus with the patient. Sk-MEL29 and D10 were HLA mismatched at all loci.

Of the cells which were found to be tumour reactive, 32.6% (15/46) were from the CD38+HLA-DR+CD39- subset, 17.4% (8/46) from the CD38+HLA-DR-CD39+ subset and 50% (23/46) from the CD38+HLA-DR-CD39- subset and none from the CD38+HLA-DR+CD39+ subset (Table 3-5).

Table 3-5 Number of tumour reactive cells identified for the different activated subsets sorted

Activated subset	No. cells sorted	No. cells positive	Percentage cells positive in subset
CD38+ HLA-DR- CD39-	404	23	5.69
CD38+ HLA-DR+ CD39-	79	15	18.98
CD38+ HLA-DR+ CD39+	27	0	0
CD38+ HLA-DR- CD39+	87	8	9.19
	597	46	7.7

Relative to the number of cells sorted from each population, there appeared to be a substantial enrichment for tumour-specific cells amongst the CD38+HLA-DR+CD39- population, but not within either of the CD39+ positive populations (Figure 3-14).

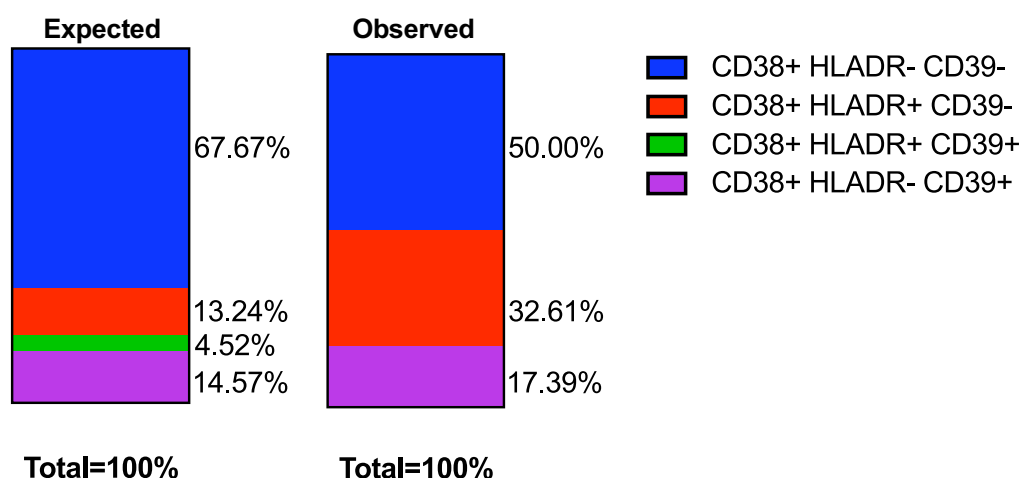


Figure 3-14. Enrichment of tumour reactive clones within different activated subsets. Expected percentage of positive clones based on frequency of different populations sorted vs observed frequency of positive clones. *p*-value 0.0006 (Chi squared test) for discrepancy between observed and expected.

TCR sequencing of all expanded clones, regardless of reactivity, was performed for this individual (Figure 3-15). Consistent with the functional data, a large number of the clones reacting to the Na8 and Sk-MEL28 tumour lines were found to share the same TCR alpha

and beta chain, consistent with them being part of a circulating expanded clonotype. Only 1 further clone of the 187 that were sequenced shared the same TCR but was not identified as reactive, possibly because of an in vitro acquired unresponsiveness of this clone to TCR stimulation. All the other reactive clones where TCR data were determined had unique TCRs, including two clones (240 and 309) which recognised the same tumour lines as the expanded TCR clone, but produced a strong interferon gamma response in addition to CD107a. From the TCR sequencing, 12 further expanded clonotypes were identified from amongst the growing clones, none of which were reactive against the tumour lines tested. These were cross-checked against a public database of common TCRs (VDJdb browser¹⁹⁸) including a number against common infectious antigens for any potential known specificity but none was found. Unfortunately since an autologous tumour cell line was not available, we could not determine whether these clones were capable of recognising neoantigens present exclusively in that patient's tumour, although it remains possible they may be recognising different tumour-associated antigens to those presented by our limited selection of tumour lines.

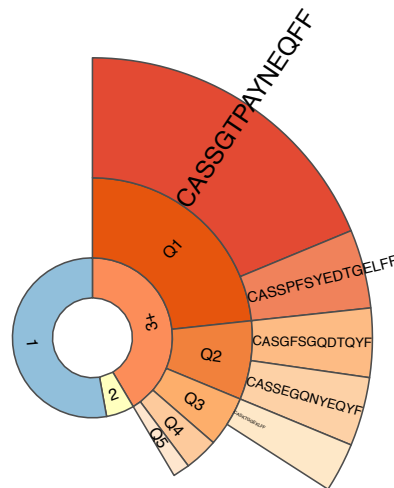


Figure 3-15. TCR β repertoire from all established clones for patient A015. Inner circle shows frequency of TCR β of T cell clones by whether they were singletons (1 - seen once), doubleton (2 – seen twice) and expanded (3+ - seen three times or more). Middle layer displays the abundance of the top 20%(Q1), and subsequent quantiles of clonotypes in the high-order set. The outer layer shows the individual abundance of the top 5 clonotypes. The most expanded clonotype (CASSGTPAYNEQFF) is that which accounted for the majority of the tumour reactive clones detected for this patient.

Overall, considering that we were testing against a relatively limited panel of tumour-associated antigens and allogeneic cell lines, the high frequency of tumour-specific T cells found amongst clones isolated from CD38+HLA-DR+ cells in patients treated with checkpoint inhibitors suggest that these tumour-specific cells are enriched within the population of cells activated by checkpoint inhibitor therapy.

3.5 Activation is selective for tumour but not microbial-specific cells

Having determined that melanoma specific T cells were present within the CD8+ population activated by therapy, we investigated whether immune check point inhibitor treatment lead to the selective activation of tumour-specific T cells, or whether it induced an antigen independent accumulation of activated cells. To determine this, we performed staining of serial PBMC samples from the HLA-A*02:01 positive individuals from our initial CyTOF cohort with a pool of tetramers loaded with melanoma-associated peptides and common microbial antigens. We first looked at the frequency of tumour tetramer+ and microbial tetramer+ T cells detectable in the peripheral blood and whether these changed over the course of treatment. In 4 of the 5 individuals we analysed, a transient increase in the proportion of tumour tetramer+ cells as a % of the total CD8+ population was observed at 3 weeks on therapy (Figure 3-16 A). By contrast, we did not see any consistent changes in the proportion of microbial tetramer+ cells detectable over therapy (Figure 3-16 B). Frequencies of both tumour and microbial tetramer+ cells over time for individual 03284 are shown in Figure 3-16 C.

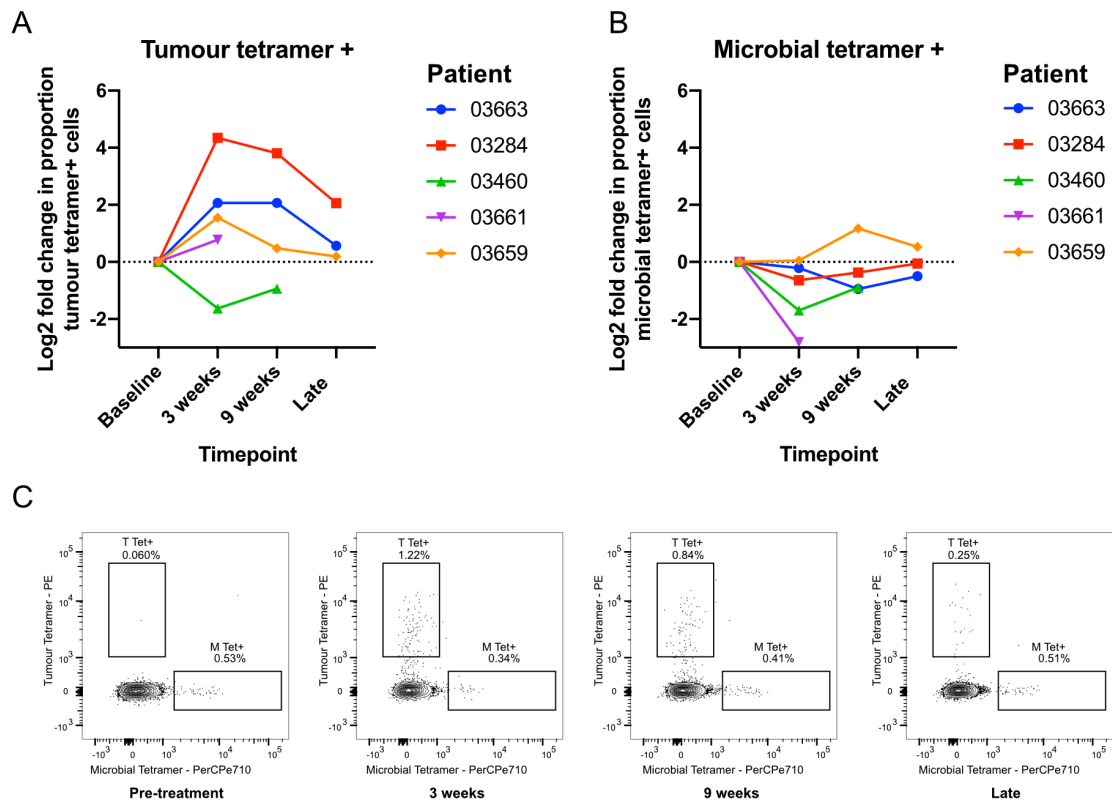


Figure 3-16. Frequency of tetramer+ cells during treatment. A) Log₂ fold change in tumour and B) microbial tetramer+ cells as proportion of total CD8+ at different timepoints on treatment. Different patients are indicated by different colours. No significant changes in log₂ FC were detected for any individual timepoint compared with baseline for either tumour or microbial tetramer+ positive cells ($p > 0.05$, two-tailed paired t-test). C) Biaxial plots for one individual (NPB_03284) demonstrating change in frequency of tetramer positive populations over time.

We then looked at the activation status of the tetramer positive cells as defined by the expression of CD38 and HLA-DR. Whilst the percentage of activated microbial tetramer+ cells did not change over treatment, there was an increase in the percentage of activated tumour tetramer+ cells in some individuals although, due to wide per-patient variation, this was not statistically significant (Figure 3-17 A-C). Inspection of biaxial overlay plots for individual patients demonstrated clear evidence of tumour tetramer+ but not microbial tetramer+ activation in response to treatment (Figure 3-17 D).

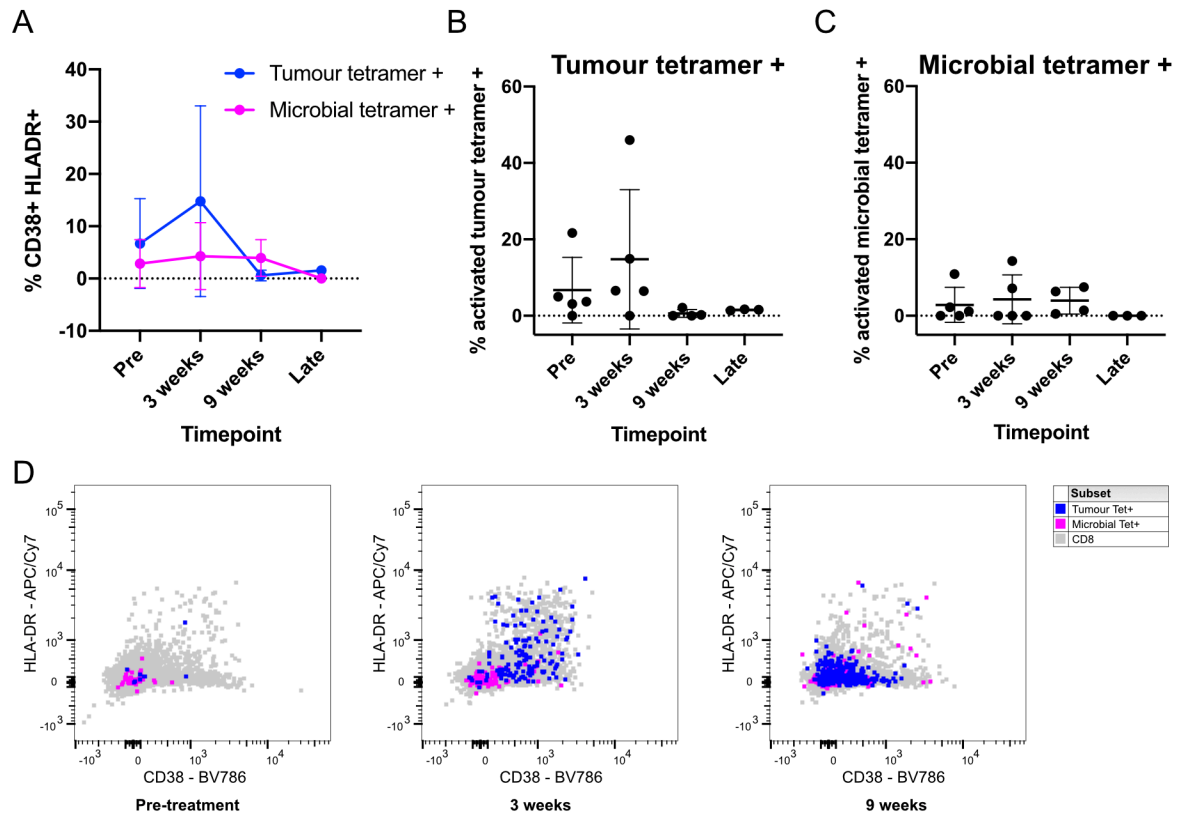


Figure 3-17. Activation status of tetramer+ cells during treatment. A) Mean CD38+HLA-DR+ tumour tetramer+ (blue) and microbial tetramer+ (pink) cells over time (mean $\pm$ SD) for 5 patients. B) Per patient % tumour tetramer+ activated over time. C) Per patient % microbial tetramer+ activated over time. No statistically significant differences were detected between 3 weeks and pre-treatment for either tumour or microbial tetramer % activated or between tumour and microbial % activated at 3 weeks ($p > 0.05$ paired two-tailed t-test). D) Biaxial dot plots for patient NPB_03284 displaying total CD8+ cell activation over time (grey) with overlay of tumour tetramer+ cells (blue) and microbial tetramer+ cells (pink).

3.6 Combination checkpoint inhibitor therapy is associated with an increase in the breadth of the anti-tumour response and diversification of TCR usage of existing tumour-specific T cells

Given that we observed an increase in frequency of tumour tetramer+ cells during treatment, this raised the question of whether this was being driven by expansion of existing tumour-specific T cells or the emergence of new tumour-specific T cells. In the experiments described in Section 3.5, we used a cocktail of tetramers loaded with different TAA peptides. In order to tease apart the response to each individual peptide, we performed

single cell sorting of tumour tetramer+ cells from the 5 individuals analysed previously and microbial tetramer+ cells from 3 of these individuals, and established T cell clones from these cells. Using the same combinatorial barcoding approach described in Section 3.4, we tested established clones against the individual constituent peptides from the pool of tetramers based on which they were sorted. Across all tetramer-based sorting/cloning experiments for these 5 patients, 1886 single cells were sorted and 728 clones were established (expanded beyond 96 well plates) giving an overall cloning efficiency of 38.6%. A breakdown of cloning efficiency by patient, specificity and timepoint is shown in Appendix B, Table B-1 & B-2. Across all patients, no statistically significant differences in cloning efficiency were detected between tumour tetramer positive and microbial tetramer positive cells overall (35.4% vs 46.6%), or at individual timepoints (Appendix B, Figure B-1 & B-2). However, variation of cloning efficiency was seen over time, with cloning efficiency at 3 weeks being significantly lower for both tumour and microbial tetramer+ cells compared to the late on-treatment timepoint in both cases, and compared to pre-treatment for microbial tetramer+ cells (Figure 3-18).

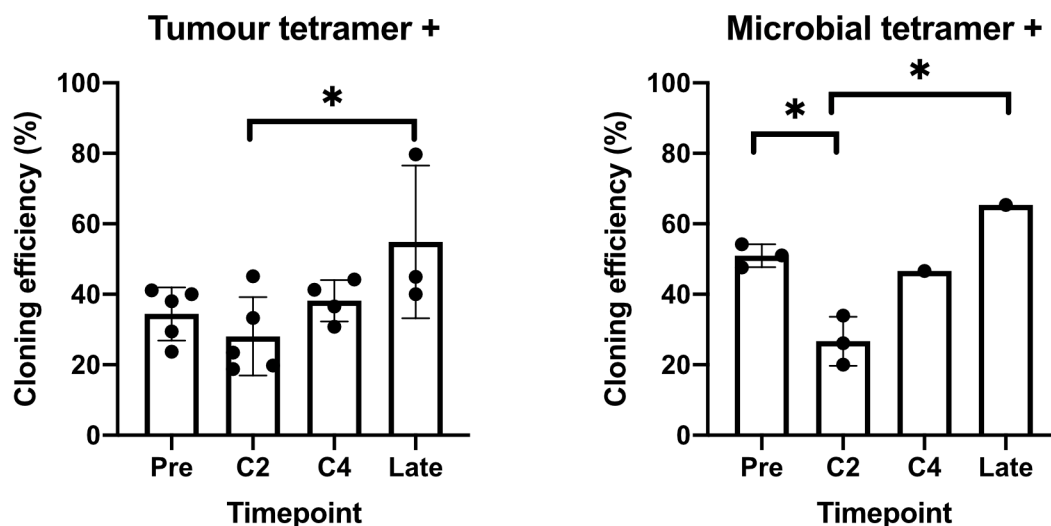


Figure 3-18. Cloning efficiency of tumour tetramer+ and microbial tetramer+ cells sorted at different timepoints on treatment. Cloning efficiency (mean±SD) by tumour or microbial specificity and timepoint across 5 patients. Tumour tetramer+ at C2 vs Late adjusted p -value = 0.0452. Microbial tetramer+ at C2 vs Pre adjusted p -value = 0.0272, C2 vs Late adjusted p -value = 0.0211 (One way ANOVA with Holm-Sidak's multiple comparisons test).

A total of 430 clones were successfully maintained and tested for their specificity. 84/430 (19.5%) clones sorted based on tetramer positivity were not found to be reactive to any of the peptides tested, suggesting either that their tetramer positivity was due to background staining, or that they were not antigen responsive after ex vivo expansion. For the remaining 80.5% we were able to determine a specificity to either tumour (293) or microbial peptides (53). Tumour-reactive T cell clones were found for all 5 patients, with reactivity observed against a total of 12 different tumour peptides from our panel of 25 (Table 3-6). Despite being public antigens, the only antigen for which we were able to isolate reactive T cells in all patients was MART-1, while 9 of the reactivities detected were found in only one patient. However, since the clones isolated represent only a very small fraction of the circulating T cell repertoire we cannot exclude the presence of T cells specific for these antigens in the peripheral blood of the tested patients.

Table 3-6. Specificities of tumour peptide reactive clones detected

Peptide	Antigen	Peptide sequence	No. positive clones detected	No. patients in which detected
3	gp100	ITDQVPFSV	1	1
4	gp100	KTWGQYWQV	2	1
5	gp100	YLEPGPVTA	4	3
6	gp100	VLYRYGSFSV	1	1
10	LAGE-1	MLMAQEALAFI	1	1
13	MAGE-A10	GLYDGMEHL	35	1
16	MAGE-A3	KVAELVHFL	1	1
18	MAGE-C2	ALKDVEERV	17	1
20	MAGE-C2	LLFGLALIEV	27	2
21	MART-1	ELAGIGILTV	186	5
22	Meloe-1	TLNDECWPA	2	1
26	SSX2	KASEKIFYV	15	2
28	Tyrosinase	YMDGTMSQV	1	1

Microbial antigen reactive T cells were identified in all patients on whom microbial tetramer sorting was performed. Reactivities were seen against the peptide for the Flu M1 antigen for 2 of the 3 patients, CMVpp65 in 1 individual and the dust mite antigen Der p 1A in 1 individual. The number of clones of different specificities isolated for each patient at each

timepoint are shown in Figure 3-19 and Figure 3-20 for tumour and microbial-specific clones respectively.

Antigen	Peptide	3284				3640		3659				3661		3663			
		Pre	C2	C4	Late	Pre	C2	Pre	C2	C4	Late	Pre	C2	Pre	C2	C4	Late
gp100	ITDQVPFSV		1														
gp100	KTWGQYWQV													1			1
gp100	YLEPGPVT		1		1							1			1		
gp100	VLYRYGSFSV						1										
LAGE-1	MLMAQEALAF		1														
MAGE-A10	GLYDGMEHL															5	26
MAGE-A3	KVAELVHFL											1					
MAGE-C2	ALKDVEERV		3	11	3												
MAGE-C2	LLFGLALIEV		3	12	8										1	3	
MART1	ELAGIGILT	11	18	29	30	6	5	12	14	9	19	7	12	3	3	2	5
Meloe-1	TLNDECWPA						2										
SSX2	KASEKIFYV	1	2	5	6			1									
Tyrosinase	YMDGTMSQV				1												
Total		12	29	57	49	6	8	13	14	9	19	7	14	4	10	32	9

Figure 3-19. Reactivities of tumour-specific T cell clones. Number of T cell clones isolated specific for tumour peptides listed are shown per patient and timepoint for 5 individuals. Green = 1 clone, yellow = 2-5 clones, red = >5 clones. Numbers in boxes indicate exact number of clones with total positive in bottom row.

Antigen	Peptide	3284			3661		3663	
		Pre	C2	C4	Pre	C2	Pre	C2
Flu M1	GILGFVFTL	20	5	10			8	1
Der p 1A	MMIEEYPYV							1
CMVpp65	NLVPMVATV				7	1		
Total		20	5	10	7	1	8	2

Figure 3-20. Reactivities of microbial-specific T cell clones. Number of T cell clones isolated specific for tumour peptides listed are shown per patient and timepoint for 3 individuals. Green = 1 clone, yellow = 2-5 clones, red = >5 clones. Numbers in boxes indicate exact number of clones with total positive in bottom row.

Newly detected tumour peptide specific responses were detected at C2 after 3 weeks of treatment in 4 of the 5 patients tested (median 2 new responses, range 0-5). Aside from a single isolated Der p 1A specific clone, no new microbial responses were detected after 3 weeks of treatment. Regarding the newly detected anti-tumour responses, in some patients these were maintained, whilst in others they disappeared. For example, in patient 03284, newly detectable MAGE-C2 responses were observed after 3 weeks and persisted through

ongoing treatment, however newly detected gp100 and LAGE-1 responses appeared transient. In patient 03663, we were also able to newly detect MAGE-C2 responses at 3 weeks on treatment, however in this individual they were maintained until 9 weeks but no longer detectable in a late on-treatment sample. Overall this data is consistent with evidence of diversification of the anti-tumour, but not anti-microbial, response during combination checkpoint inhibitor treatment similar to what has been observed with Ipilimumab alone¹³⁷. However, as the lower frequency of tumour tetramer+ cells prior to treatment limited the number of cells we were able to sort and clone from this timepoint, we cannot be certain whether these responses were present, but just not detectable by this approach before treatment. It also does not rule out concurrent expansion of pre-existing clones, as we do not know the absolute frequencies of the various different specificities amongst the total population.

Whilst this data has demonstrated an increase in the number of different antigen-specific responses detectable following treatment, we hypothesised that diversification of the TCR repertoire for individual pre-existing antigen-specific responses might also occur in response to treatment. To answer this question, we performed TCR sequencing of the tumour peptide reactive clones generated from these sorting experiments and looked at changes in TCR usage for clones that were detectable both before and during treatment. For MART-1 tumour reactive T cell clones, we observed a number of new TCR $\alpha\beta$ combinations after just 3 weeks of treatment, with further remodelling occurring over time (data from one individual shown in Figure 3-21). Some of these newly detected TCR $\alpha\beta$ clones were maintained and could still be detected in the blood late-on-treatment, whilst others were detectable only transiently and then no longer found.

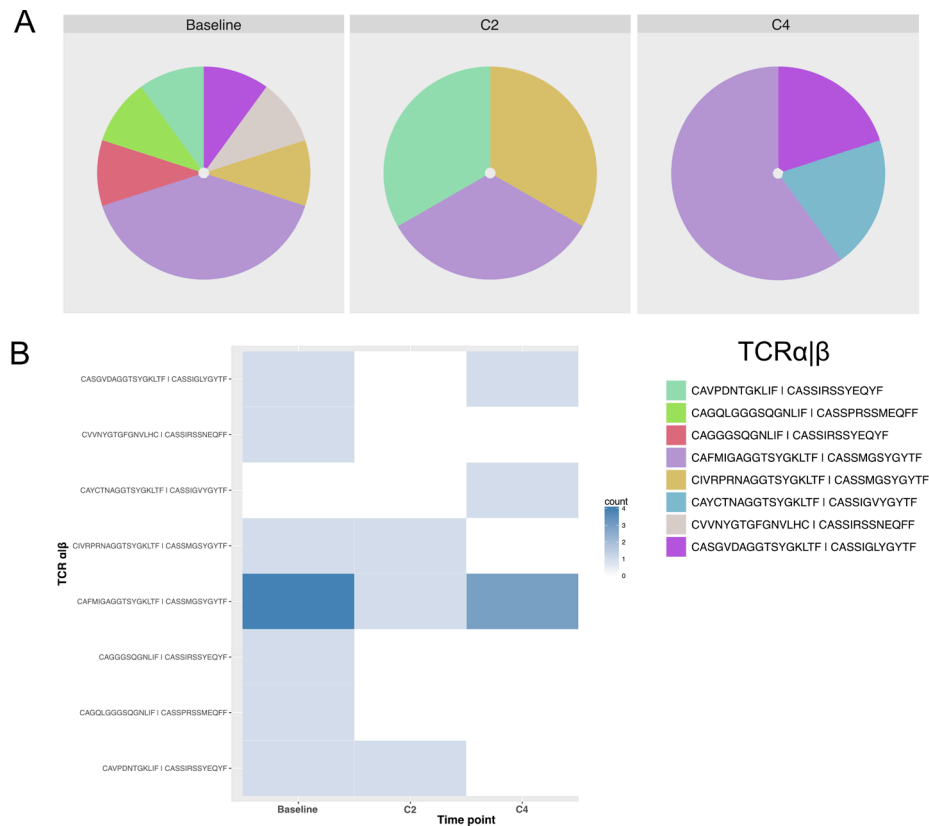


Figure 3-22. Flu-specific T cell clones as defined by TCRαβ pairs for patient NPB_03284. A) Pie chart representing each TCRαβ clone as a proportion of all T cell clones expanded at each timepoint over the course of therapy. B) Tracking plot showing individual clones and number of counts at each timepoint.

3.7 Changes in T cell functionality are observed with treatment and appear to be independent of the TCR

So far we have considered whether treatment affects the breadth or depth of the antigen-specific T cell response, however this does not capture whether treatment induces functional changes in the T cells themselves. Previous work by Simon *et al.*¹⁴³ in patients receiving anti-PD-1 therapy demonstrated amplification of MART-1-specific Vβ subfamilies in responding patients and that these emerging clonotypes displayed a higher functional avidity for their cognate antigen than clonotypes not amplified upon treatment. In their study, PBMCs were stimulated with MART-1 peptide for 14 days before isolating expanded polyclonal T cell populations via tetramer-based sorting, performing further expansion and then identification of the Vβ sequence. This approach leads to intra-clonal competition, and

does not allow the characterisation of functional capacity of individual T cells. Whilst our cloning approach might provide different sources of bias, we reasoned that it might more accurately reflect the range of different functionalities in vitro. For patients where we had multiple clones of the same specificity derived from T cells obtained at different timepoints on treatment, we therefore tested their functional avidity as determined by the percentage of cells producing IFN- γ in response to reducing concentrations of peptide as described in Chapter 2.9.4. The concentration at which 50% of the maximal IFN γ for each clone was produced (EC_{50}) was determined from non-linear regression curves fitted to the data and compared between clones derived from different timepoints on treatment (Figure 3-23 A).

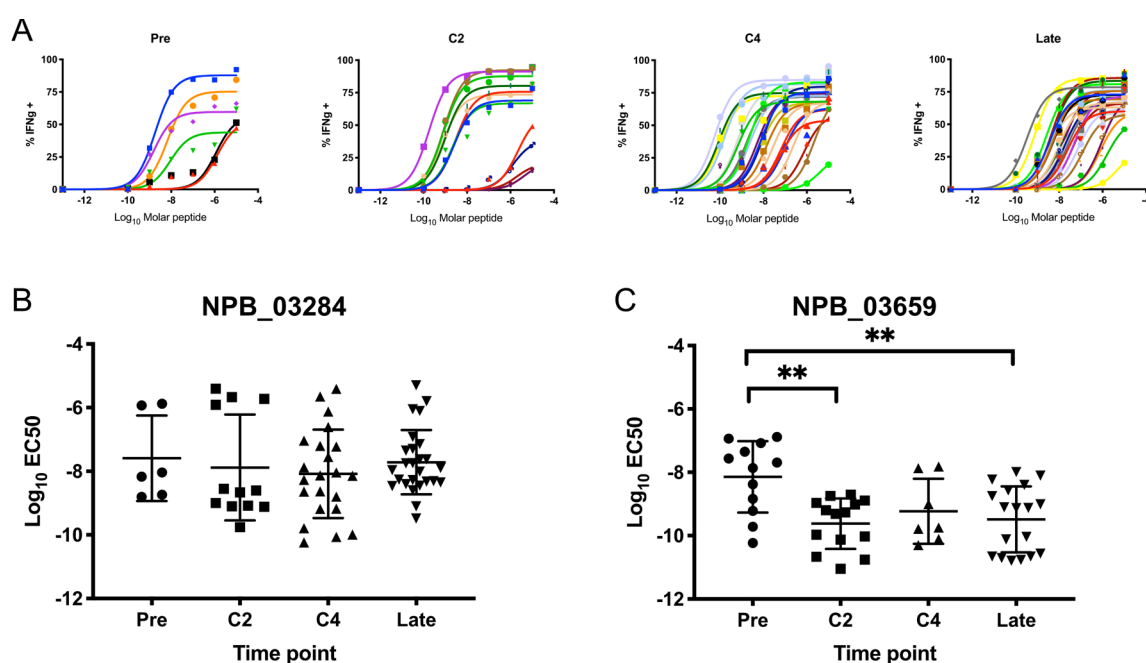


Figure 3-23. Functional avidity testing of MART-1 clones. A) Example log-agonist response curves for one individual (NPB_03284) at different timepoints on treatment showing percentage of cells producing IFN- γ in response to reducing concentrations of peptide. Each curve represents a single MART-1 specific T cell clone. B) Log₁₀EC₅₀ values for MART-1 clones from each timepoint for patient NPB_03284 and C) NPB_03659. Individual values and mean $\pm$ SD. Adjusted p -value for NPB_03659 Pre vs C2 0.0014, Pre vs Late 0.0022, one-way ANOVA with Dunnett's multiple comparison test.

For the two individuals (NPB_03284 and NPB_03659) where we had sufficient numbers of MART-1 clones over time, there appeared to be differences in the functional avidity as defined by the EC_{50} value between clones isolated at different times (Figure 3-23 B & C).

In patient NPB_03659 there was a significant reduction in EC_{50} value (i.e. a lower peptide concentration required to produce 50% maximal IFN- γ) for clones isolated at C2 and C4 compared with those from pre-treatment. Similar changes were observed for a subset of clones for patient NPB_03284, but there appeared to be substantial heterogeneity with two discrete groups evident at C2.

To better quantify the differences in cells of different functional avidities over time, we performed hierarchical clustering of all clones for each patient from all timepoints on the basis of their IFN- γ production at different timepoints (Figure 3-24 A-D). Clones were then split into 4 groups based on the clustering of low, medium, medium/high and high functional avidity. The percentage of cells at each timepoint present in each of these groups was then represented in a 4 x 4 matrix (Figure 3-24 E & F). This demonstrated that there appeared to be a shift towards increasing proportions of higher avidity clones over the course of therapy.

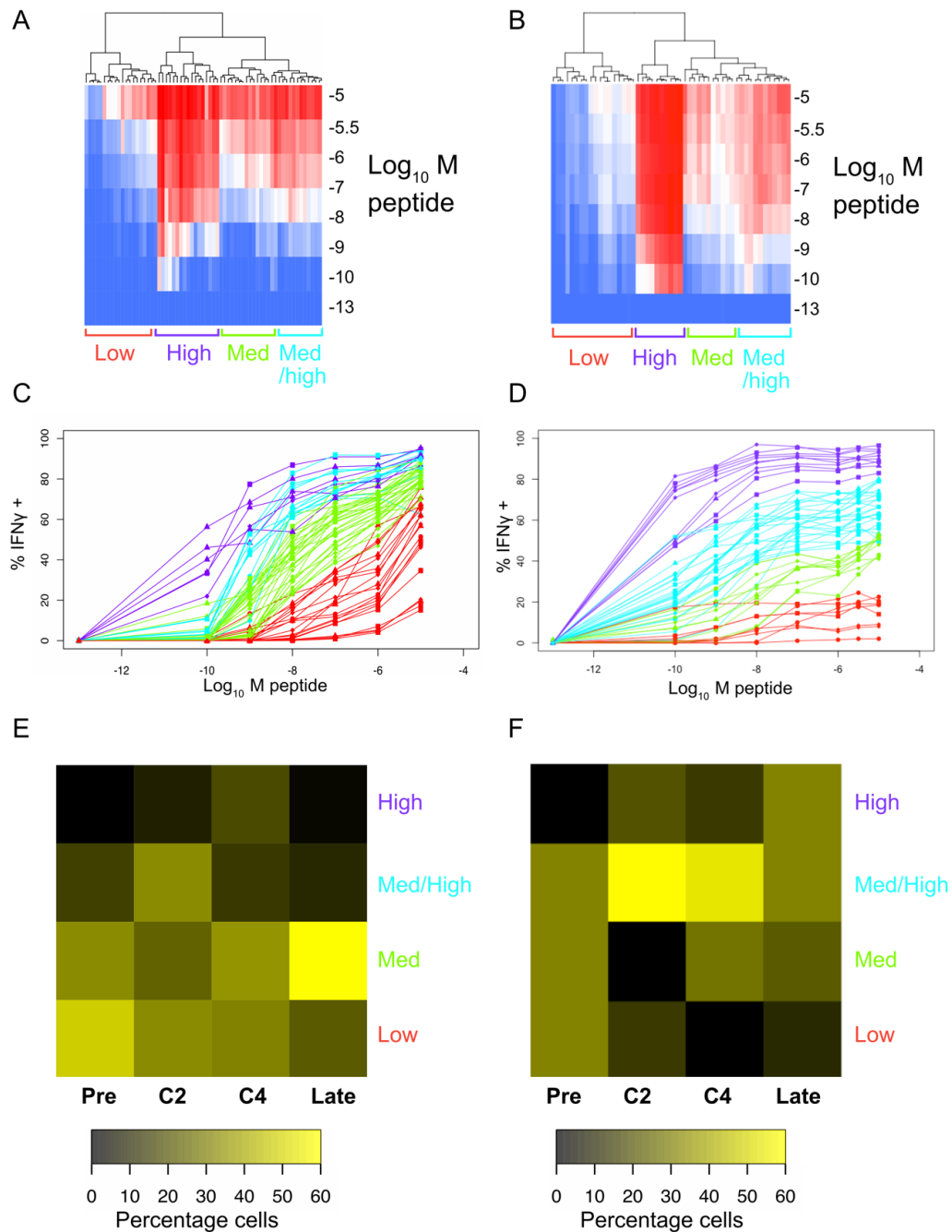


Figure 3-24. Hierarchical clustering of all clones based on functional avidity. A & B) Hierarchical clustering of clones from Patient NPB_03284 (A) and NPB_03659 (B) based on percentage IFN- γ produced in response to reducing concentrations of peptide. Based on this, clones are divided into 4 clusters – low, med, med/high and high functional avidity. C & D) Log-agonist curves of all clones from all timepoints for patients NPB_03284 (C) and NPB_03659 (D) coloured by functional avidity cluster. E & F) Percentage of cells (clones) found in each functional avidity cluster at different timepoints on treatment for NPB_03284 (E) and NPB_03659 (F).

In order to try and determine whether this functional avidity was related to the TCR sequence, we grouped cells by clonal group on the basis of shared TCR $\alpha\beta$ for patient NPB_03284 and looked how the EC₅₀ value changed during treatment (Figure 3-25). Insufficient clonally expanded TCRs were found for patient NPB_03659 to allow similar analysis. T cell clones with the highest functional avidity appeared to consist of emergent TCR clonal groups that we had not been able to detect prior to treatment, with some being seen only transiently and others persisting.

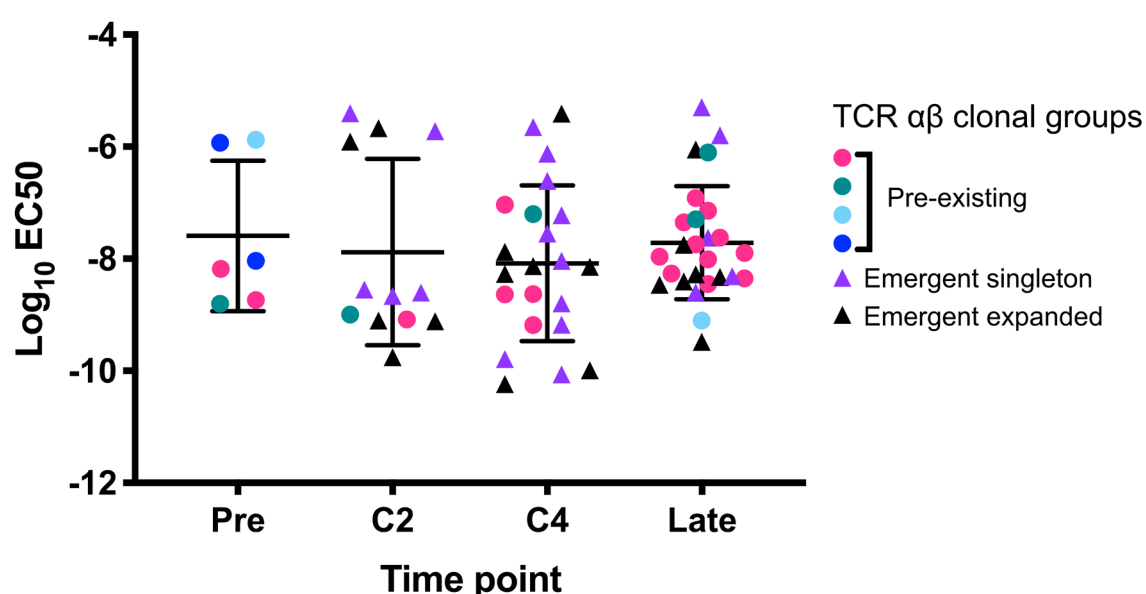


Figure 3-25. Functional avidity of MART-1-specific clones by TCR. MART-1-specific T cell clones for patient NPB_03284 coloured by whether TCRs were pre-existing or emergent and whether they were expanded or observed only once (singletons). Circles = clones detectable prior to treatment. Triangles = clones newly detectable on therapy.

Interestingly, changes in functional avidity within clonal groups over time were observed, including of those that were detected prior to treatment. Given that these differences cannot be accounted for by the TCR, this could be consistent with therapy induced changes in T cell functionality, and this might be dependent on the history of each individual clone rather than on the sequence of its T cell receptor.

3.8 Attempts to extend our observations beyond tumour-associated antigens

Whilst in our previous experiments we had used T cells specific for tumour-associated antigens to measure the tumour-specific responses to checkpoint inhibitor treatment, evidence suggests that neoantigen-specific T cells play an important part in mediating response¹³⁶. In an attempt to extend our observations beyond tumour-associated antigens, and to see whether neoantigen-specific responses behave in the same manner, we identified two patients where tumour samples had been obtained prior to commencing treatment with anti-PD-1 monotherapy and performed haplotyping (Table 3-7).

Table 3-7. Clinical characteristics and haplotypes of patients with pre-treatment tumour samples

Patient ID	Sex	Treatment received	HLA-Class I haplotype
P20c	F	Pembrolizumab	A*02:01/11:01 B*15:01/35:01 C*03:04/04:01
A009	F	Pembrolizumab	A*02:01/03:01 B*40:01/40:02 C*02:02/03:14

For patient P20c, a single cell suspension had been previously generated from an excised tumour metastasis and frozen. From this we were able to generate a melanoma tumour cell line, as confirmed by positive flow cytometry staining (Figure 3-26) for expression of melanoma-associated chondroitin sulfate proteoglycan (MCSP) antigen, known to be expressed on the majority of human melanoma tissues and cell lines but not on fibroblasts. Staining for the common tumour-associated antigens MART-1 and gp100 however was negative (Figure 3-26), so in order to confirm the identity of the melanoma cell line generated, and identify tumour antigens shared between this line and the original tumour sample, we performed RNA and exome sequencing on the tumour line, together with exome sequencing of the patient's germline DNA and the original single cell suspension of the excised tumour.

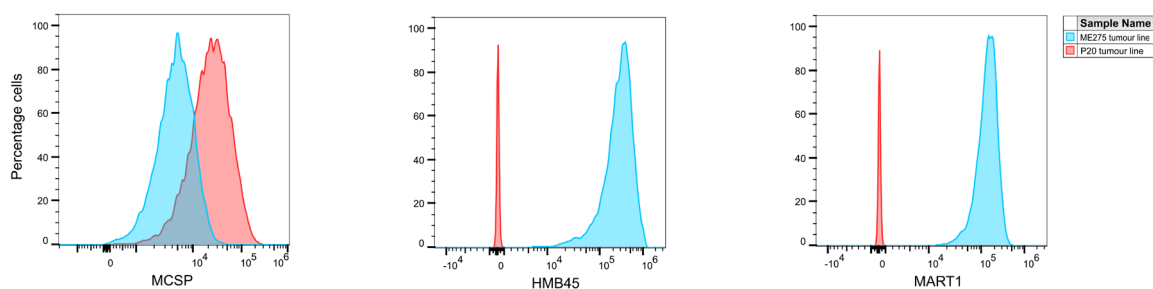


Figure 3-26. Tumour line staining for tumour-associated antigens. Histograms showing expression of MCSP, HMB45 (gp100) and MART-1 by FACS for P20 tumour cell line (red) and known established melanoma cell line ME275 (blue). ME275 tumour line is positive for all three markers, P20 tumour line positive for MCSP only.

This allowed us to determine somatic variants present in the original tumour sample single cell suspension but not in the germline and then compare the overlap of these somatic variants between the single cell suspension and the tumour line generated. The tumour mutation burden was similar for both the original tumour and the tumour cell line (4 vs 4.03 somatic mutations per megabase respectively) with a total of 1493 filtered somatic variations detectable in the primary tumour sample and 1418 detectable in the tumour line (Table 3-8).

Table 3-8. Somatic variants detected in P20 Primary tumour and P20 tumour line

Sample	Total somatic variants (n)	Unique somatic variants (n)	Shared somatic variants (n)	Shared somatic variants (%)
Original tumour	1493	252	1062	71%
Tumour line	1418	208	1062	75%

Of these, 1062 were shared between both the original tumour and the tumour cell line amounting to 71% of the mutations in the original tumour being seen in the tumour cell line and 75% of those seen in the tumour cell line being detectable in the original tumour. To check if there was any further evidence for the non-shared mutations detectable in the tumour line being present in the primary tumour, we looked for read evidence in the unfiltered mutations from the primary tumour and found that 84% of the mutations present in the tumour line could be detected in the original tumour. Whilst the overlap remains

incomplete, the discrepancy can likely be explained by insufficient read coverage to detect low-frequency mutations, or genetic drift of the tumour cell line.

We also went on to look at the expression of 145 known shared melanoma-associated antigens¹³⁷ in the tumour cell line based on the RNA-seq expression data (Table 3-9). Seventy-eight of these had detectable expression in the tumour cell line, although notably consistent with our FACS staining, *MLANA*(MART-1) expression was negligible. Taken together, these data provide evidence in support of this being an autologous tumour line with reasonable representation of the primary tumour.

Table 3-9. Expression of selected melanoma-associated genes in the P20 tumour cell line

Gene	Transcripts per million
<i>PRDX5</i>	576.592813
<i>PRAME</i>	147.098576
<i>BRAF</i>	39.5459349
<i>MAGEA4</i>	32.630333
<i>PMEL</i>	4.48361481
<i>MLANA</i>	0.16124472
<i>MAGEA1</i>	0
<i>MAGEA10</i>	0
<i>MAGEA2</i>	0
<i>MAGEA3</i>	0
<i>MAGEC2</i>	0

Having established an autologous tumour line for this patient, we then went on to perform single cell sorting of CD8+ T cells from PBMCs activated (expressing CD38+HLA-DR+ or CD38+CD39+) after 3 weeks on therapy. In total, 76 T cell clones were established and tested for reactivity against both allogeneic tumour cell lines, tumour and microbial peptides as well as the autologous cell line. A total of 10 clones were found to be tumour-reactive, 6 of which were reactive against allogeneic tumour cell lines, 3 which reacted to both allogeneic tumour cell lines and the autologous cell line, and 1 which reacted to the autologous cell line only (Figure 3-27). No reactivity was observed for any clones against TAA peptides.

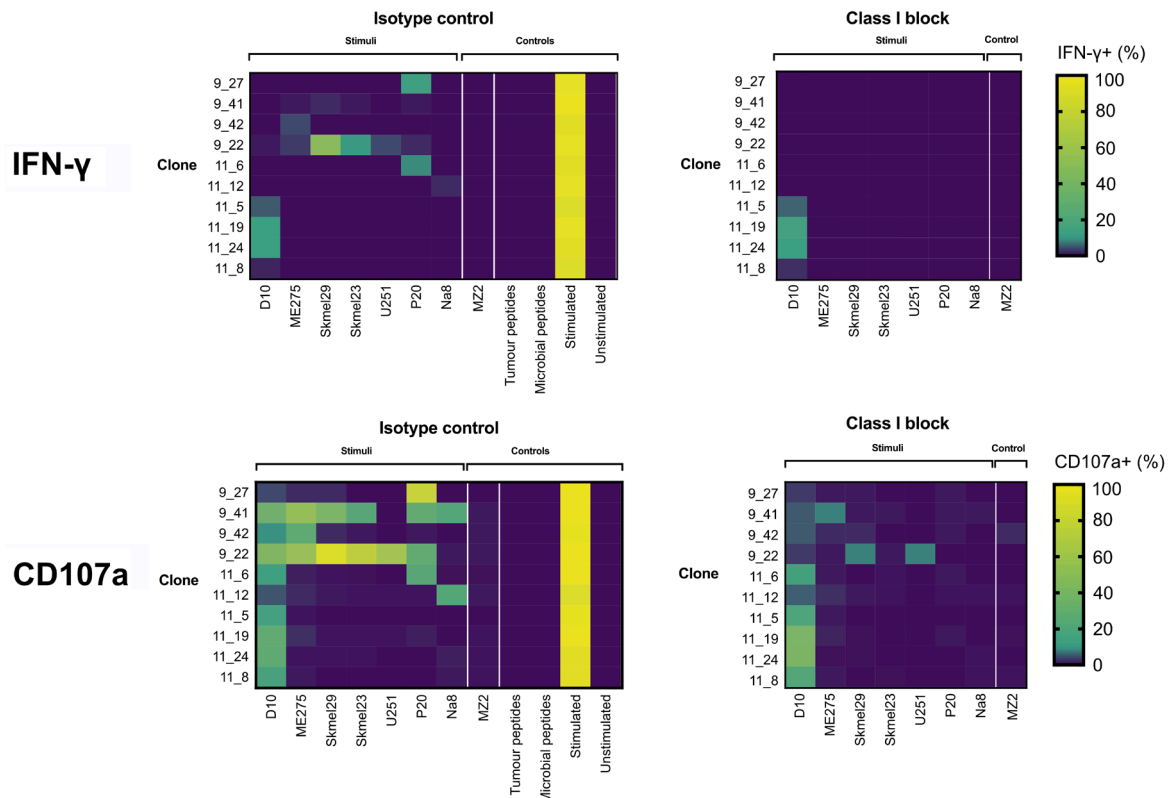


Figure 3-27. Heatmap displaying reactivity of tumour reactive clones from patient P20. Production of IFN-γ (top) and CD107a (bottom) in response to stimulation with various tumour cell lines, peptides and controls in the presence of an IgG2a isotype control antibody (left), or class I blocking antibody W6/32 IgG2a (right). Clone 9_27 demonstrates class I dependent reactivity against an autologous cell line but not allogenic cell lines.

Interestingly, as well as again seeing T cells capable of recognising the Na8 cell line (clone 9_41 and clone 11_12) for which no TAA have been previously documented, one of the clones that we tested (9_22) was also able to recognise a glioblastoma cell line, possibly representing reactivity to a TAA shared between both the melanoma and glioblastoma cell lines. Whilst the clones reacting to both the allogeneic and autologous cell lines are likely to be recognising shared melanoma-associated antigens not included in our peptide pool, it is possible that the T cell clone reacting against only the autologous tumour line might be neoantigen reactive, although we cannot be certain it is not reacting against a tumour-associated antigen expressed on the autologous cell line but not any of the other cell lines tested.

As we had been unable to demonstrate the presence of a significantly expanded T cell population reactive against the autologous cell line in patient P20, or to conclusively demonstrate the presence of neoantigen-specific cells within the activated population, we tried an alternative approach testing specifically for neoantigen-specific T cells. For this we used PBMC samples and sequencing data from patient A009 provided by our collaborator (Christian Ottensmeier, University of Southampton) who had performed germline and tumour exome sequencing together with RNA sequencing of this individual before and during Pembrolizumab therapy.

Using the data from the tumour and germline exome sequencing we were able to identify 2583 somatic variants present in the tumour but not the germline (tumour mutational burden 11.06 mutations/Mb). A total of 664 non-synonymous mutations (643 missense, 2 in frame deletions, 4 in frame insertions and 15 frameshifts) were then considered together with the patient's HLA-type to determine 6539 HLA-A2 restricted potential 9mer neopeptides. HLA binding affinity predictions, together with RNA expression data, were then used to rank the peptides for their potential antigenicity and the top 50 by priority score chosen for synthesis. We then performed single cell sorting and cloning of cells activated in response to treatment based on CD38 $\pm$ HLA-DR $\pm$ CD39 expression, although activation for this individual was relatively modest compared to others we have studied (Figure 3-28).

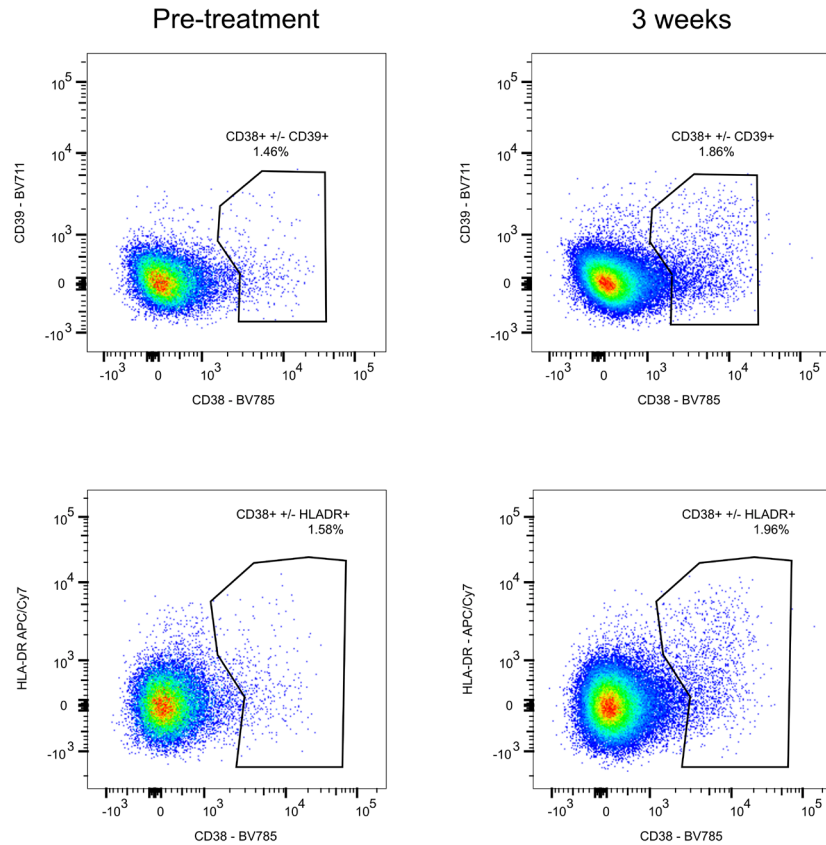


Figure 3-28. Expression of activation markers CD38, HLA-DR and CD39 before and during treatment for patient A009. Biaxial plots of CD38 vs HLA-DR or CD39 expression on CD8+ T cells pre-treatment and at 3 weeks on anti-PD-1 monotherapy checkpoint inhibitor treatment for patient A009.

Unfortunately, we were only able to successfully test a total of 34 clones against pools of the predicted neoantigen peptides as well as allogeneic HLA-matched tumour cells lines and TAA and microbial peptides. Whilst 6 clones reactive against allogeneic tumour cell lines were found, none tested were reactive to the neoantigen, TAA or microbial peptides. Given the relatively low numbers of clones available for testing it is possible that this lack of neoantigen peptide responsiveness could have been a false negative. We therefore went on to synthesise tetramers for these peptides and performed direct ex-vivo tetramer staining of PBMCs for this patient both before and at 3 weeks on timepoint. Tetramers were dual labelled with 2 different fluorophores to increase specificity. Minimal positive signal was seen in the tetramer positive channel, despite signal being evident in the positive control of

a flu tetramer synthesised in the same exchange reaction tested against PBMCs with flu specific T cell clones spiked-in (Figure 3-29).

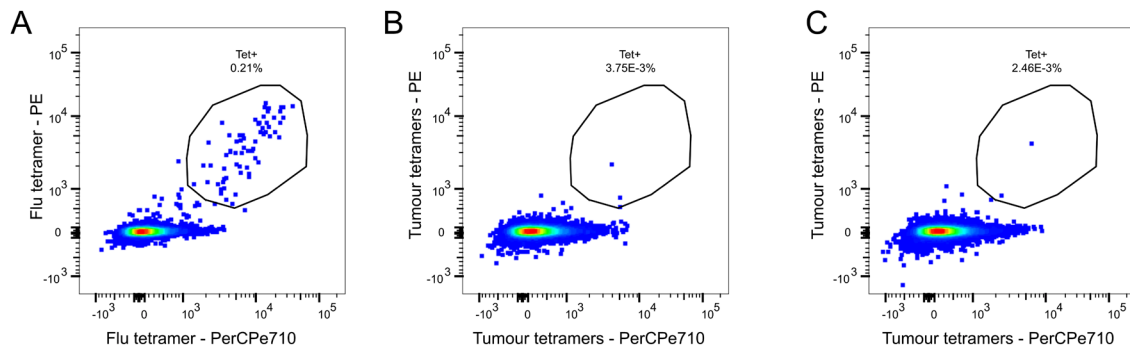


Figure 3-29. Tetramer staining for patient A009. A) PBMCs with flu specific T cell clones spiked-in stained with dual-labelled PE/PerCPe710 Flu tetramer made in the same tetramer exchange as the tumour peptides. B) CD8+ T cell population for Patient A009 pre-treatment and C) on-treatment stained with dual-labelled PE/PerCPe710 tetramers made with peptides for predicted neoantigens.

A total of 3 cells across the two timepoints which were sorted based on neoantigen tetramer positivity were successfully expanded to form T cell clones. These were then tested against the pools of neoantigen predicted peptides to try and confirm antigen reactivity. No reactive clones were found by this method, and repeat tetramer staining of the expanded clones was negative. Together with the extremely low frequency, these results are most consistent with the few tetramer positive cells seen being false positives, and overall no reactive T cells being detected. Despite our attempts, we were therefore unable to validate our previous observations for TAA in the neoantigen setting. A number of possible explanations exist for this. It is of course possible, that neoantigen-specific T cells behave in a different manner to TAA-specific T cells, however these results must be considered in the context of the challenges associated with accurately predicting neoantigens, and the limited number of both peptides and cells being tested. As such, it is entirely possible we did not have the right predictions or that the patients tested did not have a substantial neoantigen-specific response.

3.9 TCRs identified from activated CD8+ T cells in the periphery can found within the tumour

One question that arises when studying the antigen-specific response in the peripheral blood is whether these changes reflect intra-tumoural changes. In an attempt to determine whether the T cell clones we had isolated from the periphery could be found within the tumour we looked to see if we could find evidence of TCRs from the T cell clones generated from activated single cells within tumour from the same individuals, and whether these were tumour-reactive. To do this we performed TCR sequencing of RNA extracted from bulk FACS sorted TILs for two individuals (P20 and A009) and cross-referenced the TCRs from the clones generated in the previously described experiments against those found in the tumour (Figure 3-30). For patient A009, whilst we were able to find some isolated alpha (TRA) or beta (TRB) sequences within the TIL population matching to peripheral T cell clones, only one T cell clone from the periphery was found where both the alpha and beta chain could be found amongst the TILs (clone B247). This was however seen at a relatively low frequency within the TIL population (frequency of 0.5% amongst TIL TRA reads and 0.2% amongst TRB reads) and none of the highly expanded TIL clones (the largest of which occupied 24% of TRA and 26% of TRB TIL reads) were detected amongst the clones we isolated in the periphery. None of the TCRs from T cell clones that we identified for this patient that were reactive to allogenic tumour cell lines were found within the TIL population, although in the absence of an autologous tumour line it is not possible to know whether clones such as B247 would be reactive against this.

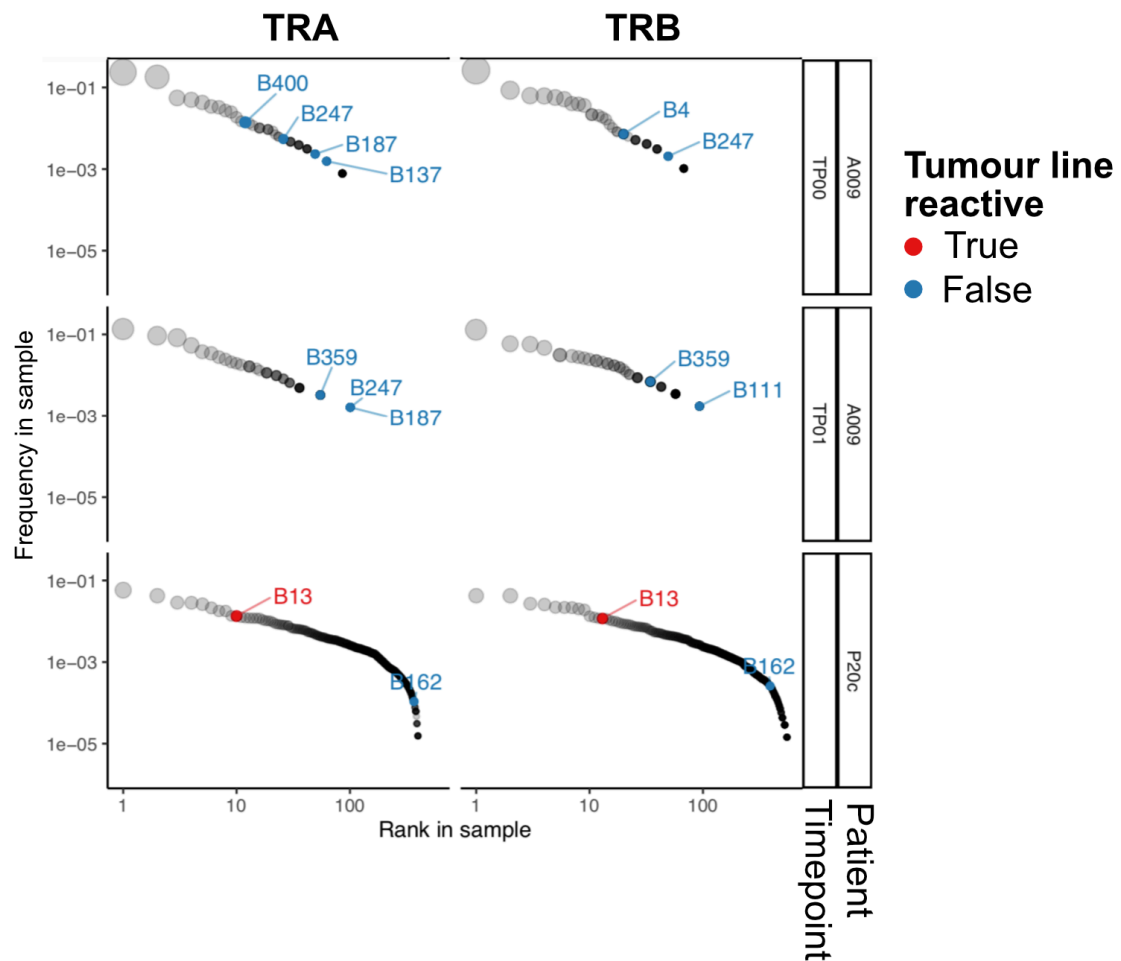


Figure 3-30. Matching of TCRs from peripherally isolated T cell clones with TCRs found within the tumour for patient P20 and A009. T cell receptor alpha (TRA) and T cell receptor beta (TRB) sequences found within the tumour are plotted by frequency within the total TIL population and by rank within the sample. Matches at the respective alpha or beta chain with T cell clones in the periphery are labelled by their clone ID with colour indicating whether they were tumour cell line reactive. For patient A009 TP00 is a pre-treatment and TP01 is after three weeks of anti-PD-1 therapy. Size of the circle is proportional to frequency of the TRA or TRB reads within the TIL population.

By contrast, for patient P20, although we only identified 10 tumour reactive T cell clones amongst those established from sorting of activated cells, the TCR $\alpha\beta$ sequence for one of these (clone 9_27/B13) was found at a relatively high frequency amongst both the TRA and TRB chains isolated from pre-treatment TILs, ranking 10th most frequent out of 402 TRA-TIL sequences and 13th most frequent out of 559 TRB-TIL sequences. Interestingly, this was the only T cell clone we isolated which reacted to the autologous tumour line but not to allogeneic tumour lines, suggesting it could indeed be a neoantigen-specific clone.

3.10 Summary

In this chapter, we have described in-depth immune monitoring for a total of 9 patients receiving checkpoint inhibitor therapy, characterising both phenotypic changes and antigen-specific responses. We have demonstrated that combination anti-CTLA-4 and anti-PD-1 checkpoint inhibitor treatment produces activation of both CD4+ and CD8+ T cells, and that the activated CD8+CD38+HLA-DR+ T cell population contains cells capable of recognising allogeneic melanoma cell lines or melanoma-associated peptides. Whilst increased expression of HLA-DR within the total CD8+ T cell population has been previously described in response to anti-CTLA-4^{108,113-115,119} and anti-PD-1 monotherapies^{129,132}, and in a small study in combination therapy¹¹⁸, direct evidence that this activated population contains tumour-specific T cells has previously been lacking. Furthermore, in addition to showing that this population contains tumour-specific T cells, we have demonstrated that activation appears to be selective for tumour-specific over microbial-specific T cells, suggesting that activation might be a defining characteristic of the anti-tumour response to checkpoint inhibitor immunotherapy.

In addition to activation, we observed transient increases in the frequency of tumour-reactive but not microbial-reactive T cells detectable in the periphery following combination checkpoint inhibitor treatment. Whilst this expansion was partially accounted for by the emergence of T cells with newly detected specificities, consistent with observations of an increase in the breadth of the anti-tumour response in previous studies of anti-CTLA-4 monotherapy^{115,137}, we also saw expansion of pre-existing tumour-reactive T cells. Amongst the expanding pre-existing tumour-reactive T cells we observed, as defined by their TCR, both expansion of pre-existing T cell clones and the emergence of expanded T cell clones utilising different TCR $\alpha\beta$ chains. Together this suggests that combination therapy is capable both of inducing novel anti-tumour responses, likely through the contribution of CTLA-4 blockade, and increasing the depth of the pre-existing anti-tumour response, likely as a result of PD-1 inhibition.

As well as studying changes in T cell phenotype and specificity in response to treatment, we also looked at T cell functionality as a result of checkpoint inhibitor therapy. Previous work by Simon *et al.*¹⁴³ using ex-vivo stimulated PBMCs has shown that MART-1-specific T cell clones amplified after 2 months of anti-PD-1 therapy have altered functional avidity compared to T cell clones detectable pre-treatment and those that did not expand upon therapy. Using unstimulated T cell clones expanded from single ex-vivo MART-1-specific T cells isolated at different timepoints during combination checkpoint inhibitor therapy, we were able to see similar changes in the functionality of MART-1-specific T cells. However, our time-course analysis showed that this effect appeared to be transient and was not sustained with ongoing therapy. Additionally, sequencing of the TCRs of these expanded clones showed that changes in functional avidity could be observed over time between cells bearing the same TCR, suggesting that some inherent difference in the phenotype or functionality of the T cells induced by the treatment was driving this, independent of the TCR.

Whilst limited by availability of tumour samples, in an attempt to expand our observations beyond tumour-associated antigens we also tried to ascertain whether neoantigen-specific cells displayed similar behaviour in response to treatment. Although we were not able to prove this definitively, we were able to isolate for one individual a T cell clone amongst the peripheral CD8⁺ T cell population activated by anti-PD-1 treatment which displayed selective reactivity to an autologous tumour line and could also be found amongst pre-treatment TILs isolated from this individual. Even though we cannot be certain that this was a neoantigen-specific T cell response, the observation that T cells isolated in the periphery can also be found intra-tumourally provides support for the relevance of peripheral immune monitoring to the anti-tumour response.

As well as providing insight into the antigen-specific immune response to combination checkpoint inhibitor therapy, these data also demonstrate how the analysis of activated T cells in the peripheral blood of patients receiving immunotherapy might represent a source of tumour-specific T cells for treatments such as adoptive cell therapies, as well as an antigen unbiased approach for studying the composition of tumour-specific responses. The observation that T cell functionality might be dependent on the history of each individual clone rather than the sequence of its TCR might also have implications for the choice of T cell clones used for adoptive cell therapy, as distinct clones bearing the same TCR sequence might have distinct functionality. Furthermore, isolation of T cell clones for adoptive therapy during immune checkpoint therapy might provide not only a larger pool of tumour-specific clones, but also clones with better functionality.

There are however a number of limitations posed by these analyses. Whilst we have characterised changes in a great level of detail, these analyses are confined to a very small number of individuals. In addition to the small number, differences observed between patients may be confounded by the potentially differing doses of Ipilimumab and Nivolumab received within different clinical trial settings. Prior studies have however demonstrated that doses of Nivolumab as low as 1mg/kg lead to complete saturation of PD-1 receptor on circulating T cells⁸⁹ so this is unlikely to cause substantial differences between patients in this cohort treated with 3mg/kg or 1mg/kg of Nivolumab. However, previous work on patients treated with escalating doses of Ipilimumab showed that whilst HLA-DR expression on both CD4+ and CD8+ lymphocytes increased with escalating doses of Ipilimumab from 3mg/kg to 9mg/kg, this only became significant at 5mg/kg and 9mg/kg for the CD8+ population¹¹⁹, so the extent of potential differences between the patients who received 3mg/kg and 1mg/kg is unknown. A further limitation is that we have focused our analyses predominantly on HLA-A*02:01 restricted melanoma-associated antigens, as these are the more comprehensively documented. Whilst we do not know if the observations we have made hold true for other HLA restricted T cells, that we are able to observe a relatively high

number of tumour-reactive responses when restricted to just this one HLA epitope, also provides support for the activated T cell population potentially containing a much larger proportion of tumour-reactive T cells if we were able to consider all relevant HLA restrictions.

Chapter 4. Single-cell RNA sequencing of tumour-specific T cells during immunotherapy treatment

4.1 Introduction

In Chapter 3 we established that combination immune checkpoint inhibitor therapy produced activation of tumour-specific T cells, and also caused remodelling of the TCR repertoire. We also observed changes in the functional avidity of MART-1-specific T cell clones isolated from patients at different timepoints on therapy, which were not accounted for by differences in their TCR, suggesting that treatment may cause both phenotypic and functional changes to the antigen-specific response. To explore this further, we performed single-cell RNA sequencing of tumour tetramer+ cells isolated from patients at different timepoints on therapy to allow us to quantify in greater detail changes in their functionality as defined by their gene expression profile, and to correlate these changes with the TCR to see if different clones behave differently in response to treatment. We were also keen to see whether long term changes in T cells could be observed following therapy, or whether after the transient activation observed, cells reverted to their previous phenotype.

4.2 Patient selection and single cell sorting

Three patients identified from the analyses described in Chapter 3 were chosen on the basis of a relatively high frequency of melanoma-associated antigen-specific T cells detectable, and on the availability of late on-treatment blood samples for analysis. Although MART-1 was the most commonly detected antigen across patients, in view of the high number of naïve cells that exist for this antigen, and to see whether cells of different specificities behaved in different ways, we focused on the top two T cell specificities for each patient detectable from previous tetramer staining (Table 4-1). In the case of patient 3 (NPB_03659) only MART-1-specific T cells were detectable, so our analyses were limited to a single antigen specificity in this patient.

Table 4-1. T cell specificities sorted from 3 individuals for single-cell RNA-seq analysis

Patient	ID	Specificity 1 Peptide sequence	Specificity 2 Peptide sequence
1	NPB_03284	MART-1 ELAGIGILTV	MAGE-C2 LLFGLALIEV
2	NPB_03663	MART-1 ELAGIGILTV	MAGE-A10 GLYDGMEHL
3	NPB_03659	MART-1 ELAGIGILTV	NA

Tetramer staining of PBMCs pre-enriched for CD8+ T cells by immunomagnetic negative bead selection from serial timepoints on treatment was performed, using dual-labelled tetramers to increase specificity and reduce false positives (Figure 4-1).

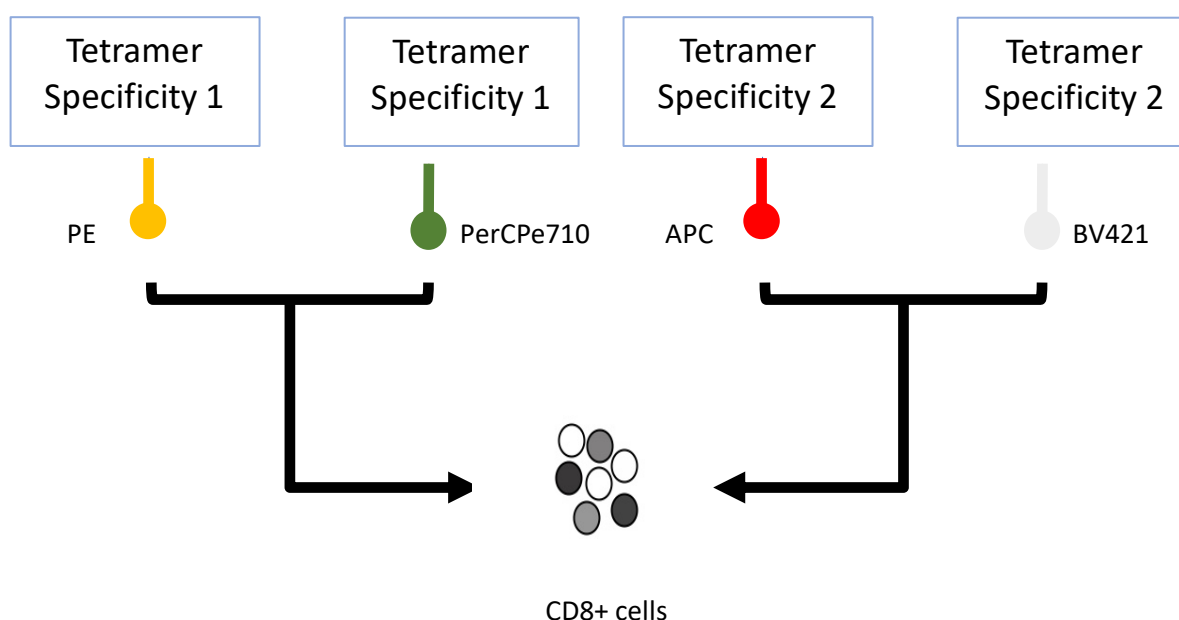


Figure 4-1. Dual labelled tetramer staining scheme. CD8+ T cells were stained with tetramers for two different specificities. Each tetramer was dual-labelled with two different, non-overlapping fluorophores as indicated.

Single cells were then sorted based on their tetramer staining (Figure 4-2) and full length RNA sequencing performed using a modified version of the smart-seq2 protocol¹⁶⁶ as described in Chapter 2.11.3.

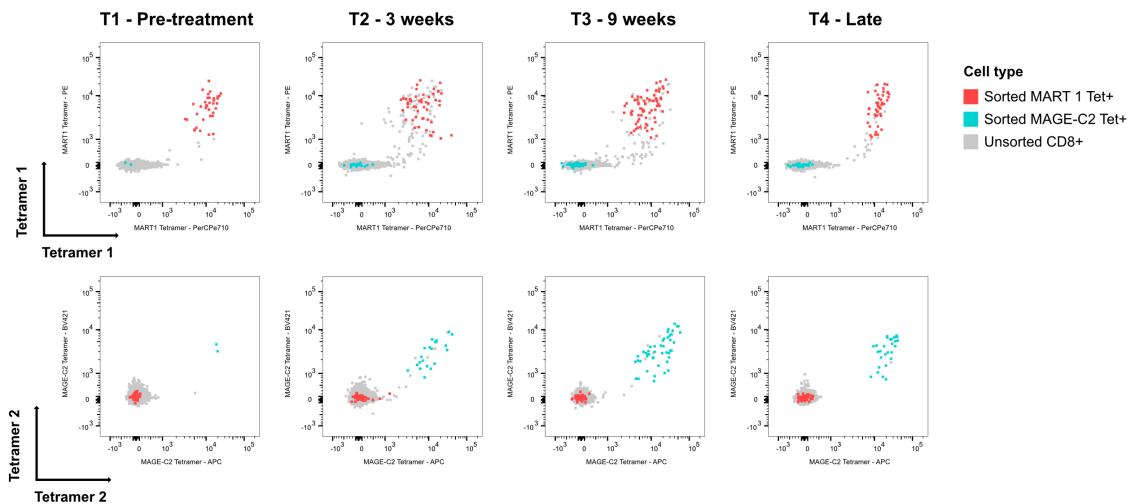


Figure 4-2. Example dual-labelled tetramer staining. CD8⁺ T cells from Patient 1 stained with dual-labelled tetramers. MART-1 tetramer is labelled with PE and PerCPe710 and cells sorted based on dual-positive staining for these fluorophores are coloured red. MAGE-C2 tetramer is labelled with BV421 and PE and cells sorted based on dual-positive staining for these fluorophores are coloured blue. All sorted cells stain dual positive for one tetramer and dual negative for the other.

A total of 672 single tumour tetramer⁺ cells were sorted across three patients and four timepoints. After applying quality control filters as described in Chapter 2.11.4.2, a total of 497 high-quality cells and 10372 expressed genes were available for downstream analyses (Appendix C). The breakdown of cells passing QC by patient and specificity is detailed in Table 4-2.

Table 4-2. Number of cells passing QC filters per patient and timepoint by specificity

Patient	Specificity	Number of cells				Total
		T1	T2	T3	T4	
1	S1	29	24	45	46	144
	S2	2	10	28	26	66
2	S1	17	26	13	13	69
	S2	0	24	53	21	98
3	S1	8	47	40	25	120

4.3 Analysis of effects of treatment over time upon gene expression

As a preliminary validation of our previous CyTOF and FACS based analyses, we performed an initial assessment of the effect of treatment upon gene expression over time

to see if we were able to confirm at a transcript level, changes that we observed previously at a protein level. Upregulation of both Ki67 (*MKI67*) and HLA-DR (*HLA-DRA*) was observed at timepoint 2 relative to pre-treatment (Figure 4-3). *CD38* expression was relatively low across the majority of cells, although where it was observed, this was typically in cells isolated at timepoint 2 and 3 on treatment.

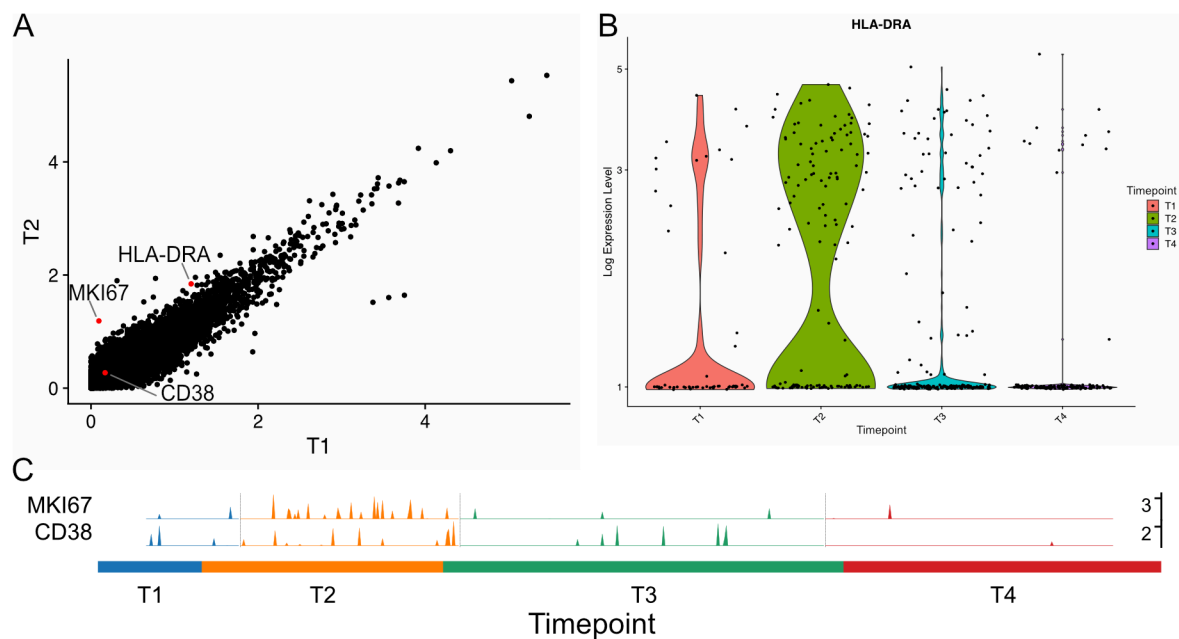


Figure 4-3. Expression of activation and proliferation markers over time. A) Scatter plot of gene expression at T2 vs T1. Points above the diagonal represent genes with higher expression values in T2 than T1. B) Violin plot of *HLA-DRA* expression at different timepoints on treatment. C) Plot showing expression of *MKI67* and *CD38* at different timepoints on treatment. Each spike represents a cell with the height indicating the expression level.

Furthermore, unsupervised differential analysis testing between cells from different timepoints identified *HLA-DRA* and *MKI67* as two of the top three most upregulated genes at timepoint 2 (Figure 4-4 A).

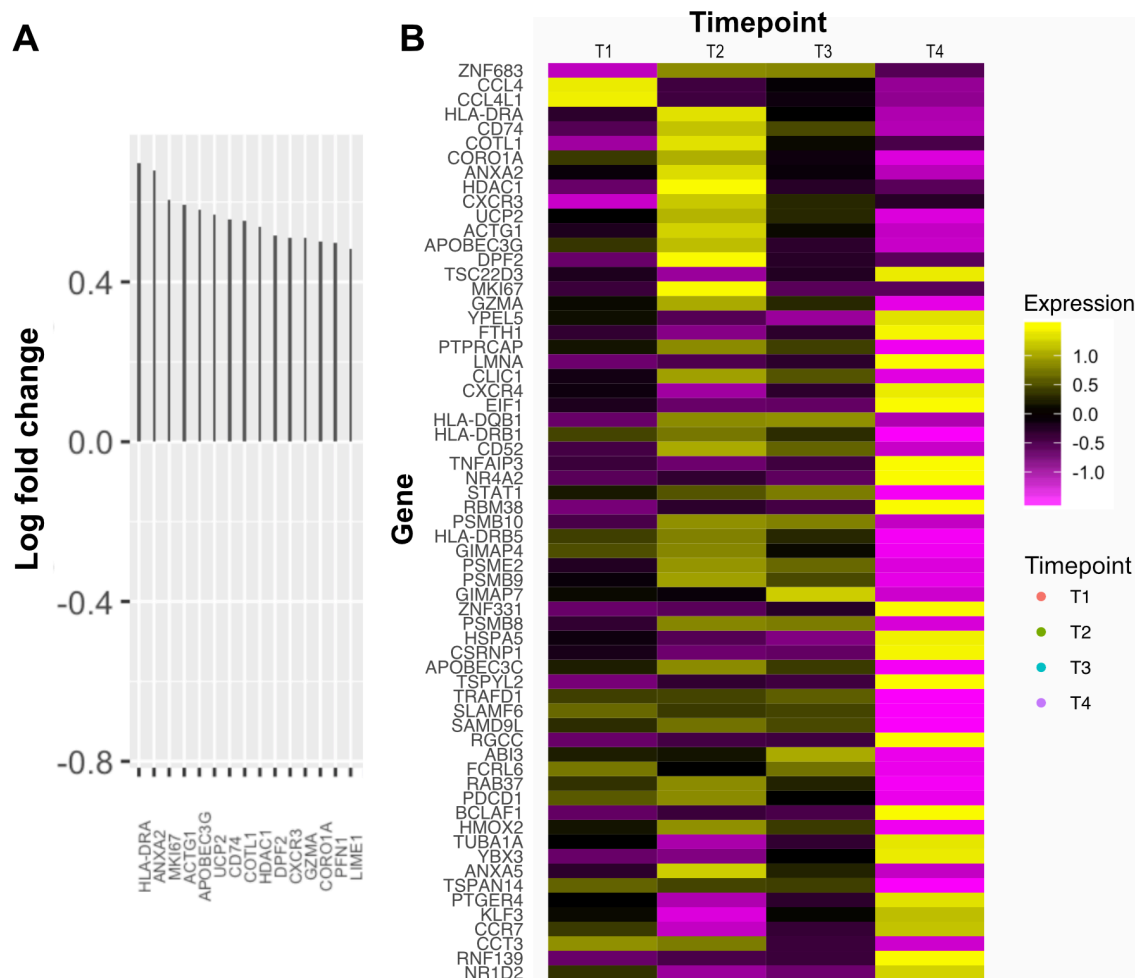


Figure 4-4. Differentially expressed genes over time. A) Top 15 significantly differentially expressed genes at timepoint 2 by log fold change. B) Genes significantly differentially expressed over time ($p < 0.05$, $\log_2 FC > 0.5$).

Amongst other genes differentially expressed over time (Figure 4-4 B) those also upregulated at timepoint 2 included *ANXA2* which has been implicated in cellular proliferation¹⁹⁹, *CD74*, the invariant chain of HLA-DR; *LIME1* which links T-cell receptor stimulation to downstream signalling pathways, and *CXCR3* which is important for lymphocyte trafficking²⁰⁰. Expression of *PDCD1* (PD-1) was noted pre-treatment and highest at timepoint 2 but reduced at later timepoints on treatment.

4.4 Clustering identifies phenotypic heterogeneity over time and between patients at a single-cell resolution

In order to explore heterogeneity between single cells over time and between individuals, the top 1500 highly variable genes for each patient were identified (Figure 4-5) and those present in at least 2 individuals (953 unique genes) were used to perform dimensionality reduction and unsupervised clustering across patients as described in Chapter 2.11.4.4.

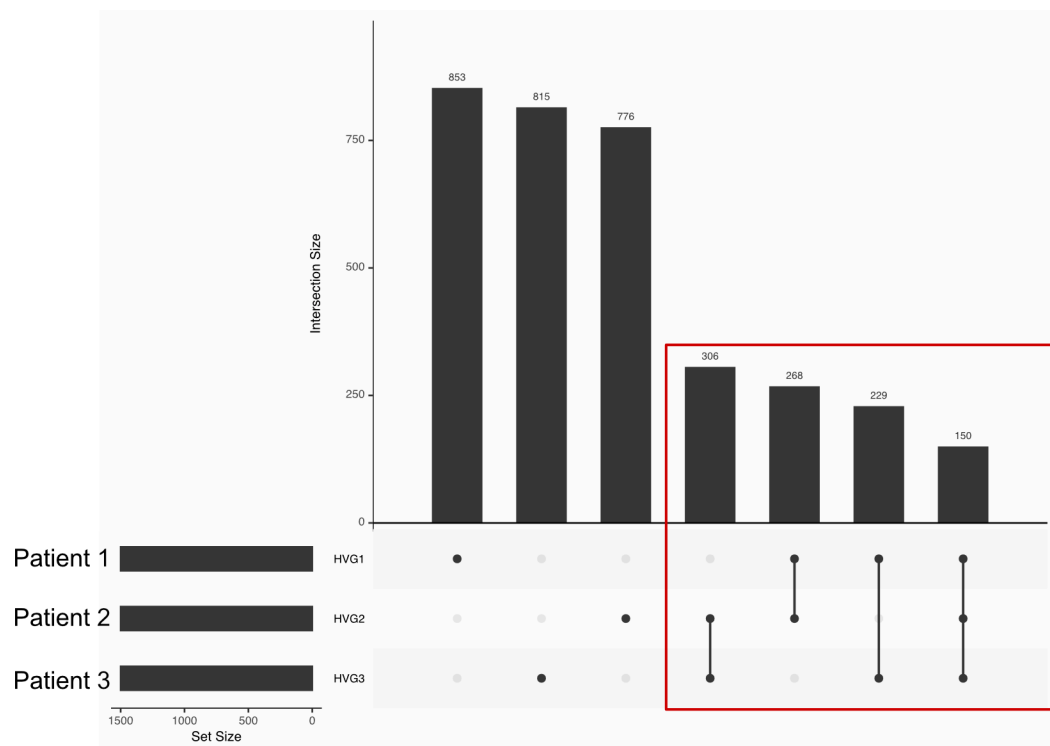


Figure 4-5. Number of highly variable genes per patient. Top 1500 HVGs taken per patient and those found in at least 2 individuals (red box) used for downstream analysis.

A total of 7 clusters were identified within the data with the distribution of these clusters by patient, timepoint and specificity shown in Figure 4-6. Whilst there was some inter-individual variation in the proportion of cells found in different clusters, for example a large number of cells from patient 1 being found in clusters 1 and 6, cells from all patients were detectable in all clusters, pointing towards relevant biological phenotypic differences.

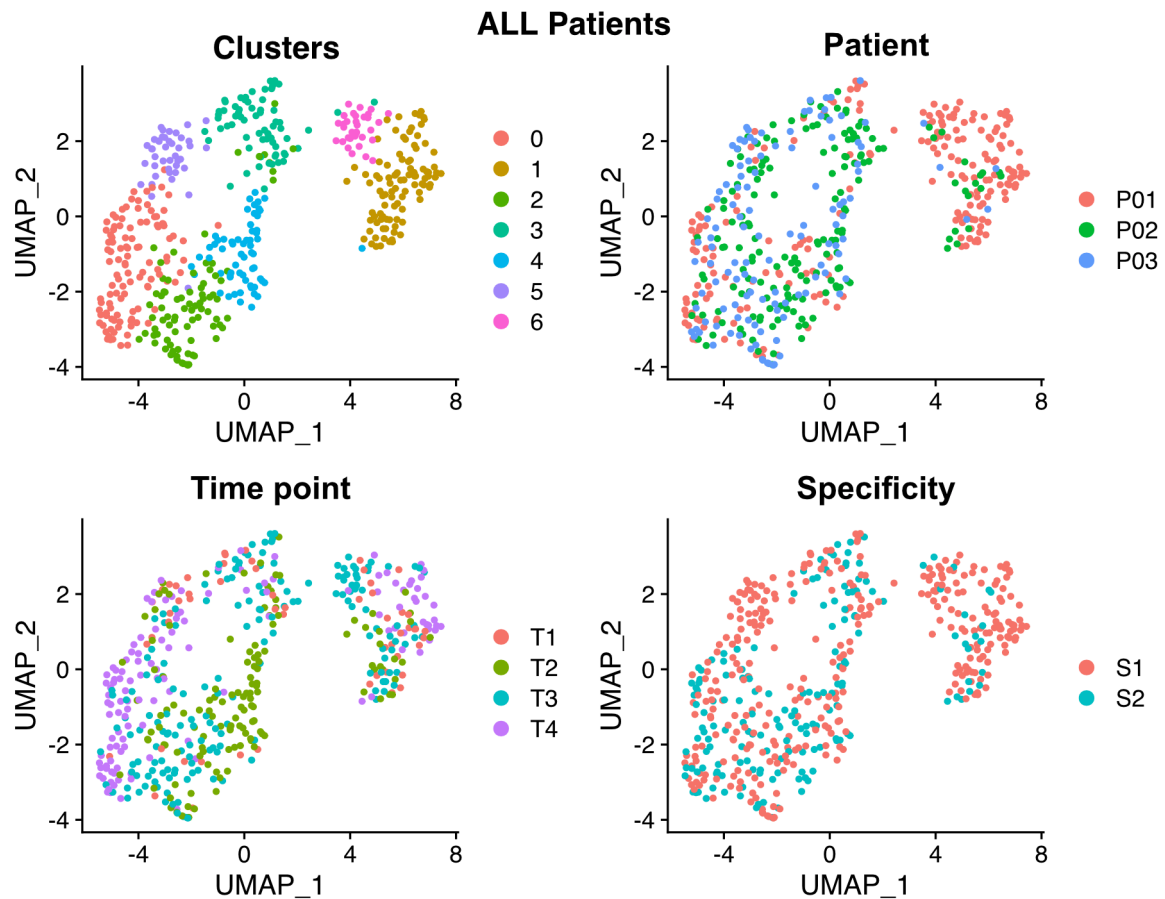


Figure 4-6. UMAP showing annotation of single cells from all patients by Louvain cluster, patient, timepoint and specificity. UMAPs and clustering were both performed using the first 5 principal components of the data, with a resolution of 0.8 for the clustering.

Clear associations were observed between some of the clusters and the different timepoints on therapy that the cells were isolated from (Figure 4-7). For example, cluster 4 consisted almost exclusively of cells isolated at timepoint 2 (after 3 weeks on treatment), whilst others such as cluster 5 consisted of cells from all timepoints, changed minimally in frequency over the course of therapy and consisted almost entirely of MART-1-specific T cells.

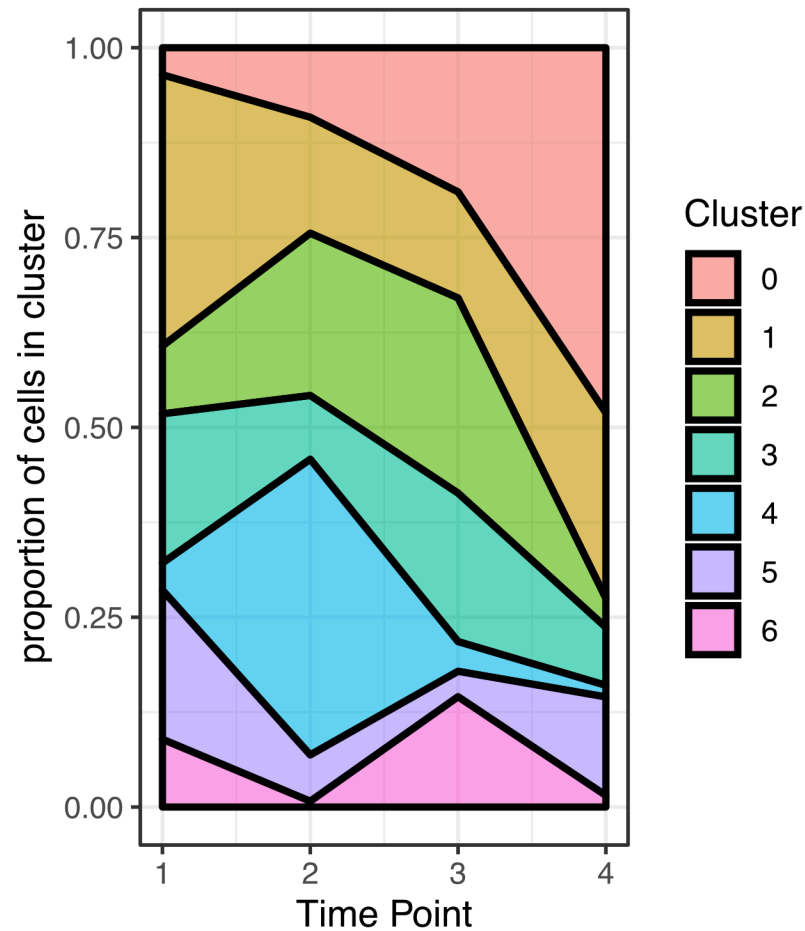


Figure 4-7. Proportion of cells in each cluster at different timepoints on treatment.

Differences in the distribution of cells of different specificity from different patients across the clusters was also evident. MART-1-specific T cells isolated from patient 1 were mostly found in clusters 1 and 6, whilst those from patient 2 and 3 displayed a more diverse distribution, with an increasing frequency of cells in clusters 2, 3 and 0 being seen towards later timepoints (Figure 4-8). For MAGE-C2 and MAGE-A10 specific cells, a much smaller proportion were observed in clusters 1 and 6 than for MART-1, and again an accumulation in cluster 0 appeared at the late on-treatment timepoint.

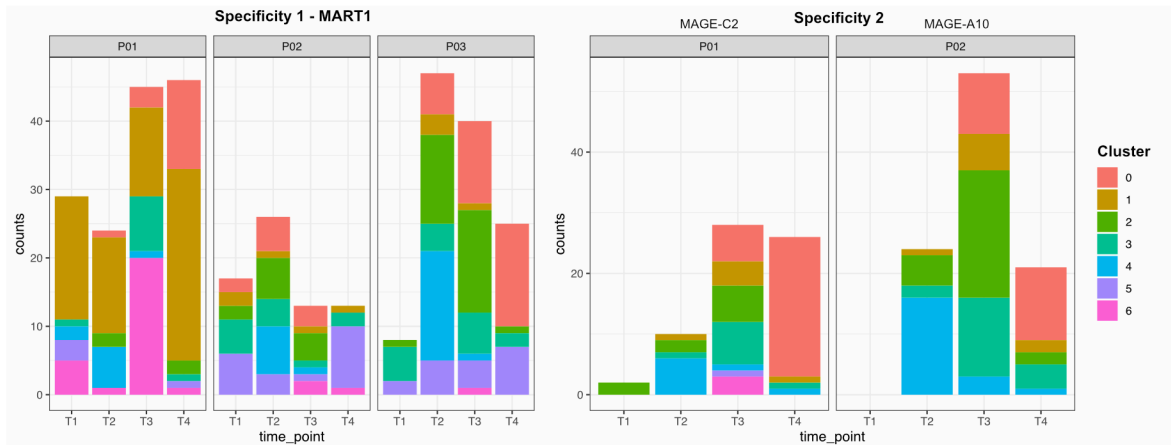


Figure 4-8. Distribution of cells in different clusters over time by specificity and patient.

4.4.1 Phenotypic characterisation of cells in different clusters based on gene expression

To determine the phenotypic characteristics of these distinct clusters, analysis of differentially expressed genes between clusters was performed identifying a total of 287 unique genes being significantly differentially expressed (adjusted p -value < 0.001 and $\log_e$ fold change $> \pm 0.4$) between any individual cluster and the rest. The top 25 differentially expressed transcripts (by fold change) for each cluster are shown in Figure 4-9.

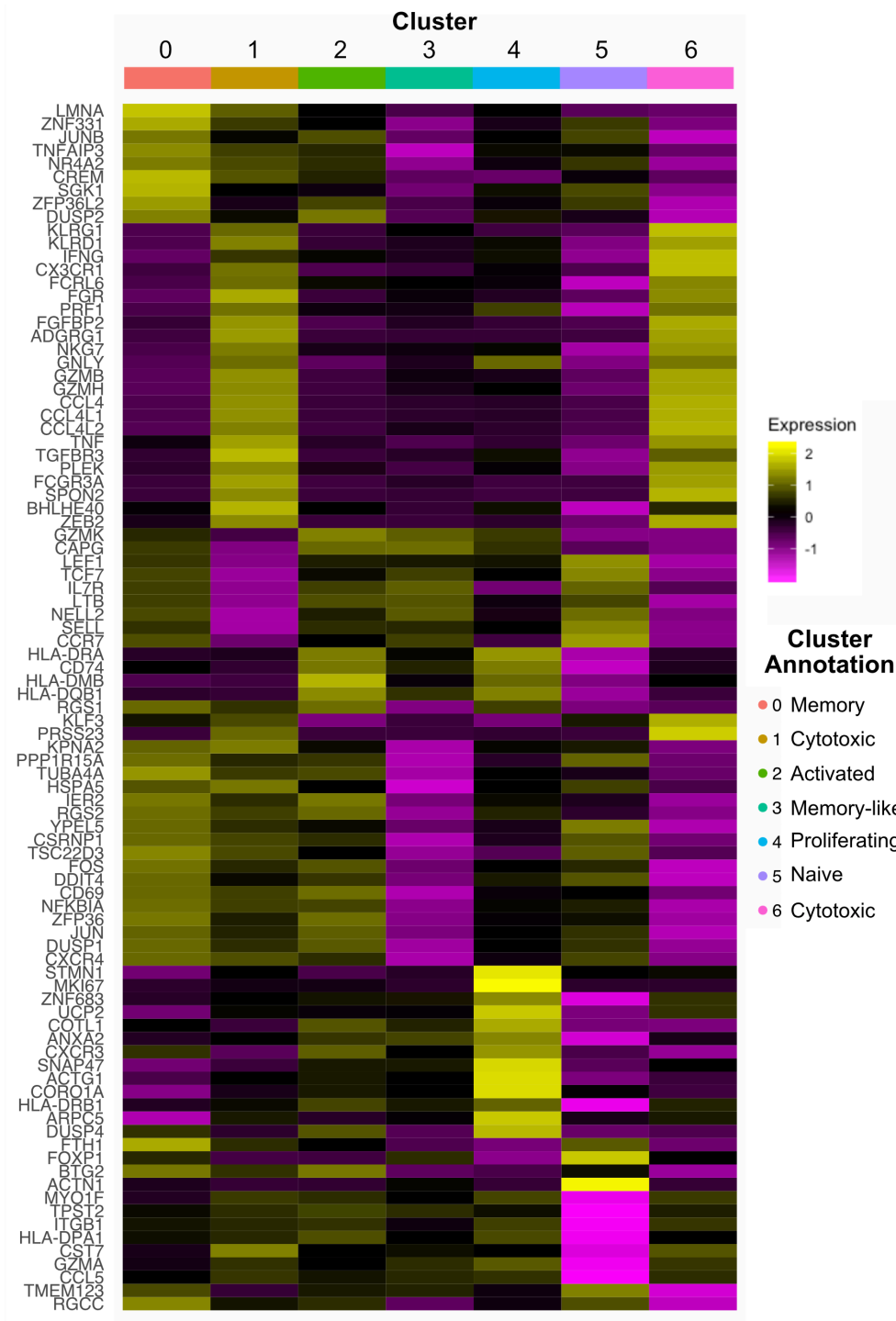


Figure 4-9. Heatmap showing average expression of top 25 differentially expressed genes per cluster. In cases where less than 25 significantly differentially expressed genes are found per cluster, all significantly differentially expressed genes for that cluster are shown.

Based on the gene expression profile of the respective clusters, and with reference to the published literature, we could see that each of these clusters corresponded to distinct phenotypic subsets. Cluster 5, expressed high levels of a number of genes which together

are associated with naïve T cells including, *CCR7*, *SELL* (CD62L), *LEF1*, *TCF7* and *FOXP1*²⁰¹⁻²⁰⁴. Consistent with these being naïve cells, the specificity of these cells was restricted to MART-1. Cells in cluster 4 were characterised by high expression of the proliferation marker *MKI67* as well as various HLA-DR isoforms consistent with activation, and showed downregulation of *LEF1*, *CCR7* and *IL7R* consistent with recent antigen encounter²⁰². Cells in cluster 2 were phenotypically relatively similar to those in cluster 4 and although not proliferating, displayed an activated phenotype with expression of *HLA-DRA* and *CD69* as well as increased expression of Granzyme K (*GZMK*). Those in cluster 0 were found predominantly at timepoint 4 (late on-treatment) and were characterised by expression of *CCR7* and *IL7R*, likely consistent with central memory cells^{205,206}. Cells in clusters 1 and 6 had a distinctly different expression profile from those in other clusters and had high expression of a number of markers associated with cytotoxicity such as *CCL4*, *GZMB*, *GZMH*, *IFNG*, *TNF*, as well as *PRF1* and *NKG7* and the senescence marker *KLRG1*²⁰⁷ suggesting these are likely terminally differentiated effector or effector/memory cells^{204,206,208}. Clusters 1 and 6 differed for their expression of a number of the markers also associated with cluster 0 including *JUN*, *FOS*, *CXCR4* and *NR4A2*. Cells in cluster 3 were characterised by a memory-like phenotype (positive for *IL7R*, *CCR7*, *TCF7*) but downregulated the majority of genes relative to the other clusters.

To further explore the characteristics of these clusters, we performed analysis of gene ontology (GO) terms of the biological processes associated with the top 50 most differentially up and down-regulated genes for each cluster (Appendix D). These were largely consistent with the phenotypic characteristics assigned above. For example, upregulation was seen of genes associated with mitotic cell cycle arrest for cluster 0, lymphocyte mediated immunity and cellular defence response for cluster 1 and 6, antigen processing and presentation for cluster 2 and negative regulators of immune system processes for cluster 3. Downregulation of genes relating to T cell differentiation was seen for clusters 1 and 6, further suggesting that these might be terminally differentiated.

In addition to our unsupervised analysis, as the concept of T cell exhaustion is integral to the rationale for checkpoint inhibitor-based immunotherapy, we went on to further characterise the cells in different clusters based on their exhaustion status. We looked first at the expression of known T cell inhibitory receptors which are commonly used to define T cell exhaustion (Figure 4-10).

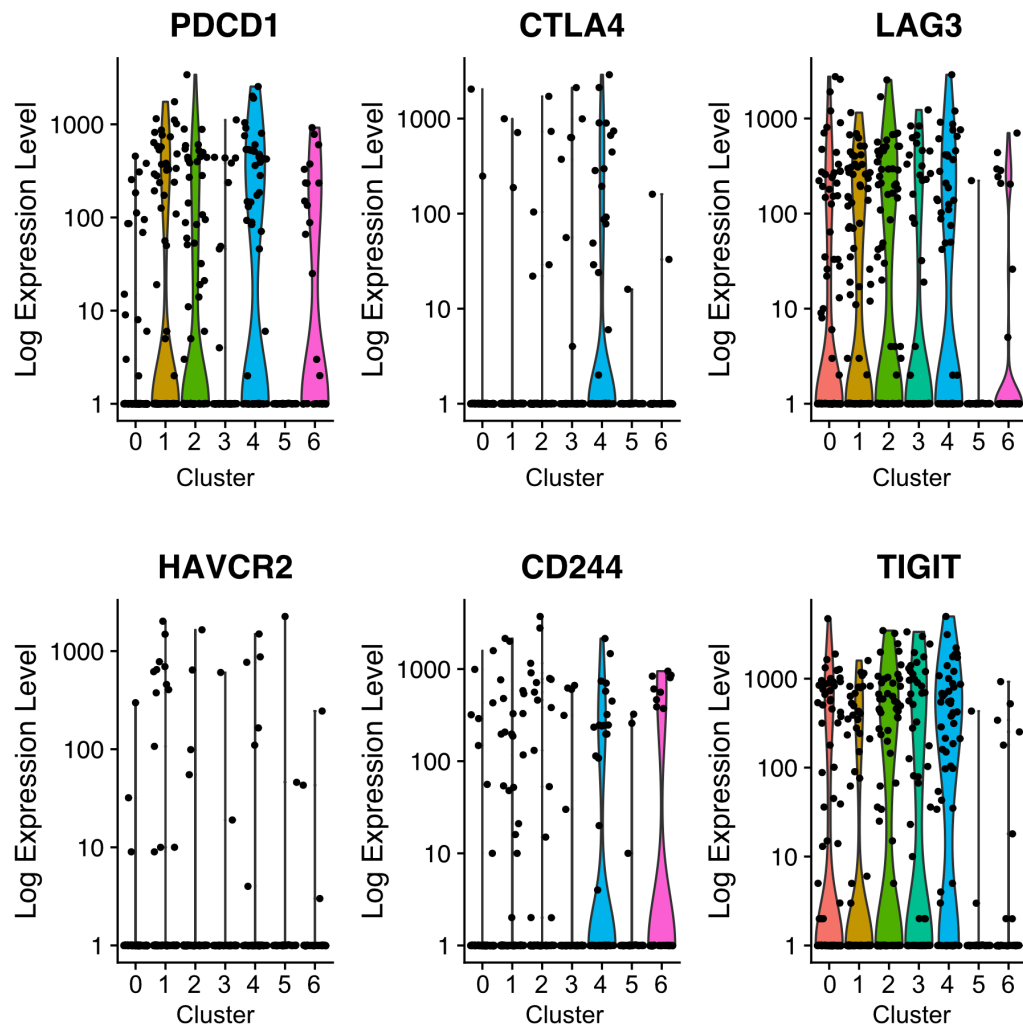


Figure 4-10. Violin plots showing expression of exhaustion markers across clusters. (*PDCD1* = PD-1, *HAVCR2* = TIM-3, *CD244* = 2B4).

Expression of different inhibitory receptors varied across the clusters, and in many cases co-localised with clusters containing cells with a proliferating/activated state. This is consistent with the previously reported concept of 'activation-dependent exhaustion marker expression'^{208,209} and differentiating true dysfunction from inhibitory marker expression associated with different differentiation states is therefore important²¹⁰. As such, we scored

each cluster against published gene sets that have been reported to characterise exhausted cells (Figure 4-11). We used gene sets from two published studies defining exhaustion states from CD8+ T cells infiltrating melanomas, one which characterised exhaustion based on inhibitory marker expression alone²¹¹, and another which identified an ‘activation-independent’ exhaustion gene signature²⁰⁸.

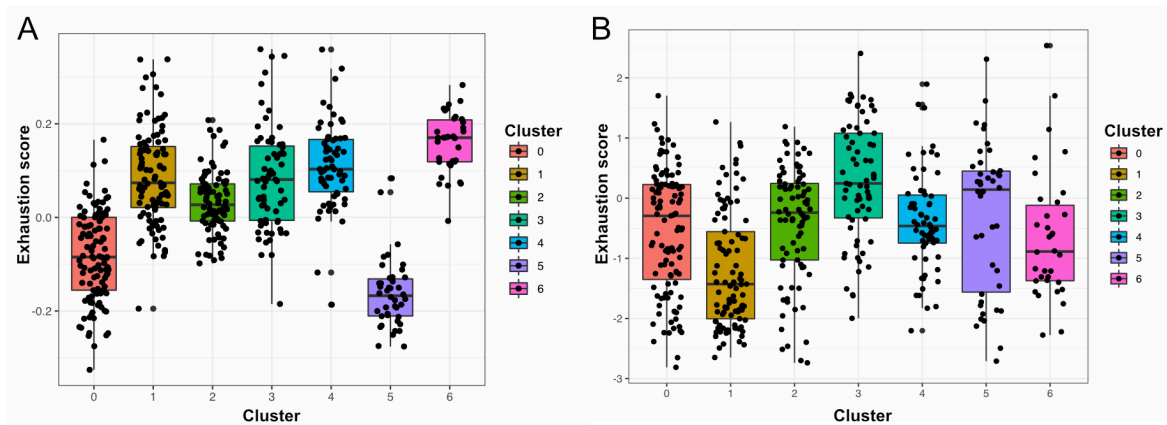


Figure 4-11. Exhaustion scores by cluster. A) Exhaustion score derived from inhibitory marker based exhaustion signature from Sade-Feldman *et al.*²¹¹ B) Exhaustion score derived from activation independent gene signature from Tirosh *et al.*²⁰⁸

From these analyses we could see that based on inhibitory marker expression, cells in clusters 1,3, 4 and 6 appeared to have the strongest exhaustion phenotype, whilst clusters 0 and 5 did not display features of exhaustion. However, when activation was accounted for, cluster 3 appeared to display the strongest exhaustion signature, which appeared to be largely driven by the expression of *TIGIT* and *LAG3*. Exhaustion of cells in this cluster appears to be consistent with the downregulation of many of the genes observed in this population and the enrichment for GO terms relating to negative regulation of immune system processes. As these cells were under the inhibition of negative regulators other than *CTLA4* or *PDCD1*, this population might potentially be unresponsive to anti-CTLA-4 and anti-PD-1 based therapy or represent cells that they have become exhausted via other mechanisms as a result of therapy.

As well as looking at exhaustion status, a number of previous studies have tried to determine subpopulations of T cells which are able to be successfully reinvigorated by

blockade of the PD-1 axis. A population of progenitor exhausted cells expressing PD-1 and TCF1 (encoded by the gene *TCF7*) have been shown to be important for the re-expansion of chronically stimulated CD8+ T cells in response to PD-1 blockade in mouse models of chronic lymphocytic choriomeningitis virus (LCMV) infection²¹²⁻²¹⁴. These cells expressed high levels of the transcription factor *EOMES* and lower levels of T-bet²¹⁴ (encoded by *TBX21*), typically associated with regulation of memory and effector function respectively. A similar population of cells has been identified amongst T cells infiltrating human tumours²¹⁵ and the frequency of these cells has been shown to be associated with response to checkpoint inhibitor therapy in some^{211,216} but not all studies²¹⁷. Some for example have found that *EOMES* positivity in non-terminally differentiated (CD57 low) cells, but not *TCF1* status, was associated with response to combination immunotherapy treatment¹³⁵. These studies however have been mainly focused on transcriptomic analysis of TILs isolated without knowledge of the antigen specificity, so we looked to see whether these markers associated with any clusters from our peripheral antigen-specific cells (Figure 4-12).

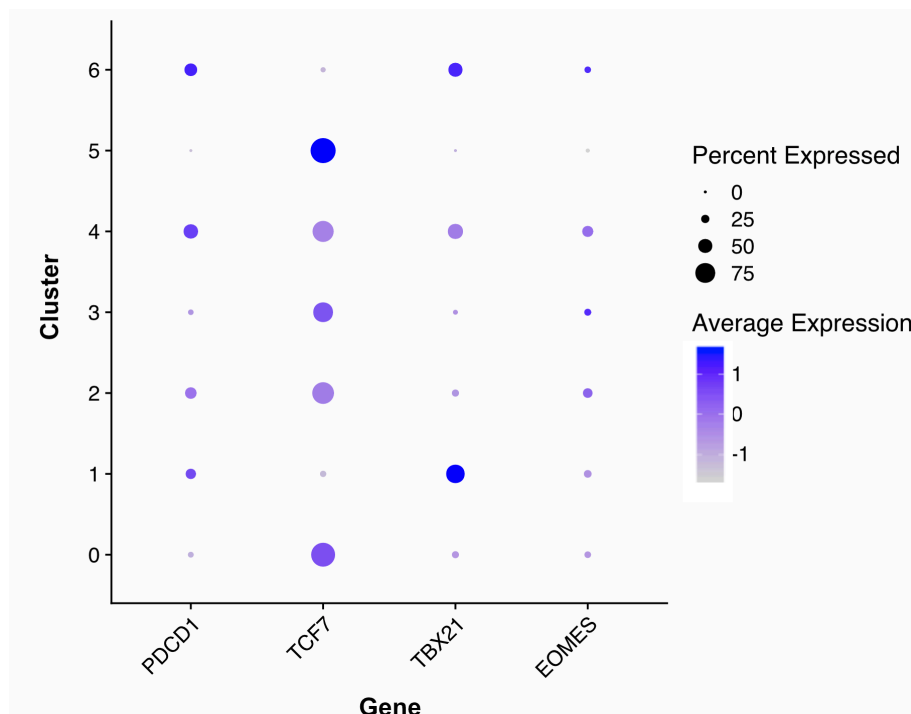


Figure 4-12. Dot Plot of the expression of selected genes implicated in responses to immunotherapy treatment by cluster. Size of circle represents percentage of cells expressing the marker within the cluster, colour indicates average expression within the cluster.

Aside from cluster 5, containing naïve T cells which are known to express *TCF7*, clusters 3 and 0 appeared to have the highest expression for this marker, while expression was low in clusters 6 and 1 which were high in *TBX21*, consistent with their more effector phenotype. *PDCD1* and *TCF7* were only seen to be co-expressed in clusters 4 and 2, which were the clusters defined by evidence of proliferation and activation. This would perhaps fit with previous observations that PD-1+TCF7+ cells are those that provide the proliferative burst in response to anti-PD-1 blockade²¹³.

4.4.2 Different phenotypic characteristics may represent a spectrum of differentiation states in response to treatment

Given the changes in the distribution of cells within clusters over time, and the observation that certain groups of genes were shared between particular clusters, we reasoned that some of these clusters might represent cells in various states along a continuum of cellular differentiation induced by therapy. To further explore potential transitions of cells from one state to another, we performed trajectory analysis using the genes differentially expressed between clusters to order cells in 'pseudotime'. This can be considered a measure of how much progress a cell has made through an underlying dynamic biological process such as differentiation and allows individual cells with potentially asynchronous behaviour to be ordered along this trajectory. From this, we found that cells in clusters 2 and 4 were predominantly located in the centre of the trajectory, with divergent branched pathways towards clusters 3, 1, 6 and 0 at the end of various branches suggesting that these might represent different cellular fates arising from a common origin (Figure 4-13). Of note, cluster 5 (naïve cells) was closely associated in pseudotime with cluster 0 cells, consistent with these being relatively similar in terms of gene expression to central memory cells. Taken together with our previous observations regarding the phenotypic characteristics of the different clusters, it seems that cells activated and proliferating in response to therapy might have a number of possible cellular outcome states, becoming either exhausted (cluster 3),

undergoing terminal differentiation into effector/cytotoxic cells (clusters 0 and 1) or forming a pool of central memory cells (cluster 0).

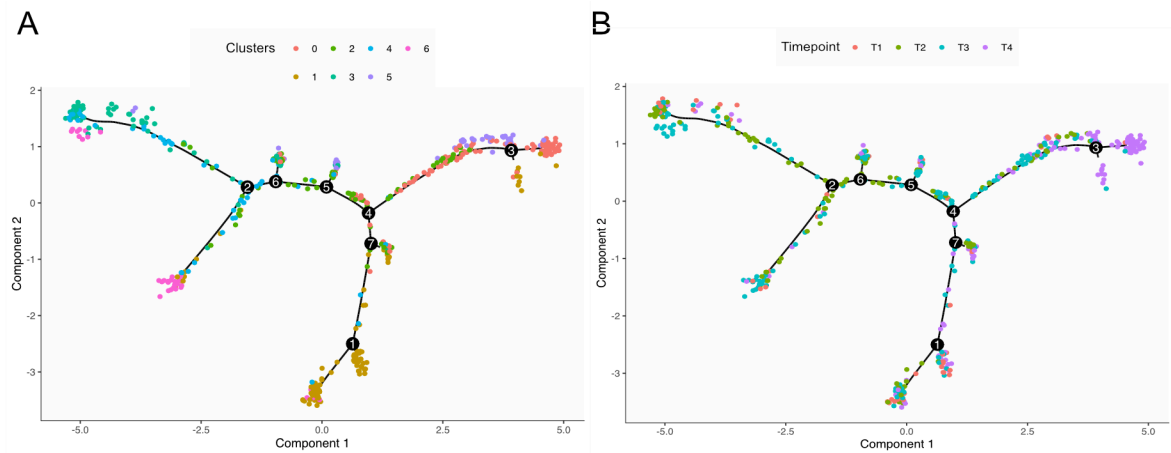


Figure 4-13. Trajectory analysis with cells ordered by pseudotime. A) cells coloured by cluster. B) cells coloured by treatment timepoint.

4.5 Phenotypic changes over time amongst clonal T cell populations

Having identified distinct clusters of cells at different timepoints on therapy, and to track transitions of cells between these clusters over time, we went on to study the clonality of these cells in relation to their TCR. In order to do this, the sequences for the TCR alpha and beta chains were established from the RNA sequencing data and TCR clones defined based on the assembly of shared alpha and beta chains. TCRs were identified for 444 of the 497 cells passing QC parameters (89.3%). Whilst the majority of cells (78%) had one alpha and one beta chain detected, 6.5% of cells had two alpha chains detectable (with or without a beta), 3% of cells had two beta chains detectable (with or without an alpha) and 0.45% of cells had two alpha and two beta chains detected (Figure 4-14). These figures are in line with those in the recent published literature using single-cell sequencing techniques^{18,20}.

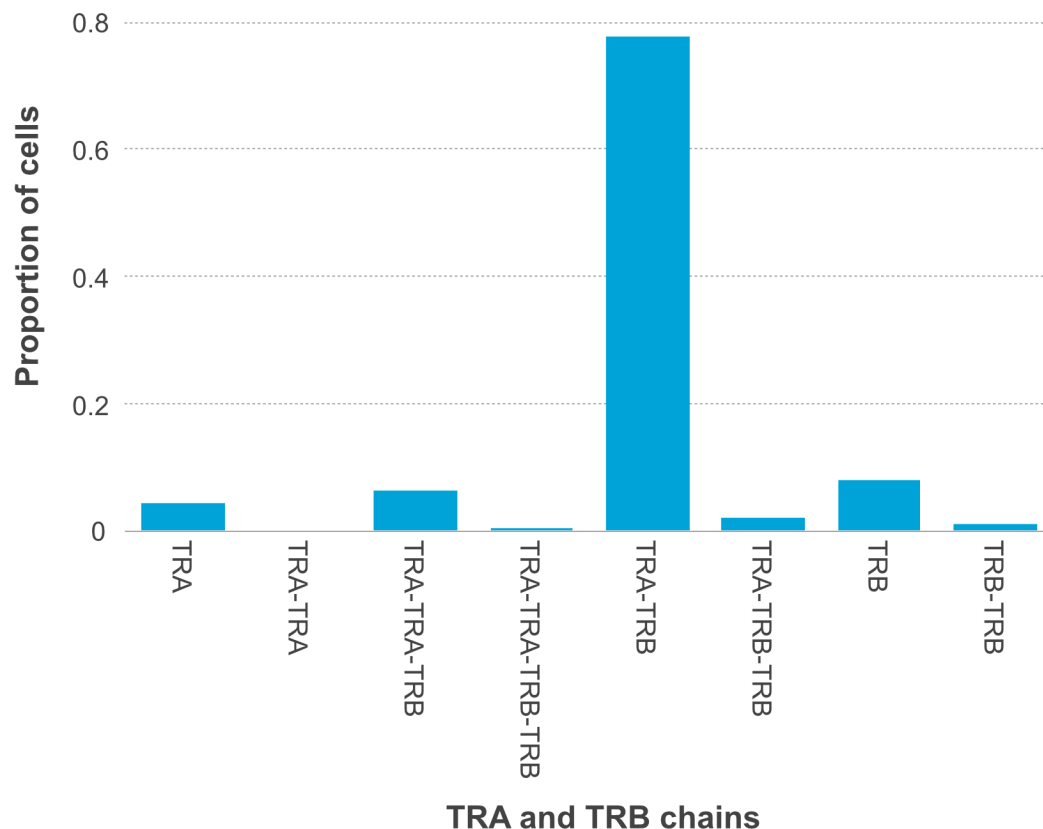


Figure 4-14. Proportion of cells with number of alpha and beta chains detected per cell.

Tracking individual clones over the course of treatment (Figure 4-15) demonstrated differences in the extent of clonal expansion between clones and also different patterns of behaviour over time. Once encountered, some clones continued to be detectable at subsequent timepoints on therapy, whilst others were present only transiently, perhaps due to trafficking of these cells into peripheral tissues/tumour or simply as a result of the limited number of cells sampled.

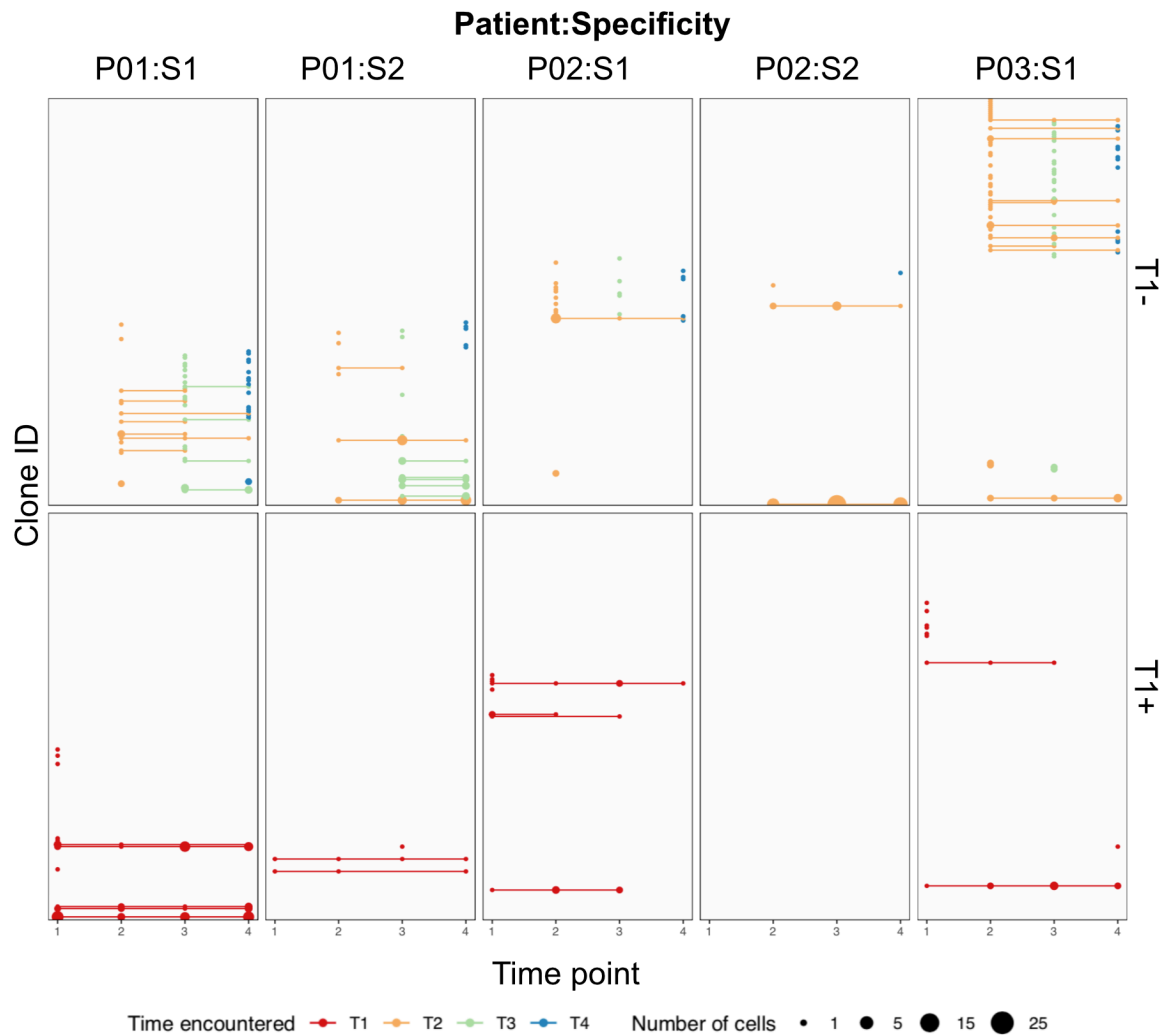


Figure 4-15. T cell clone time course. Each dot and line represents a different clone as defined by their TCR. Clones are shown by patient and specificity and split according to whether they were present from T1 (T1+) or only detectable after T1 (T1-). Clones are coloured by the time point they were first encountered with the size of each circle representing the number of cells detected with this TCR.

To try and link together cell phenotypes with clones that were pre-existing vs newly detected and the extent of clonal expansion, we generated a number of gene signatures relating to specific functions including proliferation, activation, effector function and exhaustion based on the top 50 genes correlated with *MIK67*, *HLA-DRA*, *IFNG* and *PDCD1* respectively. We then classified cells into 5 quantiles, based on the extent of their clonal expansion, and by the time they were first detected (pre-therapy or after the start of therapy), and examined the relationships between the different functional signature scores (Figure 4-16).

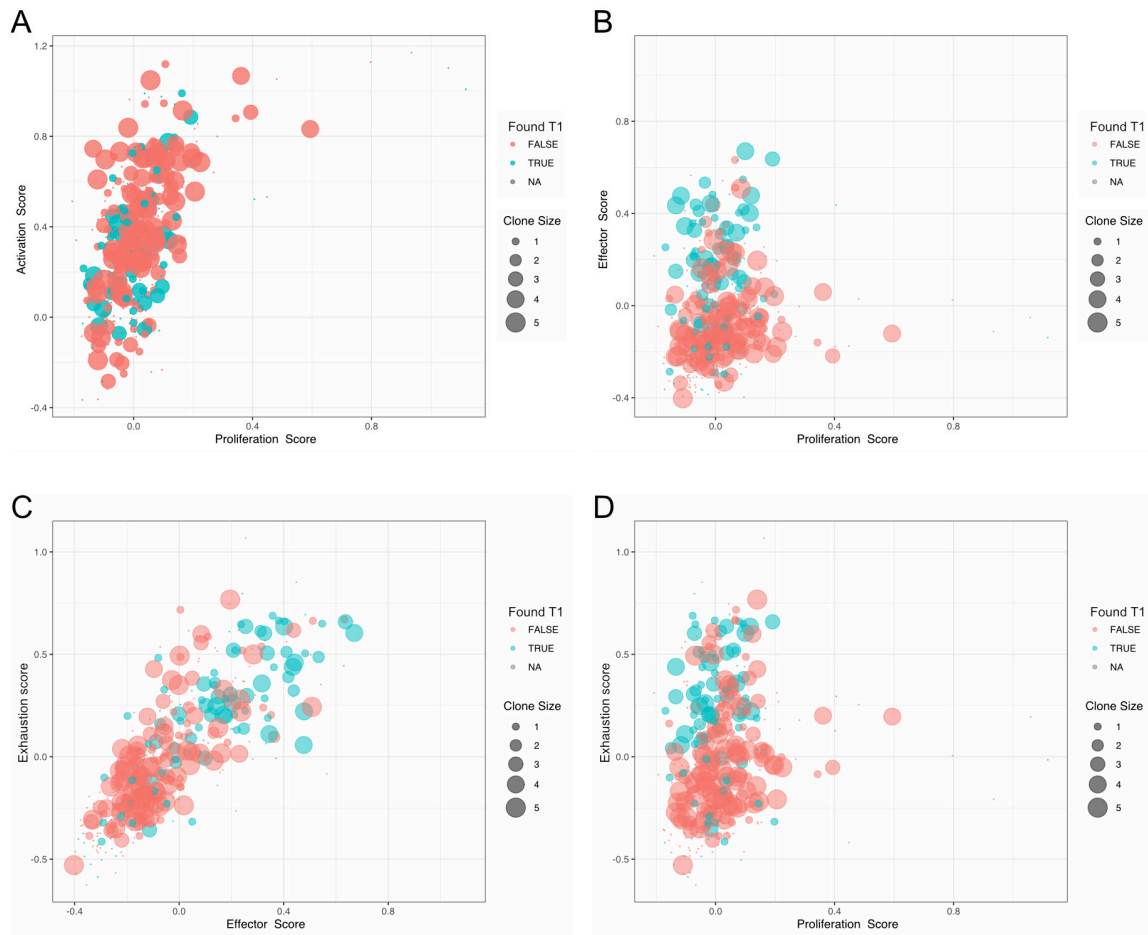


Figure 4-16. Relationships between different functional gene signatures. A) Activation score vs proliferation score. B) Effector score vs proliferation score. C) Exhaustion score vs effector score. D) Exhaustion score vs proliferation score. Each circle represents an individual T cell clone, coloured by whether clones were detectable before (found T1 = true) or only during therapy (found T1 = false) and sized by the extent of clonal expansion (clone size 5 = top most expanded quantile, 1 = least expanded quantile).

Activation and proliferation showed a degree of correlation (Figure 4-16 A), although whilst all proliferating cells showed features of activation, not all activated cells proliferated. Interestingly, whilst pre-existing T cell clones detectable before therapy could be activated, proliferation appeared to be restricted almost entirely to newly observed clones. Consistent with this, cells with an effector phenotype which were in many cases clonally expanded and detectable pre-therapy, did not proliferate in response to treatment likely in keeping with their terminally differentiated state (Figure 4-16 B). These effector clones detected pre-treatment showed higher degrees of exhaustion than non-effector clones and newly identified effector clones (Figure 4-16 C). When looking at proliferation in relation to

exhaustion, consistent with previous observations²¹², those with an intermediate state of exhaustion were able to proliferate, whilst those which were very highly exhausted did not (Figure 4-16 D).

In order to study the kinetics and phenotypes of specific T cell clones in further detail, we combined the clonal data with our clustering analysis based on RNA expression. We looked first at the MART-1-specific T cells isolated on a per patient basis and classified them as either singletons, where only one cell bearing that TCR was isolated, or non-singletons where the same TCR was detected in multiple cells within the same individual (Figure 4-17). Any TCRs detected in multiple cells can be considered to be clonally expanded in vitro, as the chance of isolating two cells of the same TCR from the naïve repertoire in this small sample size is negligible. However, TCRs seen only once (singletons) might either be naïve cells that are not clonally expanded in vitro, or clonally expanded cells which were only sampled once due to limited cell numbers isolated.

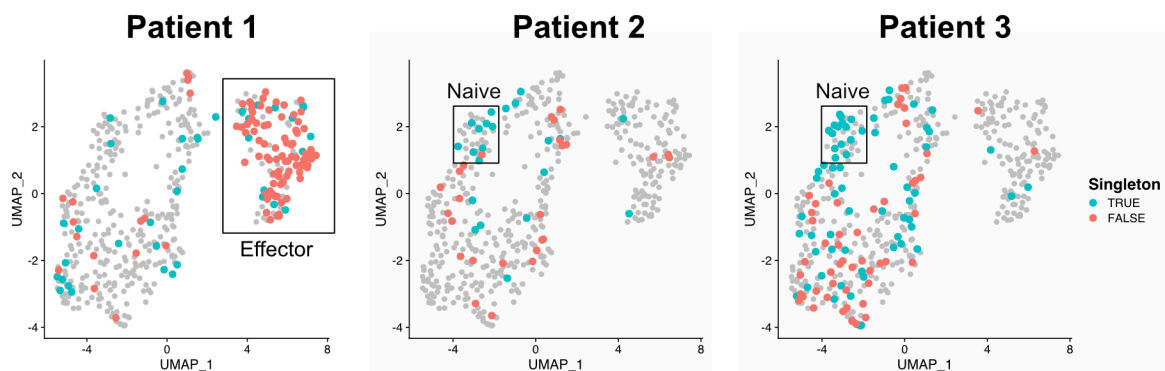


Figure 4-17. Clonal expansion of MART-1-specific T cells. Per patient UMAP plots showing MART-1-specific T cell clones coloured by whether they were found only once (Singleton = True) or clonally expanded (Singleton = False).

Clear differences were seen between the patients in the degree of clonal expansion of MART-1-specific T cells and also their distribution in relation to the previously identified clusters (Figure 4-17). Patient 1 had a large number of clonally expanded MART-1-specific T cells, mainly co-localising with clusters 1 and 6, indicating a cytotoxic effector memory phenotype, whilst those clonally expanded in patients 2 and 3 were found co-localising with

clusters 3,4,2 and 0 but typically not clusters 1 and 6. Whilst singletons could be identified across all clusters, in patient 2 and 3 a large number of these co-localised with cluster 5 which together with the phenotype of this cluster suggested that these were likely to be naïve cells, rather than expanded clones that were only sampled once.

In order to establish whether expanded T cell clones found in different clusters shared the same or different TCRs, and whether they were changing phenotype over time, we then looked at the distribution of individual expanded clones across clusters and over time (Figure 4-18). Individual expanded clones could be found in a variety of different clusters at different timepoints on treatment, although some such as a large expanded clone (clone 12) seen in patient 1 appeared to be mainly restricted to clusters 1 and 6, whilst others from this individual and patients 2 and 3 appeared more mobile over time.

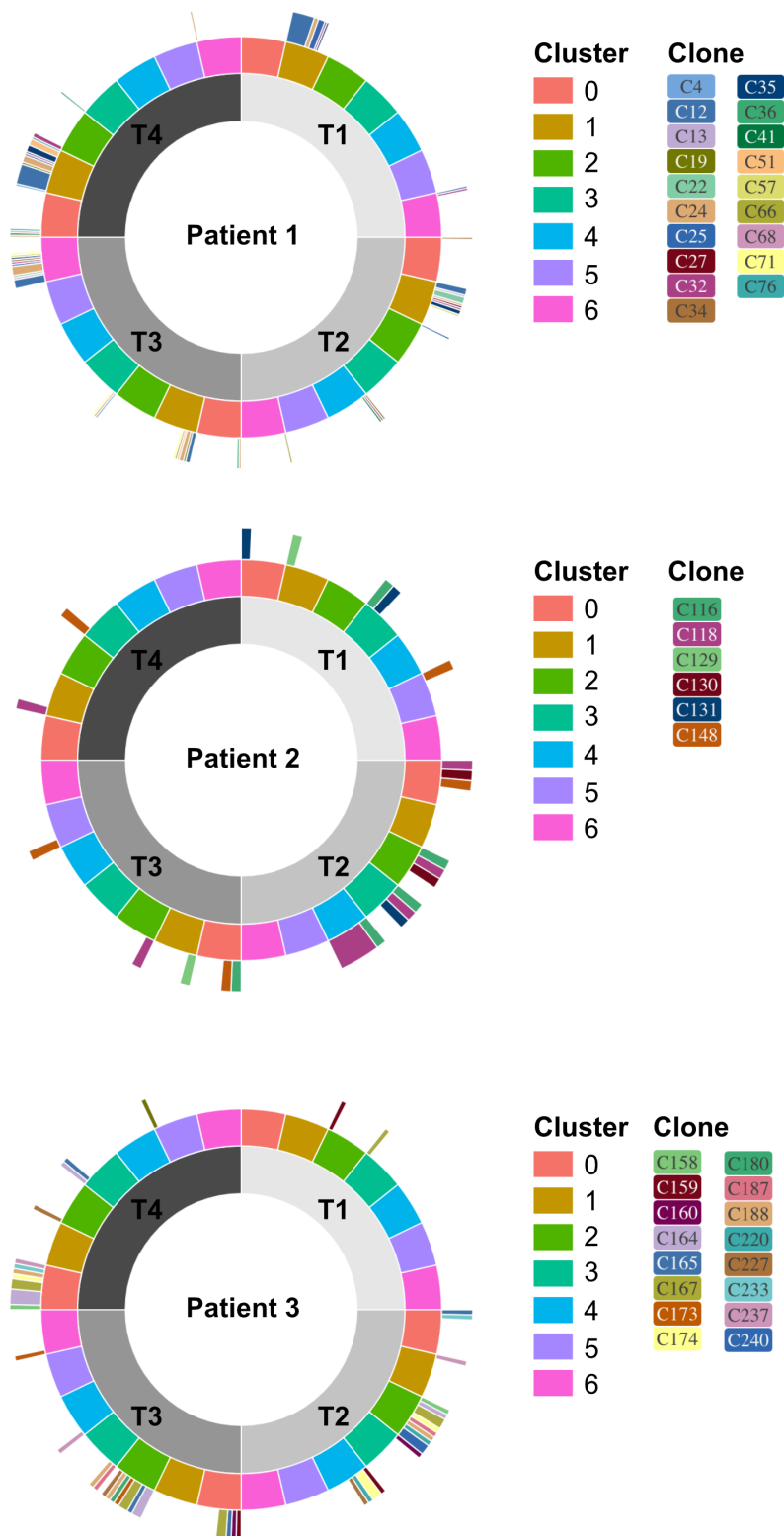


Figure 4-18. Per patient sunburst plots showing distribution of expanded MART-1-specific clones across clusters over time. Expanded T cell clones are shown in the outer segment with the size of the segment proportional to the number of cells in that clone. The phenotype of the cells by cluster (middle circle) at each timepoint they are found (inner circle) is shown.

To try and establish whether the distribution and mobility of clusters over time was influenced by whether clones were pre-existing or newly identified, we compared the behaviour of clones found at T1 with those newly detected on therapy. For patient 1 (Figure 4-19 & Figure 4-20), whilst some transition between clusters was evident over time for cells detected pre-treatment (mainly from cluster 1 to cluster 6 at timepoint 3), the vast majority of these had reverted to their pre-treatment phenotype by the late on-treatment timepoint. Similar behaviour was observed for a large proportion of the clones newly detected on therapy, which in most cases were found in cluster 1 when they were first detected, suggesting that these may actually have been pre-existing clones that were simply were not detected at baseline. Of three clones which were detected in cluster 0 at T4, only 1 of these was detected at other timepoints (clone 41, which was observed in cluster 4 at T2) pointing to these perhaps being newly generated clones that acquired a central memory rather than terminally differentiated effector phenotype.

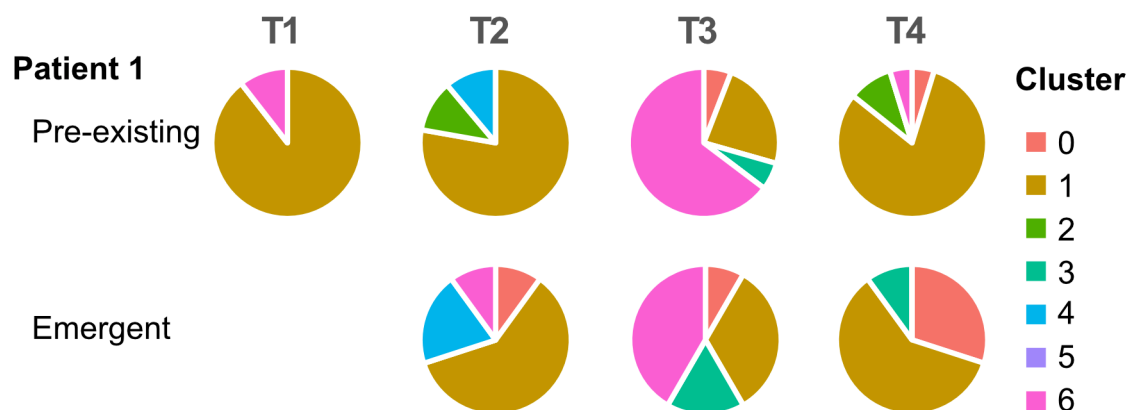


Figure 4-19. Distribution of Patient 1 MART-1-specific T cells from expanded clones by cluster over time. Clones are divided by whether they were detectable pre-treatment (pre-existing) or only after the start of therapy (emergent).

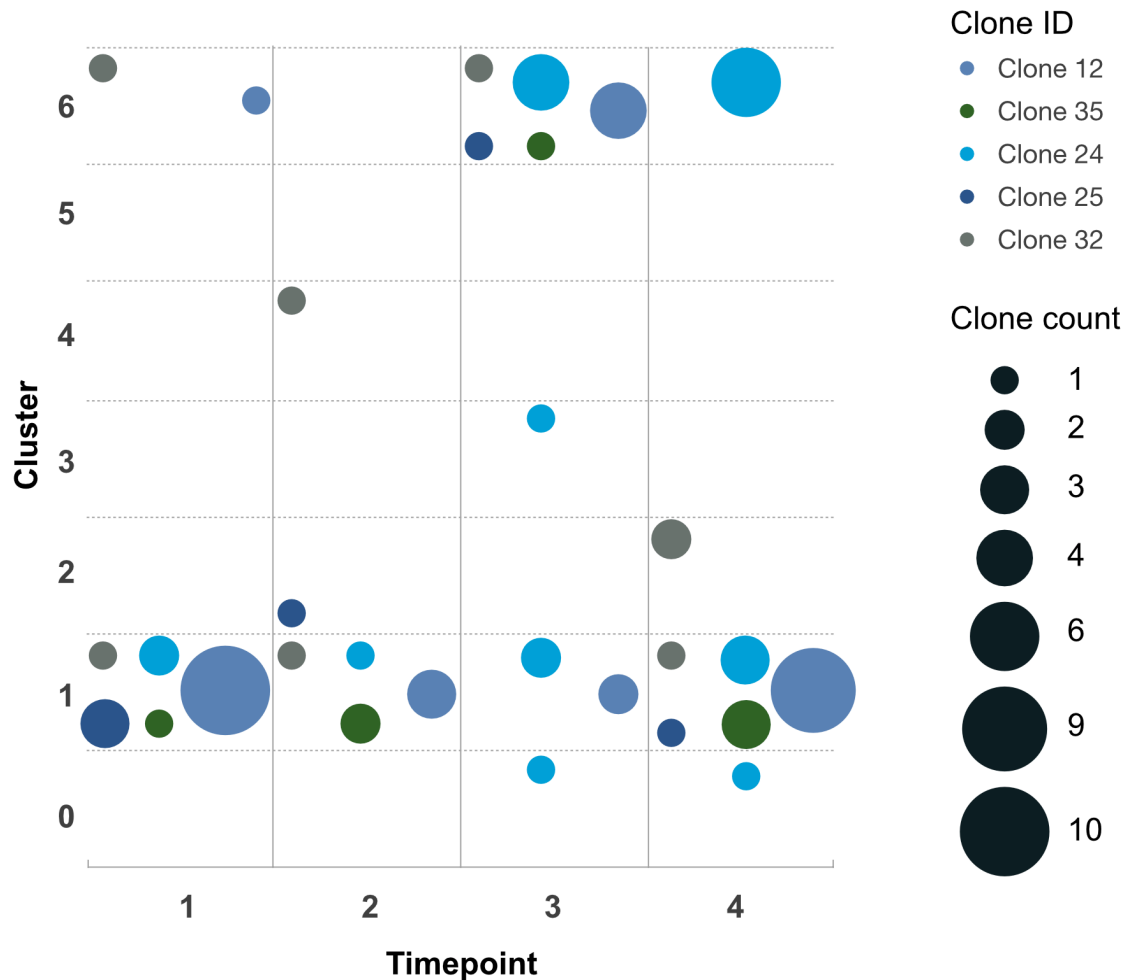


Figure 4-20. Changes in phenotype over time of individual MART-1-specific T clones detectable pre-therapy for patient 1. Each TCR clone is represented by a different coloured circle with the size indicating the number of clones detectable.

For patient 2, pre-existing MART-1 clones were predominantly located in cluster 3 prior to therapy and although some shifts were seen over time, by timepoint 4 cells had reverted to a cluster 3 phenotype (Figure 4-21 & Figure 4-22). In this individual, newly identified clones appeared to show greater mobility, transitioning from cluster 4 at timepoint 2 through to cluster 2 and eventually cluster 1. For patient 3, both pre-existing and newly detected clones appeared relatively phenotypically plastic displaying transitions between clusters over time and predominantly appearing in cluster 0 as effector memory cells at timepoint 4 (Figure 4-21 & Figure 4-22).

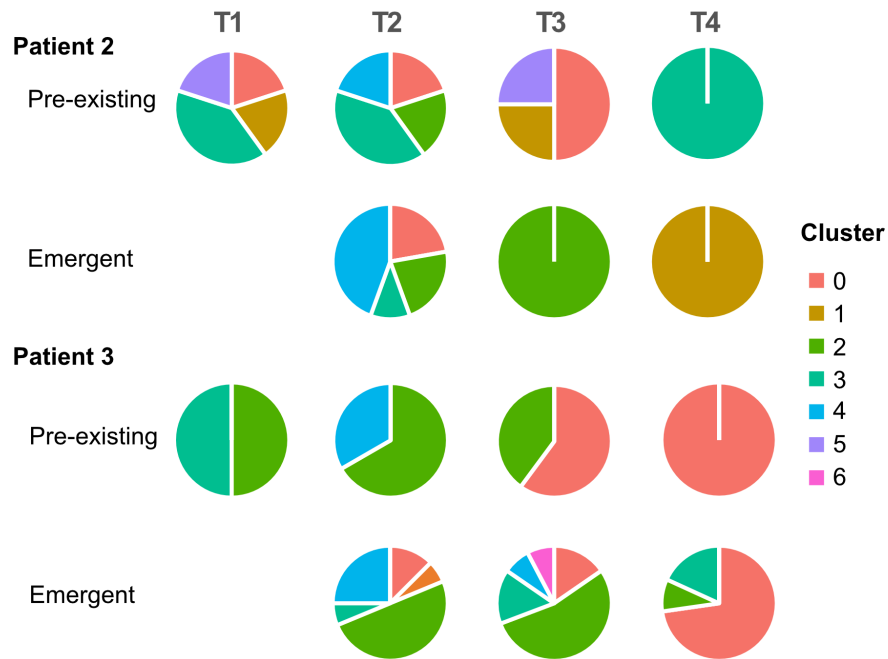


Figure 4-21. Distribution of Patient 2 and Patient 3 MART-1-specific T cells from expanded clones by cluster over time. Clones are divided by whether they were detectable pre-treatment (pre-existing) or only after the start of therapy (emergent).

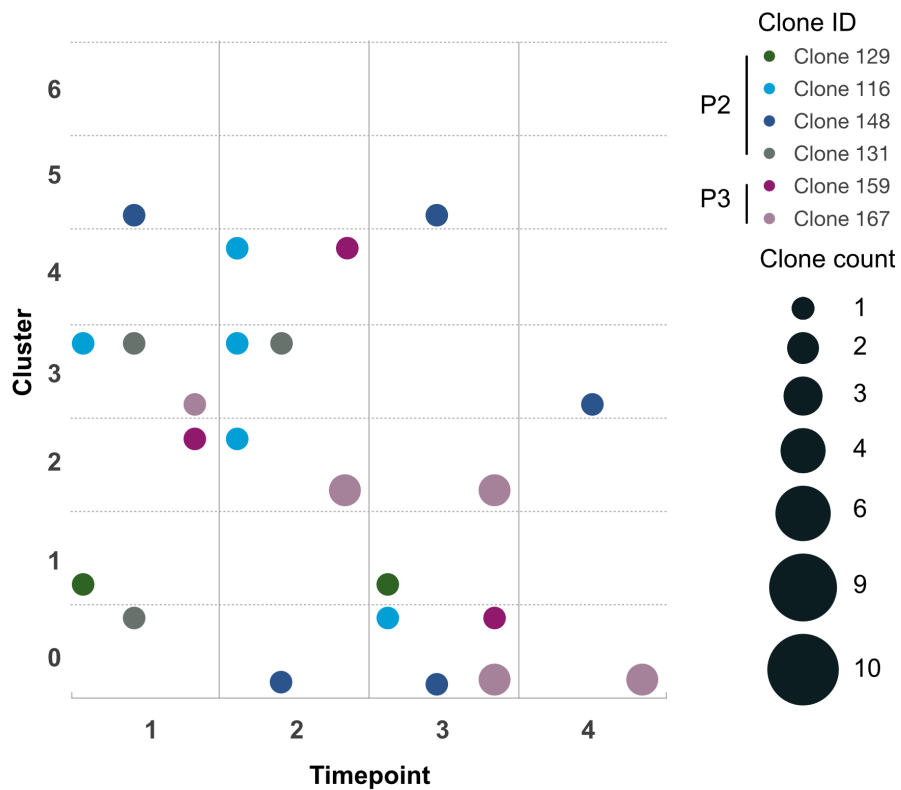


Figure 4-22. Changes in phenotype over time of individual MART-1-specific T clones detectable pre-therapy for patient 2 and 3. Each TCR clone is represented by a different coloured circle with the size indicating the number of clones detectable.

To determine whether T cells specific for antigens other than MART-1 behaved in a similar manner, we interrogated the T cells sorted based on the second specificity for patients 1 and 2 (Figure 4-23).

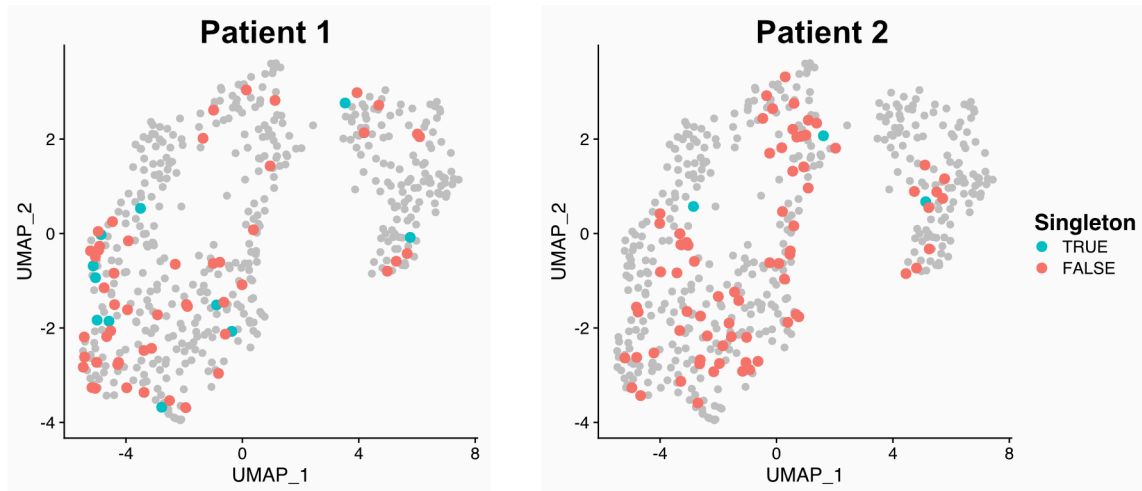


Figure 4-23. Clonal expansion of MAGE-specific T cells. Per patient UMAP plots showing MAGE-C2 (Patient 1) and MAGE-A10 (Patient 2) specific T cell clones coloured by whether they were found only once (Singleton = True) or clonally expanded (Singleton = False).

In keeping with these T cells being specific for antigens other than MART-1 (and hence without the propensity towards a large naïve repertoire), most of the MAGE-C2 and MAGE-A10 T cells isolated were clonally expanded. Whilst a few singletons were detectable, these were not of a naïve phenotype and therefore likely to represent expanded but under-sampled T cell clones. Expanded cells also demonstrated greater clonality than observed previously for MART-1-specific T cells, and in the case of patient 2, the expanded MAGE-A10 population was dominated by a single clone (115) and were mostly found in non-cytotoxic clusters (Figure 4-24).

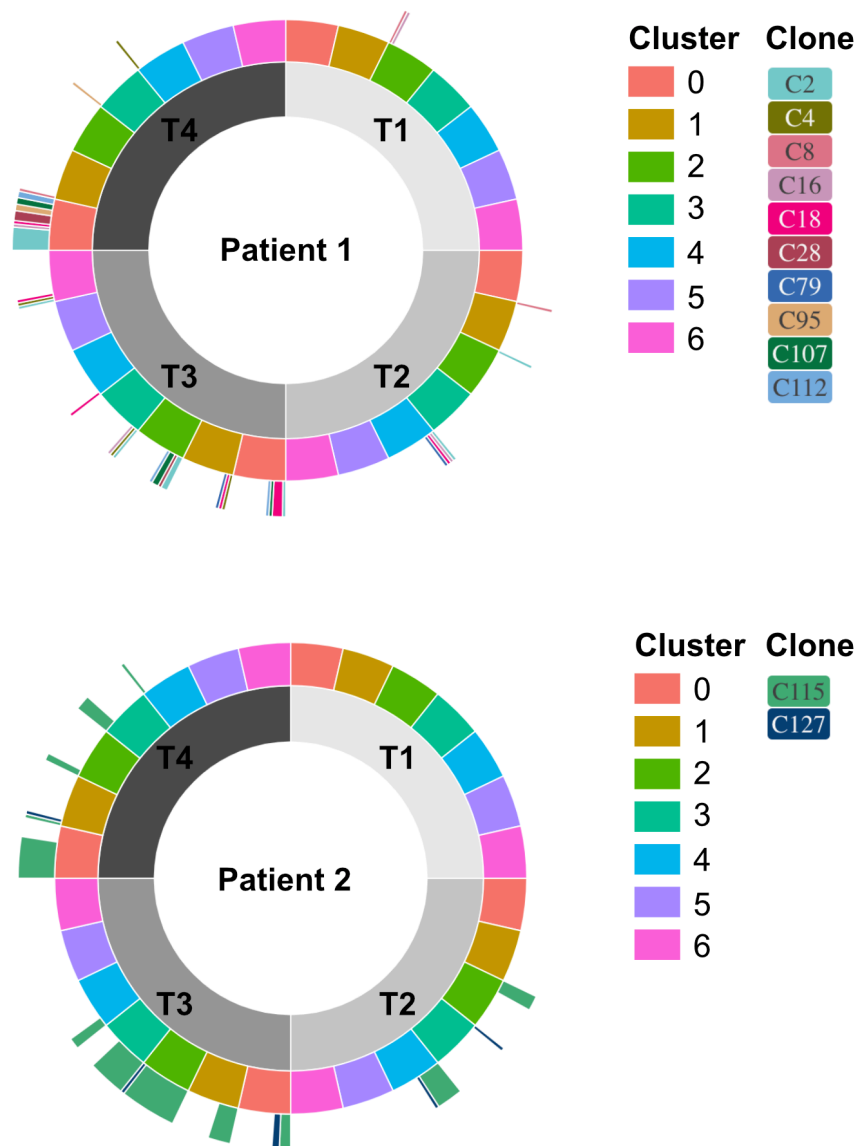


Figure 4-24. Per patient sunburst plots showing distribution of expanded MAGE-specific clones across clusters over time. Expanded T cell clones are shown in the outer segment with the size of the segment proportional to the number of cells in that clone. The phenotype of the cells by cluster (middle circle) at each timepoint they are found (inner circle) is shown.

We again tracked the movement of individual T cell clones across clusters over the course of therapy (Figure 4-25) and were able to observe transitions from cluster 4 to cluster 2 and cluster 0 for a large proportion of the clones.

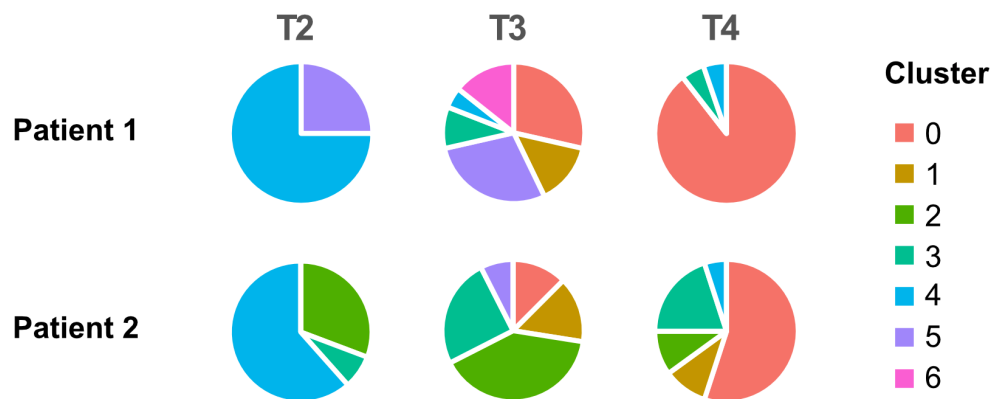


Figure 4-25. Distribution of newly detected MAGE-specific T cells for patients 1 and 2 (MAGE-C2 and MAGE-A10 respectively) from expanded clones by cluster over time.

Taken together, these data would appear to suggest that cells with an effector cytotoxic phenotype show relatively limited capacity for response to treatment, shifting only between cluster 1 and 6 before reverting to their pre-treatment state. In patient 1, the large proportion of MART-1-specific cells displaying this behaviour suggest that perhaps this individual had a pre-existing MART-1 anti-tumour response that was not particularly altered by therapy. For patient 2 and 3 however, MART-1-specific T cells appeared to follow a different trajectory, ending up as either cluster 0 or cluster 3 phenotype cells, suggesting that perhaps these were de-novo responses induced by treatment. MAGE-specific responses for both patient 1 and 2 appeared to show much greater changes over time and the vast majority of cells were found in either cluster 0 or 3 at the late on-treatment timepoint rather than as terminally differentiated effector cells. Interestingly, whilst all three patients did respond to treatment, patient 1 did not maintain his response long-term whilst patient 2 and 3 did. Whether the balance between cluster 0, central memory phenotype and effector phenotypes impacts upon this however is unknown, as although central memory type cells appeared to dominate in patient 3 at the late on-treatment timepoint, the MAGE-C2 specific cells from patient 1 also displayed this behaviour.

4.6 Identification of T cell clones from the periphery intra-tumourally

In order to determine the relevance of these tumour antigen-specific T cell responses we had identified in the periphery to the intra-tumoural response, we obtained a pre-treatment tumour biopsy for patient 1 and performed bulk TCR sequencing on RNA extracted from this. We then searched for matches of the TCRs we had identified for antigen-specific cells in the periphery with TCRs obtained from the tumour, using both TCRs identified from the single-cell sequencing of tumour tetramer+ cells and from T cell clones established for this patient as described in the experiments in Chapter 3. As the sequencing of the tumour was from bulk RNA it was not possible to establish T cell receptor alpha (*TRA*) and beta (*TRB*) pairings for individual TILs, however evidence of matches at both the *TRA* and *TRB* within the TIL TCRs was found for three distinct peripheral TCRs, strongly pointing towards the presence of these T cell clones intra-tumourally (Figure 4-26).

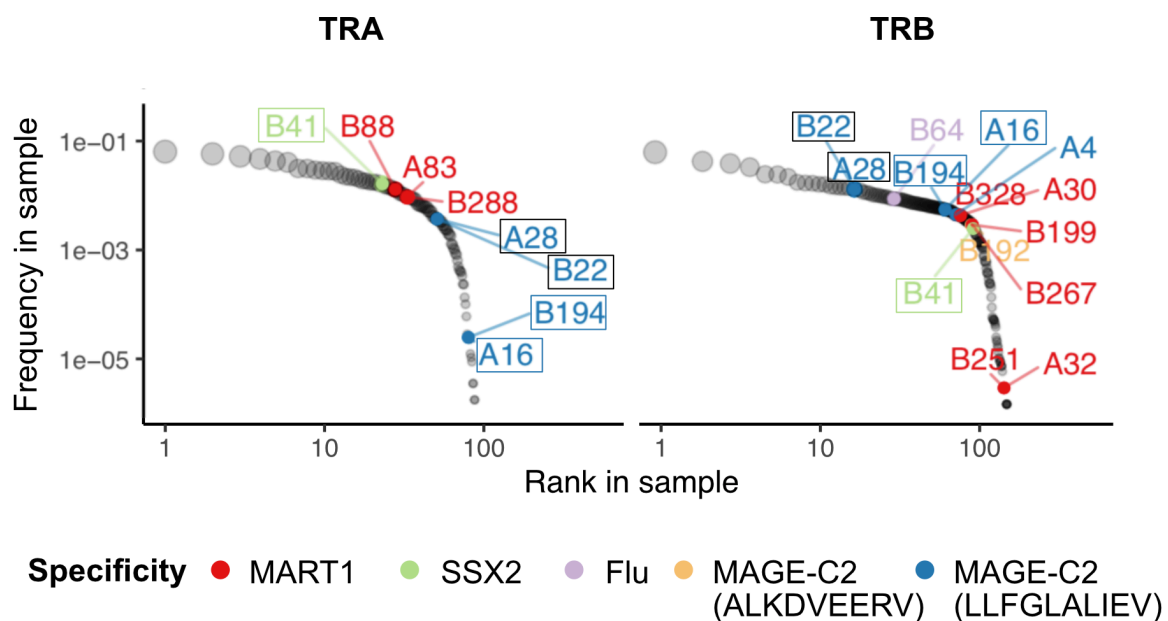


Figure 4-26. Matching of TCRs from the periphery with TCRs found within the tumour. TRA and TRB sequences found within the tumour of Patient 1 are plotted by frequency within the total TIL population and by rank within the sample. Matches at the respective alpha or beta chain with T cell clones in the periphery are labelled by their clone ID with colour indicating their specificity (prefix A = isolated from single-cell RNA-seq experiment, prefix B = isolated from T cell cloning experiments). Those highlighted in boxes are where both the alpha and beta chain from the peripheral TCR clone were found within both the TRA and TRB sequences of the tumour, with those in boxes of the same colour having the same TCR.

Two of these T cell clones were identified both from the single-cell RNA-seq data and from the expanded T cell clone experiments, and were specific for MAGE-C2 (LLFGLALIEV). Clone A28/B22 ranked 53rd most frequent amongst the TIL-*TRA* sequences and 18th highest amongst the TIL-*TRB* detected, whilst clone A16/B194 ranked 84th and 69th out of a total of 93 TIL-*TRA* and 174 TIL-*TRB* sequences detected respectively. Matches at both the *TRA* and *TRB* for a further single T cell clone (B41) specific for SSX2 were found within the TIL TCRs, ranking 24th by frequency for the *TRA* and 105th for the *TRB*. Isolated *TRA* and *TRB* reads for a number of MART-1-specific T cells were also detected, although in the absence of pairing for these, it is possible that some of these matches could be random given the high diversity of MART-1-specific TCRs. Isolated *TRB* sequences were also found for a Flu-specific T cell clone (B64) and a MAGE-C2-specific (ALKDVEERV) T cell clone (B192) identified in the cloning experiments. Using the RNA-seq data from the tumour, we looked for the expression of the tumour-associated antigens these T cells were reacting to within the tumour (Table 4-3). We were able to demonstrate expression of all three antigens, *MLANA*(MART-1), *SSX2* and *MAGEC2*, for which we were able to find specific T cells within the TIL, providing support for the expansion of these T cells being relevant to the anti-tumour response.

Table 4-3. Expression of genes for selected melanoma-associated antigens within bulk tumour RNA sequencing data for Patient 1

Gene	Transcripts per million
<i>TYR</i>	876.28
<i>MLANA</i>	727.32
<i>PRDX5</i>	489.71
<i>PRAME</i>	475.80
<i>PMEL</i>	409.12
<i>SSX2</i>	27.91
<i>MAGEC2</i>	26.38

Of the six T cell clones from the single-cell RNA-seq experiment found to match TIL TCRs at either the *TRA*, *TRB* or both (A4, A16, A28, A30, A32 and A83), 4 were seen more than

once, including both of the clones with *TRA* and *TRB* matches (A28 and A16) and two with *TRB* only matches (A4 and A32). To see whether we could identify any phenotypic features characteristic of these clones, we went back to the clustering data and looked at the assignment of these different clones across clusters over time (Figure 4-27). Phenotypically these cells were surprisingly diverse, being distributed over a variety of clusters. Clones 16 and 28 appeared to move from activated clusters to central memory phenotype over time, whilst clusters 4 and 32 were more often seen in clusters 6, 1 and 3. Notably no naïve phenotype clones (cluster 5) were found intra-tumourally. Due to the limited number of cells captured for these clones, it is hard to be able to draw any further conclusions about the phenotype or behaviour of these clones found in the tumour compared to other clones which were not.

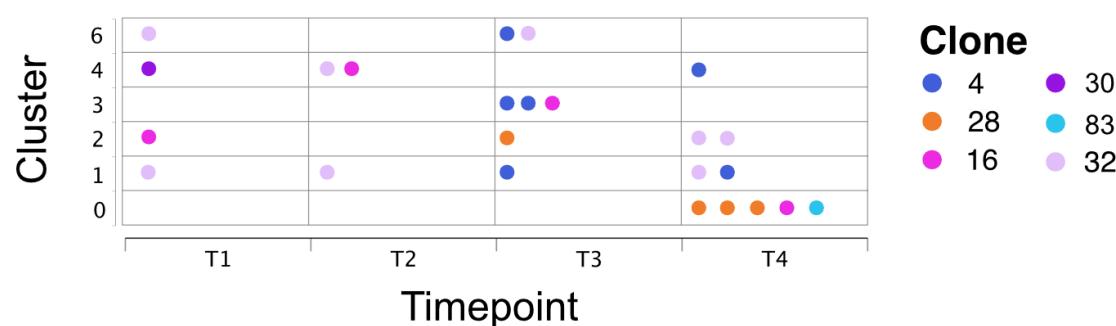


Figure 4-27. Distribution of peripheral T cell clones found intra-tumourally by cluster over time. Each cell is represented by a circle and coloured by clone ID.

4.7 Tumour-specific TCR library

Over the course of this experiment and our previous T cell cloning experiments we successfully determined the TCR sequences of over 1000 single T cells and identified 584 unique T cell clones based on their TCR. Of these, 384 had known reactivity (37 reactive to tumour cell lines, 347 reactive to peptides). For T cells specific for tumour-associated antigen peptides, a high level of concordance was seen between the data obtained from the two different experimental approaches (Figure 4-28).

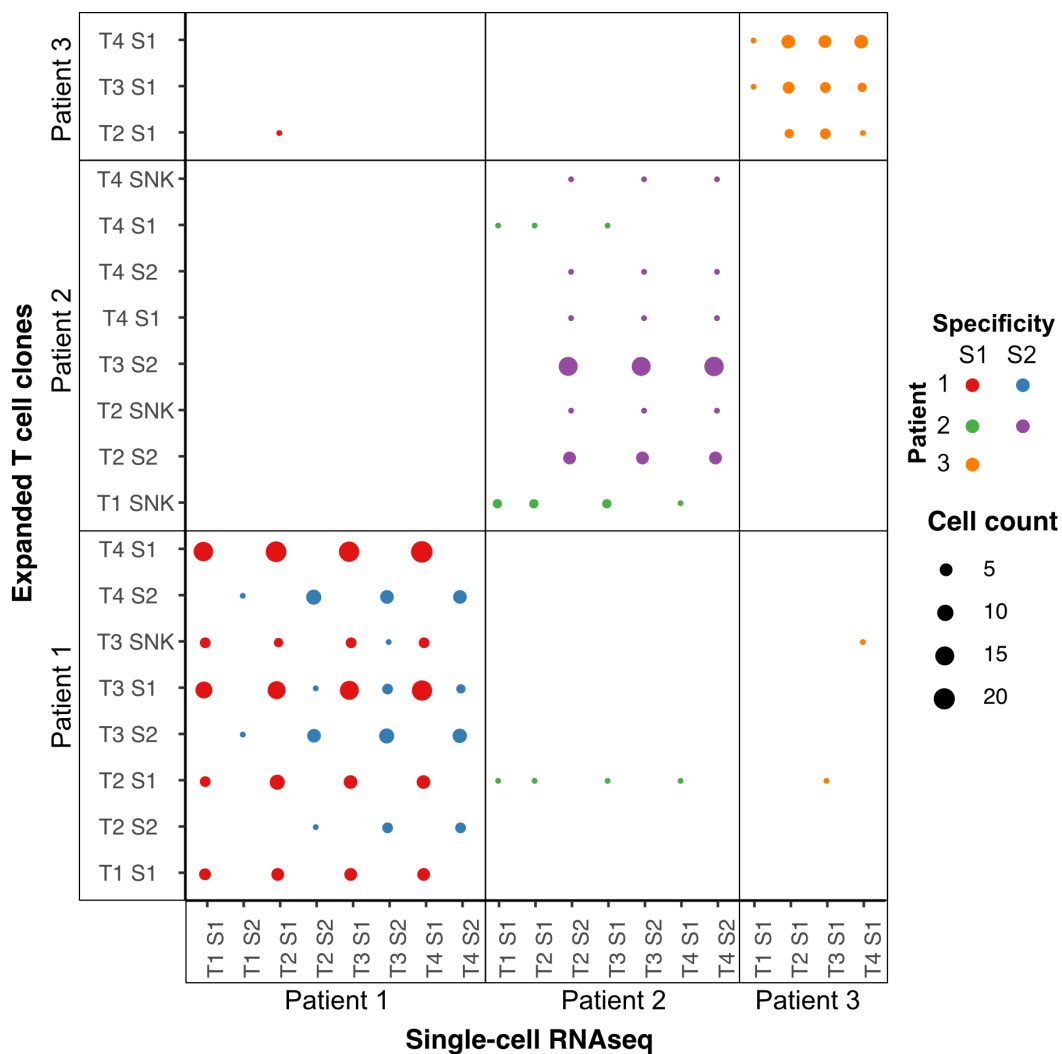


Figure 4-28. Number of cells with shared TCRs by timepoint and specificity between single-cell RNA-seq and expanded T cell clone datasets. Cells with TCRs shared between datasets are indicated by circles with the size representing the number of cells. Timepoint and specificity are indicated by T and S respectively. S1 = MART-1, S2 = MAGE-C2 (patient 1) or MAGE-A10 (patient 2), NK = where specificity was not known for expanded T cell clones but could be determined based on shared TCRs of known specificity from the single-cell RNA-seq data.

Despite a large number of these T cell clones being specific for the public antigen MART-1, very few TCRs were observed to be shared between patients. In addition, minimal evidence of cross reactivity between TCRs specific for different antigens was observed. It is possible that where this is seen, this may represent low level cross-contamination at the level of the TCR sequencing plate and work is ongoing to verify the identify and specificity of these individual clones. Whilst some of the MART-1 sequences matched those that had been previously reported in public databases such as VDJdb¹⁹⁸, those reactive to MAGE-C2 and MAGE-A10 have not to our knowledge been previously described.

Unexpectedly, TCR γ (*TRG*) (but not δ) CDR3 sequences were also identified in 16% of cells where both TCR α and/or β sequences were found. These were detectable across all three patients in the single-cell RNA-seq experiment and amongst cells of all specificities isolated. These *TRG* sequences were highly diverse and strongly linked to corresponding TCR clones as defined by the $\alpha\beta$ chain (Figure 4-29). Aside from increased expression of the gene *TARP* (TCR gamma alternate reading frame protein) which is embedded within the *TRG* locus and downregulation of *CD8B* encoding the CD8 β chain, differential gene expression analysis did not identify any other features discriminating these from cells without co-expression of *TRG*, and no distinguishing patterns of cluster behaviour over time were observed for these clones. Therefore, whether this observation is of any biological significance is currently unknown.

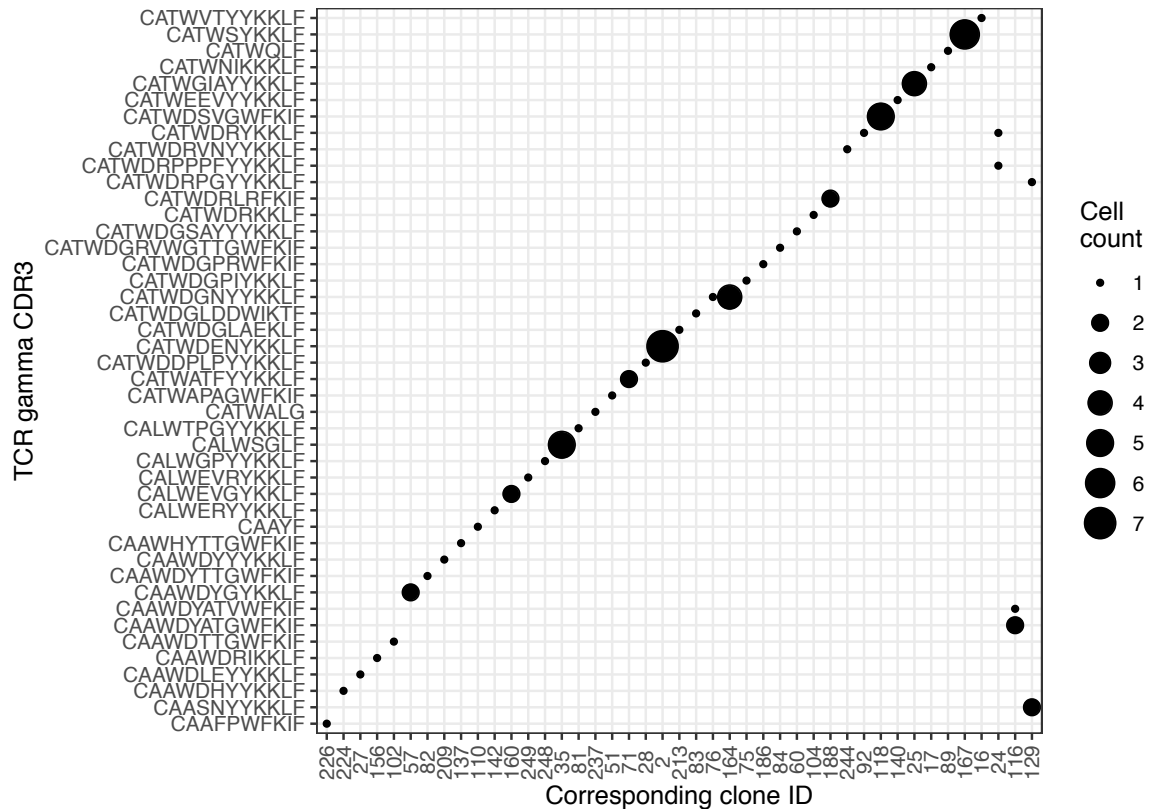


Figure 4-29. TCR gamma CDR3 sequences identified and their relationship to T cell clones as defined by their TCRA β . Each circle represents a clone with the size of the circle indicating the number of cells in that clone expressing the indicated TCR gamma CDR3.

4.8 Discussion

In the analyses presented in this chapter, we have explored the transcriptional changes in tumour-specific T cells detectable in the blood in melanoma patients during combination anti-CTLA-4 and anti-PD-1 checkpoint inhibitor treatment at a single-cell resolution. As well as validating at a transcriptomic level changes we had observed previously, including activation of tumour-specific T cells in response to checkpoint inhibitor therapy, we were able to characterise in much greater detail the phenotype of these cells over the course of therapy. Different clusters of cells based on shared gene expression profiles were identified, which represented distinct and recognisable phenotypic states. These different clusters were found to be associated with different timepoints on therapy and appeared to reflect a spectrum of differentiation states of the cells over time. Substantial heterogeneity was observed not only between individuals, but between T cells of different specificities and also

within cells of the same specificity bearing the same TCR over the course of treatment, highlighting the value of single-cell approaches in such analyses. These shifts in phenotype of cells with the same TCR over time might account for some of the functional differences we observed in expanded clones in terms of functional avidity in Chapter 3.7, with cells in different phenotypic states being associated with different cytotoxic capabilities.

As well as capturing the initial changes in response to treatment, the late on-treatment (T4) sample allowed us to study the long-term effect of therapy upon these cells. As well as displaying fewer changes in response to therapy, cells with a cytotoxic/effector phenotype which were typically present pre-treatment did not appear to maintain long-term any changes in their phenotype, and in most cases reverted to their pre-treatment phenotype despite ongoing treatment. By contrast, cells which were observed to undergo proliferation and activation, more commonly were found in the central memory cluster at the late on-treatment timepoint.

As all three patients in this cohort responded to treatment, whether the balance between central memory phenotype and effector phenotypes impacts upon treatment efficacy is unknown. As mentioned previously, although patient 1 responded to treatment initially, unlike patients 2 and 3, this response was not maintained. Whilst the MART-1-specific clones in this individual had a cytotoxic phenotype, evidence of MAGE-C2 memory cell formation was observed. MAGE-C2 expression was however, less pronounced within the tumour of this individual pre-treatment than tumour-associated antigens such as MART-1 and it is possible that either the expression could have been insufficient for MAGE-C2 clones to generate an effective anti-tumour response, or perhaps that MAGE-C2 expressing cells were relatively few in number and could have been successfully eliminated, with progression ultimately driven by tumour cells expressing different antigens. Serial biopsies from tumour samples over the course of treatment might potentially prove informative in cases such as this, to allow changes in T cell specificity and phenotype to be correlated with expression of individual antigens within the tumour.

Whether responses to checkpoint blockade are driven by reinvigoration of pre-existing or novel T cell clones has not previously been extensively investigated, and recent work suggests that expanded clones seen amongst TILs in responding tumours were novel clonotypes²⁰. Whilst cell numbers in our experiment limit the extent of conclusions we are able to draw from this data, the observation that alterations in pre-existing tumour-specific T cell responses in the periphery tended to be non-sustained compared with those in newly expanded T cell clones might support changes in novel clonotypes as the potential mediators of efficacy. However, whilst Yost *et al.*²⁰ were able to observe frequent transitions between memory, effector and activated states in response to therapy, we saw limited overlap and transition between cells with a terminally differentiated cytotoxic effector phenotype and other phenotypic states. This may be due to differences in definitions of effector and activated phenotypes or different functionality between cells in the periphery and those in the tumour microenvironment.

Previous analyses have implicated TCF7+ PD-1+^{211,216} and EOMES positive T cells¹³⁵ in the response to checkpoint inhibitor treatment. Whilst we were not able to identify a discrete cluster within our data displaying these characteristics together, these previous analyses were based on intra-tumoural T cells and compared at most paired on-therapy to pre-therapy samples without tracing these cells over time. Our data suggest that these may represent cells at different states upon a continuum in terms of cellular differentiation. It is not possible however from this small cohort of patients, all of whom responded to treatment, to establish which phenotype of cells present at which timepoints are those responsible for mediating response. Recent data using CyTOF based analysis of the phenotype of neoantigen-specific tetramer+ cells in patients receiving anti-PD-L1 treatment for NSCLC¹⁴⁰ showed that the phenotype of the majority of neoantigen-specific cells in responding patients was that of a late differentiated effector phenotype characterised by high expression of *KLRG-1*, *CD57* and *2B4*, consistent with cluster 1 and 6 in our data. Conversely, non-responders had higher frequencies of cells with a memory like phenotype

which the authors reasoned may be less effective in mediating anti-tumour responses. Other work by our colleagues¹³⁴, in a similar patient cohort, found associations between the number of large clonally expanded T cell populations seen after three weeks on therapy (defined as clones occupying >0.5% of the CD8+ T cell repertoire) and clinical benefit. Transcriptomic analysis found these large clones to have an effector memory phenotype characterised by expression of markers including *GNLY*, *GZMH*, *GZMB*, *NKG6*, *KLRD1* and *PRF1*, again a phenotype similar to that described by Fehlings *et al.*¹⁴⁰ and to cells in clusters 1 and 6 of our analysis. Although it is not stated in the work by Fehlings *et al.* at what time their on-treatment sample was obtained, it may be possible that the phenotype of cells associated with positive outcome during the initial stages of response are different from those associated with long term responses, highlighting the need for longitudinal studies capturing late on-treatment samples. In addition, whether ongoing treatment is required to sustain the changes induced by checkpoint inhibitor therapy also remains unknown, and evaluation of samples from patients with maintained response despite discontinuation of treatment, as well as patients who progressed on-treatment might shed more light upon behaviours associated with outcome. It is possible that the genes we identified that defined the different cellular populations within our data could potentially be candidate gene markers for use in such further investigations, allowing the use of more targeted but higher throughput techniques in a wider cohort of patients than we used in this experiment.

As well as allowing us to define and track individual T cell clonotypes over the course of therapy, our TCR analyses identified some unexpected findings in relation to the co-expression of the *TRG* genes encoding the T cell receptor γ in cells also expressing the genes for *TRA* and *TRB*. Whilst a subset of CD8 $\alpha\beta$ + $\gamma\delta$ + T cells have been reported by one group with a potential role in inflammatory bowel disease²¹⁸, co-expression of *TRG* and *TRB* in the absence of *TRD* is not something that is currently recognised within the current paradigm of T cell receptor rearrangement. Work in CD4+ V δ 1+ $\gamma\delta$ T cells has shown that

these may have the potential to develop into functional $\alpha\beta$ T cells in a process of extrathymic T cell development²¹⁹, suggesting that TCR gene expression may be more flexible than previously thought. Further work to establish whether these *TRG* chains are expressed at the cell surface would be the first step towards validating this observation and determining whether further investigation of this is warranted.

In addition, the library of nearly 400 tumour-specific TCRs that we have identified over the course of this experiment and those described in Chapter 3, provides a potentially rich resource for future investigations. Those where the specificity is known could provide novel TCRs for therapeutic approaches such as bispecifics, and further investigation of those reactive to tumour lines but not to our tumour antigen peptides, might reveal additional TCRs or possibly previously undescribed tumour-associated antigens. The fact that we were able to observe expanded tumour-specific T cell clones from the periphery within the tumour-infiltrating lymphocyte population adds weight to the notion that T cell clonotypes found in the blood can be representative of those within the tumour and further supports peripheral immune monitoring as a valid approach for tracking responses to immunotherapy based treatments.

Chapter 5. Comparisons of the effects of different immunotherapy treatments upon T cell populations and associations with clinical outcome

5.1 Introduction

In Chapter 3 we showed that activation of CD4+ and CD8+ T cells is seen early on-treatment with combination Ipilimumab and Nivolumab therapy, and demonstrated that the CD8+ activated population contained cells capable of recognising tumour lines or peptides. It is known that the predominant effect of anti-CTLA-4 blockade is at the level of T cell priming, whilst blockade of anti-PD-1 acts at the level of T cell effector function, however whether the immunological effects when these drugs are given in combination are additive or whether they have a synergistic effect is not clear. For example, there is some evidence that the CD28/B7 co-stimulatory pathway is required for an effective proliferative response to PD-1 therapy during chronic viral infections in mice²²⁰. Although B7 is not typically expressed on tumour cells, it is expressed on antigen presenting cells such as dendritic cells whose tumour infiltration has been implicated in the response to anti-PD-1 blockade²²¹. It is therefore possible that removing CTLA-4 induced suppression of co-stimulation by this route might augment at an immunological level anti-PD-1 responses. Furthermore, we do not know if the observations we made are specific for combination checkpoint inhibitor treatment or reflective of a more generic immunotherapy induced immune response, and whether the changes are related to clinical efficacy.

To address this, we performed CyTOF analysis in a second larger cohort of patients receiving checkpoint inhibitor monotherapy treatment, combination treatment, or the ImmTAC IMCgp100 with or without additional checkpoint blockade. We also incorporated tetramers into the CyTOF panel to allow us to track tumour-specific cells in HLA-A*02:01 patients. We looked first at the differences in the immune response to the different

treatments both at the level of CD8+ T cells and antigen-specific T cells and then went on to assess these changes in relation to clinical outcome.

5.2 Patient samples/clinical characteristics

Serial blood samples from 10 additional patients treated with combination anti-PD-1 and anti-CTLA-4 (Ipilimumab and Nivolumab) therapy, 16 patients treated with anti-PD-1 monotherapy (Pembrolizumab) and 3 patients treated with anti-CLTA-4 monotherapy (Ipilimumab) were collected. Numbers for the later were limited by the availability of previously banked samples as single agent anti-CTLA-4 treatment is rarely used in clinical practice since the development of PD-1 based therapies. In addition, we obtained samples from a small number of patients receiving treatment within clinical trials at the Churchill Hospital in Oxford with the ImmTAC IMCgp100 either as monotherapy (1 patient), or in combination with the anti-PD-L1 antibody Durvalumab (2 patients), or Durvalumab plus the CLTA-4 inhibitor Tremelimumab (4 patients). Peripheral blood mononuclear cells were isolated and frozen as described in Chapter 2.4. Samples from 34 different patients over 6 different treatment regimes, totalling 120 individual samples were collected, processed and subjected to staining with metal labelled antibodies and CyTOF analysis as described in Chapter 2.7.2.1. The clinical characteristics of the patients at baseline are described in Table 5-1.

Table 5-1. Baseline characteristics of second melanoma patient cohort

Characteristic	Ipilimumab + Nivolumab	Pembrolizumab	Ipilimumab	IMCgp100
Patients - n	10	16	3	Monotherapy 1 + Durvalumab 2 + Durvalumab & Tremelimumab 4
Total Samples - n	25	56	8	31
Median/patient	2.5	3.5	3	5
Range/patient	2-3	2-4	2-3	2-6
Sex – n (%)				
M	7 (70)	7 (43.8)	3 (100)	5 (71.4)
F	3 (30)	9 (56.2)	0 (0)	2 (28.6)
Age - years				
Median	53.5	66	57	58
Range	32-70	41-88	52-67	43-74
Melanoma type – n (%)				
Cutaneous	9 (90)	14 (87.5)	2 (66.6)	6 (85.7)
Ocular	1 (10)	1 (6.25)	0 (0)	1 (14.3)
Mucosal	0 (0)	1 (6.25)	1 (33.3)	0 (0)
BRAF status – n (%)				
Mutant	7 (70)	2 (12.5)	1 (33.3)	0 (0)
Wild type	3 (30)	12 (78.1)	2 (66.6)	7 (100)
Not available	0 (0)	2 (12.5)	0 (0)	0 (0)
Prior treatment – n (%)				
Nil	8 (80)	6 (37.5)	2 (66.6)	1 (14.3)
Immunotherapy	0 (0)	9 (56.3)	0 (0)	6 (85.7)
Ipilimumab	0 (0)	8 (50)	0 (0)	3 (42.9)
IMCgp100	0 (0)	1 (6.25)	0 (0)	1 (14.3)
Pembrolizumab	0 (0)	0 (0)	0 (0)	5 (71.4)
Ipi + Nivo	0 (0)	0 (0)	0 (0)	1 (14.3)
Targeted therapy	2 (20)	1 (6.3)	1 (33.3)	0 (0)
Chemotherapy	0 (0)	3 (18.8)	0 (0)	1 (14.3)

5.3 Dissecting the effects of different checkpoint inhibitor therapies upon the peripheral immune response

We started by comparing the differences in the immunological response to different checkpoint inhibitor therapies. The scheduling of treatment administration and blood sampling for these patients is shown in Figure 5-1.

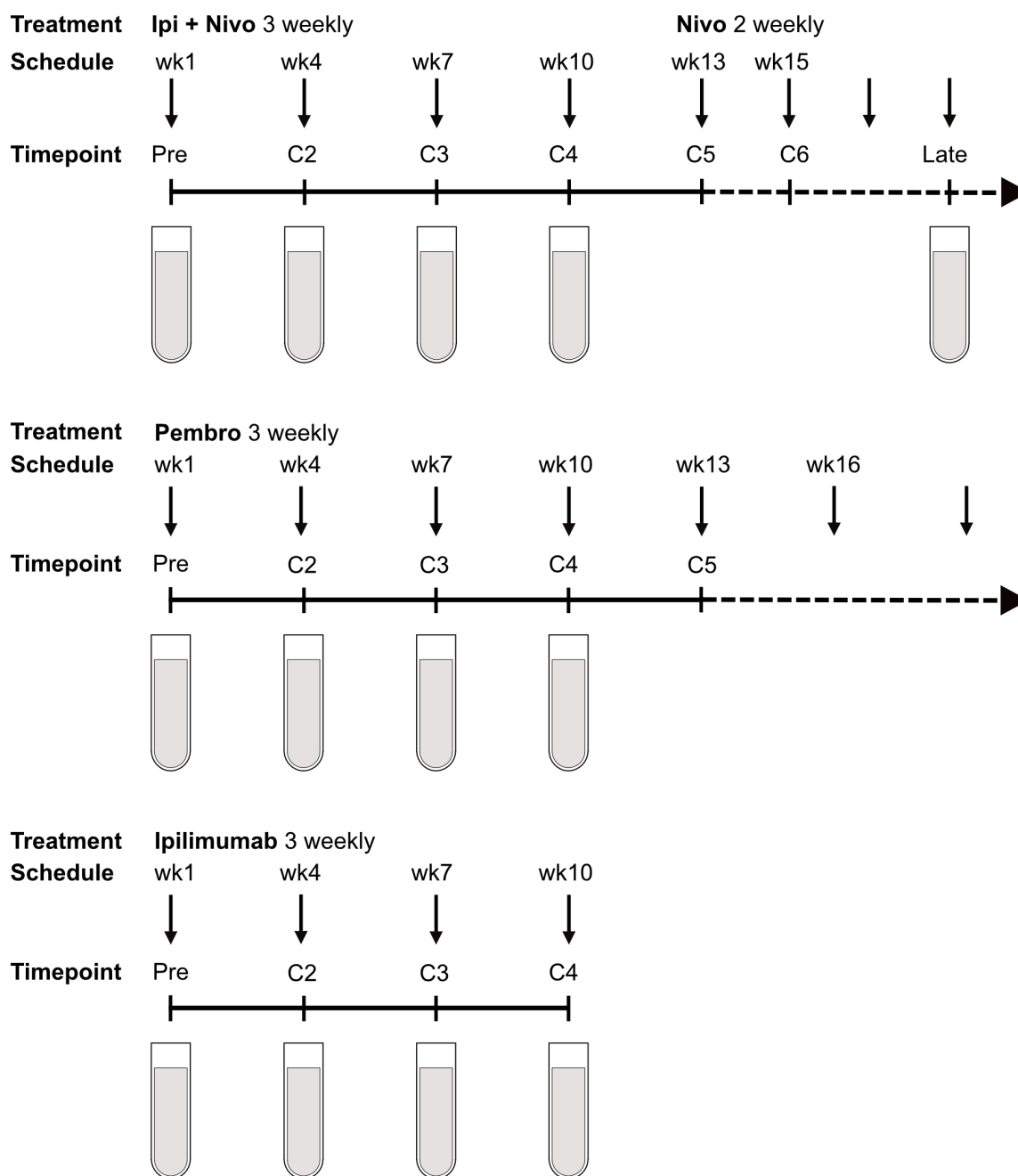


Figure 5-1. Schedule of treatment administration and blood sampling for checkpoint inhibitor treatments.

Gating of CD8⁺ and CD4⁺ populations from CyTOF stained PBMCs was performed using a combination of manual gating and clustering. To allow us to directly compare the different treatments, clustering was then performed separately on the CD8⁺ and CD4⁺ population across all 120 samples using FlowSOM, followed by further grouping into 20 metaclusters, allowing for a degree of overclustering to ensure we were able to identify low frequency populations.

5.3.1 Comparison of combination anti-CTLA-4 and PD-1 therapy to PD-1 monotherapy and CTLA-4 monotherapy

In order to determine the differences in the immunological response between patients treated with combination checkpoint inhibitor therapy and PD-1 monotherapy, we first focused on CD8⁺ cells, and looked at changes in the frequency of the 20 clusters identified (Figure 5-2 A & B) at different timepoints on treatment compared to baseline (Figure 5-2 C & D). 11 clusters were found to be significantly differentially abundant early on-treatment at C2 (after 3 weeks) and 10 clusters on-treatment at C4 (after 9 weeks) in patients treated with combination checkpoint inhibitor therapy, compared with 5 clusters at C2 and 4 clusters at C4 for patients treated with PD-1 monotherapy (Figure 5-2 E & F). All clusters that either expanded or contracted upon PD-1 monotherapy showed similar changes during combination therapy (Figure 5-2 G). However, the magnitude of change in abundance for these shared clusters was significantly greater for combination therapy than PD-1 monotherapy (Figure 5-2 H) (mean log₂ fold change 2.52 vs 1.36 at C2 and 2.29 vs 1.19 at C4, $p = 0.0128$ and 0.276 respectively for paired, two-tailed t-test).

Among the clusters differentially abundant over time across both treatments, clusters 20, 12, 13 and 1 expressed high levels of both the activation markers CD38 and HLA-DR, while cells in cluster 18 expressed CD38, CCR7 and CD27 but not HLA-DR, consistent with a more central memory rather than effector T cell phenotype. Cells in cluster 20 were found to have a high expression of the proliferation marker Ki67, high levels of CTLA-4, and expressed high levels of PD-1 prior to treatment (Appendix E, Figure E-1). Cells in cluster 1 displayed a CD103⁺Integrin β 7⁺ tissue memory phenotype similar to the population we saw changing in Chapter 3.

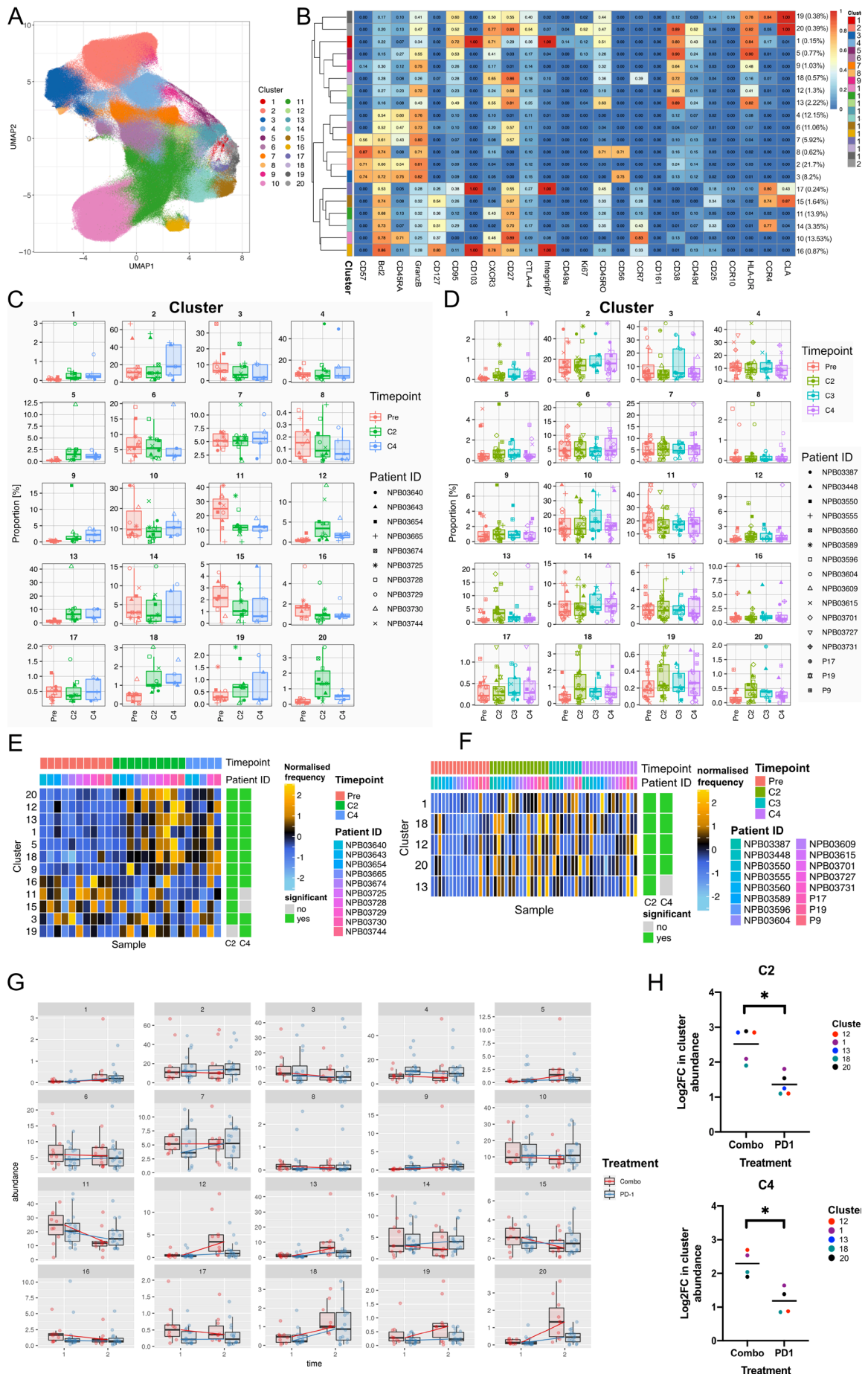


Figure 5-2. Analysis of CD8+ T cells for patients treated with combination checkpoint inhibitor therapy and PD-1 monotherapy. A) UMAP for all patients (5000 cells randomly selected per patient). Cells coloured according to metacluster. B) Heatmap of median marker intensities of CD8+ metaclusters (arcsinh, 0-1 transformed marker expression calculated over cell from all the samples). C) Proportion of cells in each cluster at different timepoints (indicated by colours) for patients treated with combination therapy (individual patients indicated by different symbols). D) Proportion of cells in each cluster at different timepoints (indicated by colours) for patients treated with PD-1 monotherapy (individual patients indicated by different symbols). E) Metaclusters with significant changes in abundance over treatment for patients treated with combination therapy. F) Metaclusters with significant changes in abundance over treatment for patients treated with PD-1 monotherapy. Heatmaps shows arcsine-square-root transformed cell frequencies normalised per cluster (row) to mean of zero and standard deviation of 1. Each column represents one patient samples from one timepoint. The colour indicates the extent of relative under-representation (blue) to over-representation (orange). Significantly differently abundant clusters (FDR adjusted p -value <0.05) are indicated in green in the bar on the right hand side. G) Comparison of change in abundance of 20 metaclusters between C1 and C2 for combination therapy (blue) and PD-1 monotherapy (red). H) Log2 FC in cluster abundance for clusters that changed in response to both combination therapy and PD-1 monotherapy at C2 and C4. Each cluster is indicated by a different colour. Mean log2 FC 2.52 vs 1.36 at C2 and 2.29 vs 1.19 at C4, $p = 0.0128$ and 0.276 respectively for paired, two-tailed t-test.

The frequency of a number of other clusters increased (clusters 19, 5 and 9) or decreased (clusters 16, 11, 15 and 3) in response to treatment, but these changes reached significance only during combination therapy and not PD-1 monotherapy. Those that increased were additional clusters of low frequency activated cells, whilst those that decreased were cells with a T_{CM} or T_{EMRA} phenotype. However, we could not confirm the contraction of CCR7+CD45RA+CD27+CDR45RO- naïve cells (cluster 10) observed in the analysis of the smaller cohort of patients described in Chapter 3.3.2.

We next went on to perform analysis of the differential abundance of the 20 metaclusters of CD4+ T cells (Figure 5-3 A-D). 9 clusters were found to be differentially abundant at C2 (after 3 weeks) of combination therapy and 6 clusters at C4 (after 9 weeks) of treatment. In contrast, only 4 clusters were differentially abundant at C2 and 5 at C4 for PD-1 monotherapy (Figure 5-3 E-F). All of the clusters that changed significantly in abundance in response to PD-1 monotherapy, with the exception of cluster 14, also changed in

combination therapy and with the same directionality but lesser magnitude, similar to what we observed in the CD8⁺ population (Figure 5-3 G). These shared clusters (11,10, 9 and 20) again all displayed the activation marker CD38, although only cells within cluster 20 also expressed HLA-DR. No strong Ki67⁺ proliferation signature was observed for any of the CD4⁺ clusters. Similar to the CD8⁺ clustering, we saw some other activated clusters (8 and 7 and 15) increased both in combination therapy and PD-1 monotherapy, but without reaching statistical significance in the later. We also saw a reduction in the small frequency of circulating cells with a CD103⁺ Integrin β 7⁺ tissue homing memory phenotype displaying the skin homing marker CLA (cluster 17).

With the exception of cluster 14, all fluctuations in cluster frequency were first apparent 3 weeks after treatment and then sustained at 9 weeks. In contrast, cluster 14 increased in frequency only 9 weeks into treatment, and only in PD-1 monotherapy. This cluster had features of Th1 central memory cells (CCR7⁺CD45RO⁺CD27⁺CXCR3⁺). In combination therapy, this cluster trended towards a reduction in response to treatment rather than an increase. Two other CXCR3 positive clusters (9 and 11) showed a transient increase at C2 in both PD-1 monotherapy and combination therapy.

No significant changes in the abundance of CD4⁺ Tregs were observed with either treatment (cluster 6). Similar to the CD8⁺ clustering, and unlike the data in Chapter 3.3.2, we could not confirm a significant reduction in circulating naïve CD4⁺ cells (cluster 18) in response to either treatment.

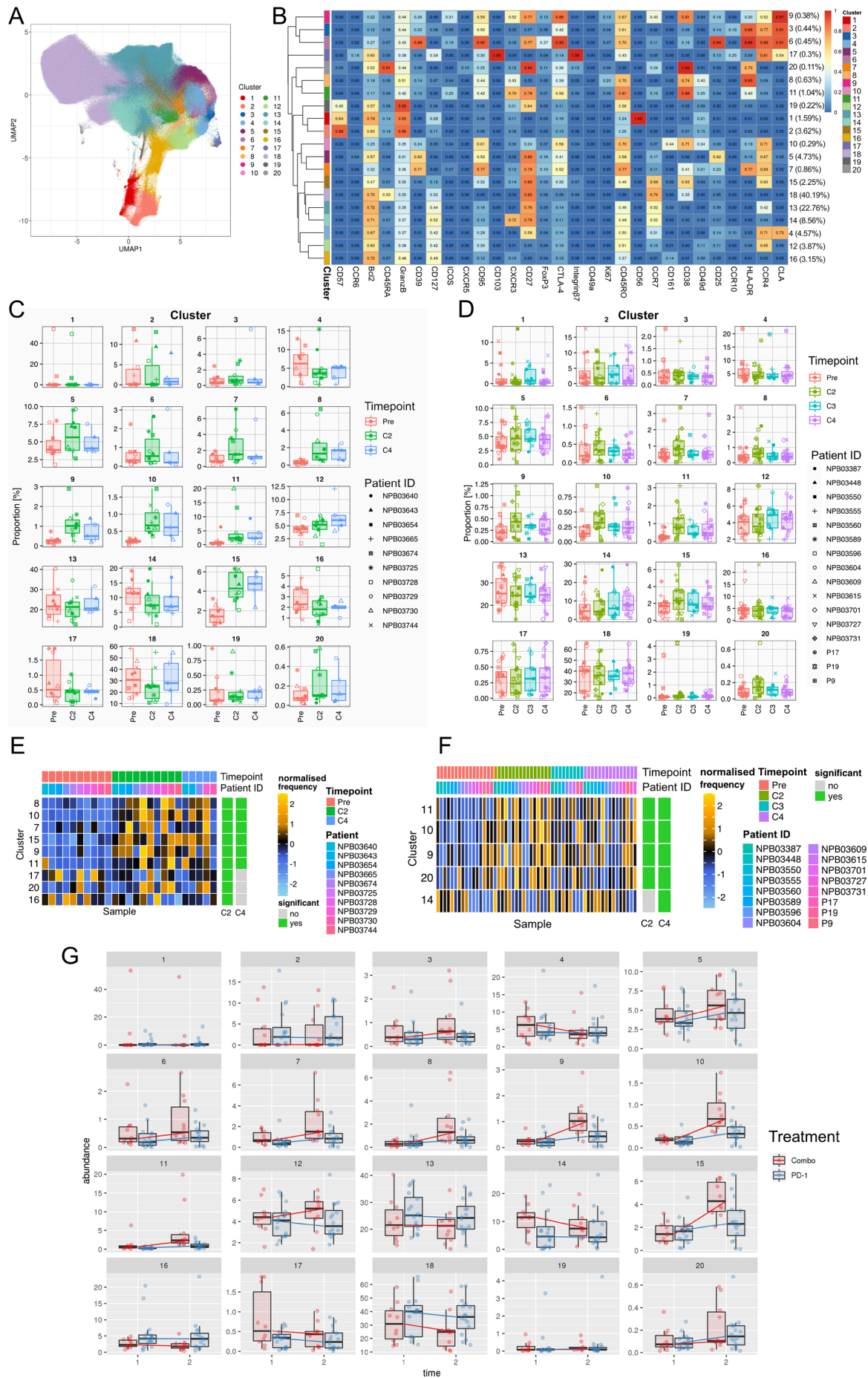


Figure 5-3. Analysis of CD4+ T cells for patients treated with combination checkpoint inhibitor therapy and PD-1 monotherapy. A) UMAP for all patients (5000 cells randomly selected per patient). Cells coloured according to metacluster. B) Heatmap of median marker intensities of CD8+ metaclusters (arcsinh, 0-1 transformed marker expression calculated over cell from all the samples). C) Proportion of cells in each cluster at different timepoints (indicated by colours) for patients treated with combination therapy (individual patients indicated by different symbols). D) Proportion of cells in each cluster at different timepoints (indicated by colours) for patients treated with PD-1 monotherapy (individual patients indicated by different symbols). E) Metaclusters with significant changes in abundance over treatment (FDR adjusted p -value <0.05) for patients treated with combination therapy. F) Metaclusters with significant changes in abundance over treatment (FDR adjusted p -value <0.05) for patients treated with PD-1 monotherapy. G) Comparison of change in abundance of 20 metaclusters between C1 and C2 for combination therapy (blue) and PD-1 monotherapy (red).

In order to extend our observations on the differences between therapies, we looked also at a small cohort of 3 patients who received Ipilimumab monotherapy treatment. CyTOF staining of these samples was performed in a separate experiment with a slightly different antibody panel from the cohort described above, so clustering of CD4+ and CD8+ lymphocytes from this experiment was performed separately (Figure 5-4). Due to the low numbers of patients and variation in timing of the available samples, for the purpose of this analysis, samples obtained from patients at C3 and C4 were considered together to represent an 'on-treatment' timepoint for differential abundance analyses.

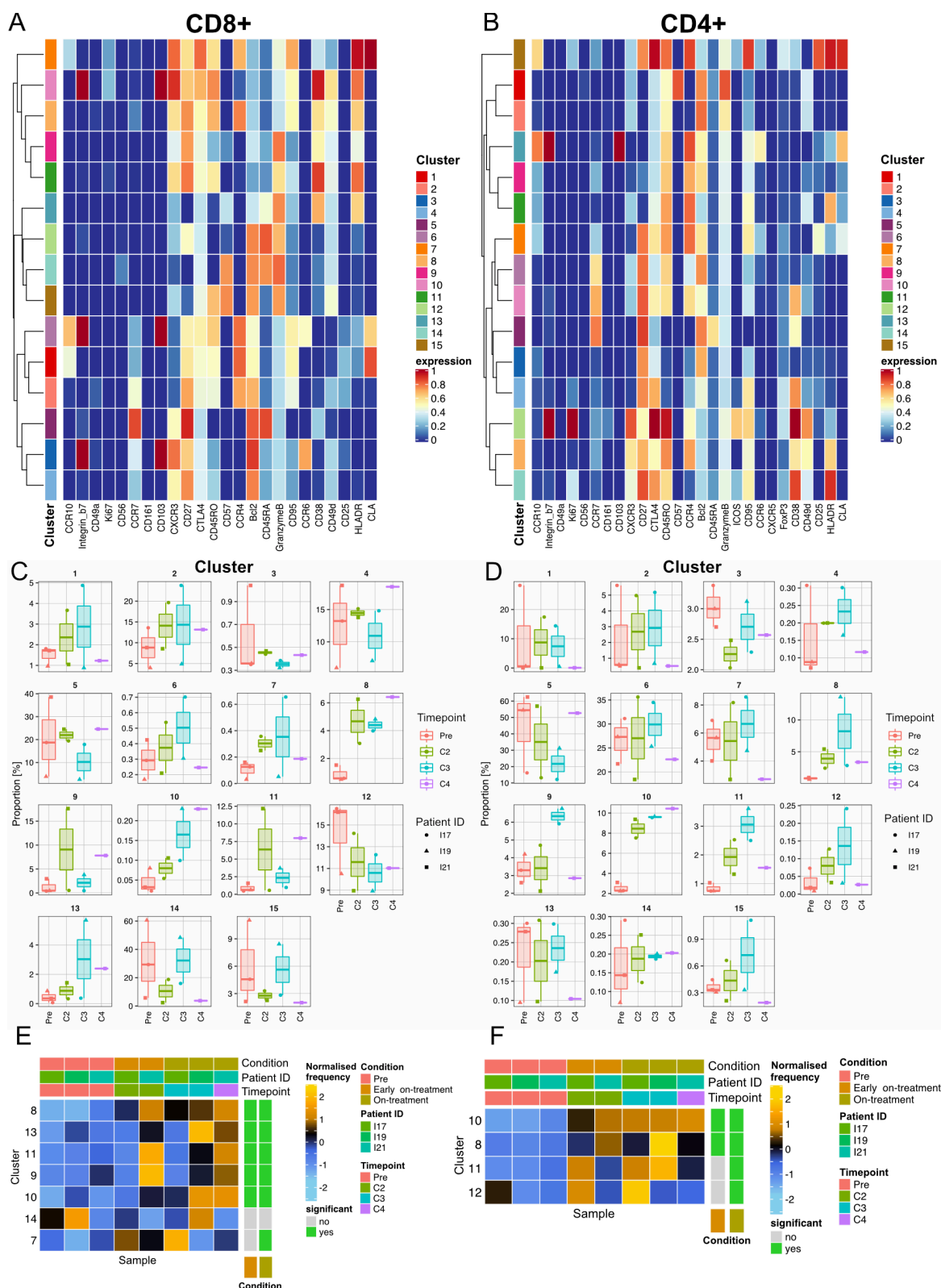


Figure 5-4. Analysis of CD8+ and CD4+ cells from patients treated with Ipilimumab. A) Heatmap of median marker intensities of CD8+ metaclusters (arcsinh, 0-1 transformed marker expression calculated over cell from all the samples). B) Heatmap of median marker intensities of CD4+ metaclusters (arcsinh, 0-1 transformed marker expression calculated over cell from all the samples). C) Proportion of cells in each CD8+ cluster at different timepoints (indicated by colours,

individual patients indicated by different symbols). D) Proportion of cells in each CD4+ cluster at different timepoints (indicated by colours, individual patients indicated by different symbols). E) CD8+ metaclusters with significant changes in abundance over treatment (FDR adjusted p -value <0.05). F) CD4+ metaclusters with significant changes in abundance over treatment (FDR adjusted p -value <0.05).

In this analysis we found 5 clusters to be differentially abundant early on-treatment (after 3 weeks) and 6 clusters on-treatment (after 6 or 9 weeks) for the CD8+ population and 2 clusters early on-treatment and 4 clusters at the on-treatment timepoint for the CD4+ population. All of the clusters that were differentially abundant across both CD4+ and CD8+ populations were high in the activation markers CD38 and/or HLA-DR. In addition, increases in cells with CD103+Integrin β 7+ phenotype were observed for both the CD8+ (cluster 10) and CD4+ (cluster 12) populations. Two of the activated clusters CD8+ clusters (10 and 7) and one of the CD4+ clusters (12) expressed the proliferation marker Ki67. Similar to our observations in patients treated with combination therapy or PD-1 monotherapy, no significant changes were seen in the abundance of CD4+ Tregs (cluster 15) in response to Ipilimumab monotherapy.

5.3.2 Comparison of ImmTAC immunotherapy to immune checkpoint blockade

To provide a comparison to checkpoint inhibitor therapy, and to establish whether the activation we observed was specific to checkpoint inhibition or indicative of a more general anti-tumour immune response, we also analysed samples from a limited number of patients treated with the ImmTAC IMCgp100. The patients analysed received IMCgp100 alone (IMCalone), IMCgp100 together with the anti-PD-L1 inhibitor Durvalumab (IMC-Durv) or IMCgp100 together with Durvalumab plus the anti-CTLA-4 inhibitor Tremelimumab (IMC-Treme-Durv). All patients receiving the combination regimes received a monotherapy run-in of 2 doses of IMCgp100 alone given at weekly intervals on day 1 and 8, before introduction of the combination therapy alongside IMCgp100 on day 15 (Figure 5-5).

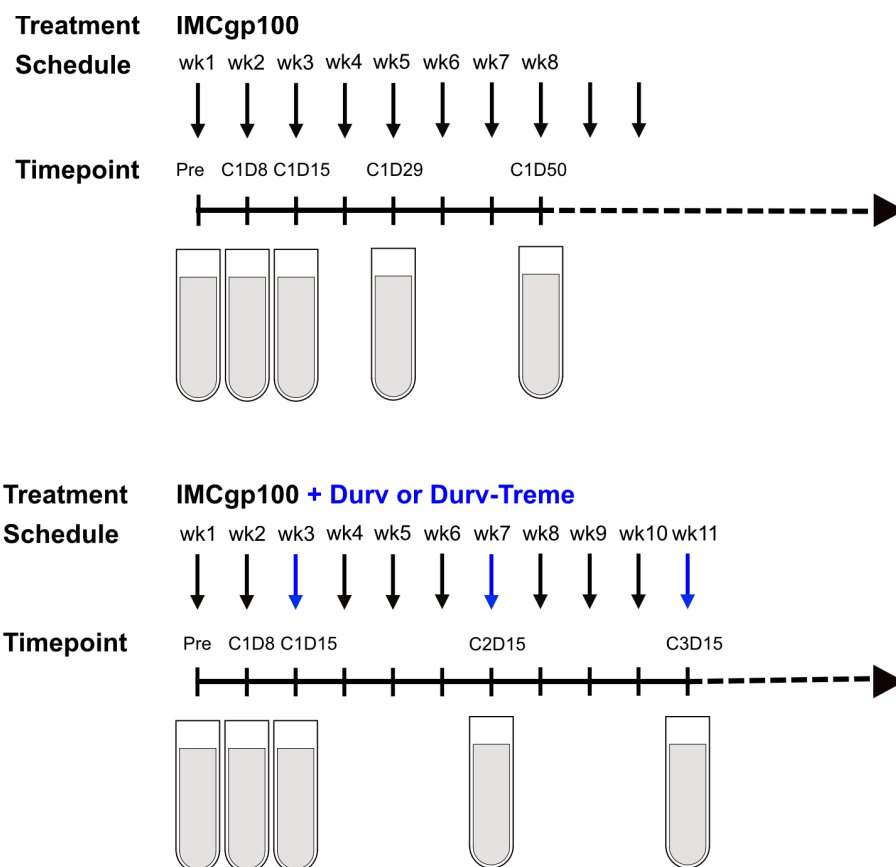


Figure 5-5. Schedule of treatment administration and blood sampling for IMCgp100 treated patients. IMCgp100 was given on a weekly basis in all treatment combinations. Timepoints where Durvalumab (Durv) or Durvalumab and Tremelimumab (Durv-Treme) were given in addition to IMCgp100 are indicated by blue arrows.

Clustering performed together with the samples from patients treated with combination checkpoint inhibitor therapy and PD-1 monotherapy, identified different changes in response to therapy during the IMCgp100 monotherapy phase than seen previously with the checkpoint inhibitor therapy (Figure 5-6).

For the CD8+ compartment (Figure 5-6 A & C), the only significant change seen during the IMCgp100 monotherapy phase was a marked and transient expansion of CD45RO+CD27+ cells expressing the skin homing markers CLA, CCR4 and CCR10 (cluster 19) at day 8 (after 1 dose of IMCgp100 treatment), which subsequently decreased at day 15 (Figure 5-6 C).

Figure 5-6. Analysis of CD8+ and CD4+ cells from patients treated with IMCgp100 or IMCgp100 combination therapies. A) Heatmap of median marker intensities of CD8+ metaclusters (arcsinh, 0-1 transformed marker expression calculated over cell from all the samples). B) Heatmap of median marker intensities of CD4+ metaclusters (arcsinh, 0-1 transformed marker expression calculated over cell from all the samples). C) Proportion of cells in each CD8+ cluster at different timepoints (indicated by colours, individual patients indicated by different symbols). D) Proportion of cells in each CD4+ cluster at different timepoints (indicated by colours, individual patients indicated by different symbols). E) Biaxial plot for one individual (01-A732) showing frequency of CD8+ cells expressing the skin homing markers CCR4 and CLA over time. F) Biaxial plot for one individual (01-A732) showing frequency of CD4+ Treg cells displaying skin homing marker CLA over time. Cells were gated on CD4+CD25+CD127- and displayed as FoxP3 vs CLA. G) Serial biaxial plots of CD8+ T cell activation as defined by CD38+HLA-DR+ cells for patient NPB_03555/4401-105 during PD-1 monotherapy followed by treatment with IMCgp100 + Durvalumab. H) Abundances of activated cell clusters for patient NPB_03555/4401105 over sequential PD-1 monotherapy and IMCgp100 + Durvalumab treatments.

This was also accompanied by an increase in CD4+CLA+ Tregs (cluster 6 – Figure 5-6 B & D) at day 8, although this did not reach statistical significance given the low sample numbers and after correction for multiple testing (FDR = 0.19). These observations were confirmed by manual gating of CD8+CCR4+CLA+ and CD4+CD25+CD127-FoxP3+CLA+ cells (Figure 5-6 E & F). Notably, these cells were only marginally expanded during combination therapy or PD-1 monotherapy (Figure 5-2 & Figure 5-3).

Aside from the expanding skin homing cluster, which expressed HLA-DR but not CD38, whilst no significant activation of CD8+ T cells was observed during the IMCgp100 monotherapy phase, we observed a significant increase in the frequency of clusters of activated CD8+ cells (clusters 5 and 20) in patients who subsequently received IMC-Durv or IMC-Treme-Durv at the first sampling point after introduction of the checkpoint inhibitor (C2D15), consistent with the previous observations in checkpoint inhibitor monotherapy or combination therapy treatment. However, unlike in the patients treated with checkpoint inhibitors alone, no significant CD4+ activation was observed in patients receiving IMCgp100 plus Tremelimumab or Durvalumab.

Within the cohort of IMCgp100 patients we studied, two individuals had received prior PD-1 monotherapy, followed by treatment with IMCgp100 plus Durvalumab after they progressed. Samples were available for these patients across both treatments which provided a unique opportunity to study the effect of the different treatments within the same individual over time. In addition, this also allowed us to establish whether immune cell activation upon first exposure to anti-PD-1 treatment can be observed upon re-challenge with the same drug. The activation profile for one of these individuals (NPB_03555/4401-105) is shown in Figure 5-6 I. Consistent with our previous observations in the wider cohort, in this individual during PD-1 monotherapy activation was observed at C2 after 3 weeks on treatment, as indicated by an increased frequency of CD38+HLA-DR+ cells, but was not sustained with ongoing therapy. Upon treatment with IMCgp100 alone, no appreciable activation of CD8+ T cells was observed, however upon introduction of Durvalumab transient activation was again induced. Inspection of the changes in cluster abundance over time for this individual (Figure 5-6 J) revealed that the activation induced by Durvalumab seemed to be reflected predominantly by an expansion of cells in different clusters than those during the previous pembrolizumab treatment (clusters 1, 5, 9 and 13 during pembrolizumab compared with clusters 12, 18, 19 and 20 in response to Durvalumab), perhaps suggesting a phenotypic shift over time. Notably, clusters 19 and 20 express high levels of CLA with the change in cluster 19 being noted at D15 and therefore reflecting the effect of IMCgp100 not Durvalumab.

5.3.3 Summary

In this section we have described the distinct effects of different immunotherapy treatments upon the peripheral T cell response. Changes in response to combination checkpoint inhibitor treatment in both the CD8+ and CD4+ compartment shared a considerable degree of overlap with changes noted in the respective anti-PD-1 and anti-CTLA-4 monotherapy treatment analyses. Both activation and proliferation of cells were observed across treatments, however the magnitude and extent of this appeared to be greater in

combination therapy than compared to either monotherapy treatment, consistent with an additive effect of the two drugs. Reductions in the frequency of other non-activated clusters in combination treatment, that were not observed in monotherapy treatment, may therefore represent a redistribution of cells from these other clusters into the activated compartment in this setting, or recruitment of cells to peripheral tissues such as tumour or sites of off-target inflammatory responses induced by the therapy. Likewise, in the CD4⁺ compartment, activation was observed in response to both anti-CTLA-4 and anti-PD-1 monotherapy, and seen also in combination treatment but at a greater magnitude. No changes in Treg abundance were observed in any of the checkpoint inhibitor treated groups.

Three distinct clusters of CD103⁺Integrin β 7⁺ memory tissue homing phenotype cells were identified in the CD8⁺ clustering; a CLA⁺CCR4⁺ subset (cluster 17), a CLA⁺CXCR3⁺CD38⁺HLA-DR⁺ subset (cluster 1), and a CLA⁺CXCR3⁺CD38⁻HLA-DR⁻ subset (cluster 16). Across all three treatments (anti-CTLA-4 monotherapy, anti-PD-1 monotherapy and combination therapy), we observed an increase in early activated CD103⁺Integrin β 7⁺CLA⁺CXCR3⁺ cells (cluster 1). No changes were seen in any treatment for non-activated CD103⁺Integrin β 7⁺CLA⁺ cells (cluster 17), but a reduction in CD103⁺Integrin β 7⁺CLA⁻CXCR3⁺ cells was observed for combination therapy, maybe reflecting a recruitment of these cells to the site of Th1 inflammatory responses.

Distinct changes were observed in patients being treated with IMCgp100 monotherapy that were not observed during immune checkpoint blockade treatment. Early increases in CD8⁺ memory cells displaying the skin homing markers CLA, CCR4 and CCR10 (cluster 19) and CLA⁺ Tregs (cluster 6) were observed in IMCgp100 treatment but not checkpoint blockade. Upon addition of anti-CTLA-4 and/or anti-PD-L1 checkpoint inhibitor treatment to IMCgp100, activation of CD8⁺ but not CD4⁺ cells was observed. In individual cases where patients had received prior anti-PD-1 treatment, this CD8⁺ activation was observed both upon the initial challenge with the drug and re-challenge, although upon the re-challenge the cells activated were phenotypically subtly different.

5.4 Analysis of antigen-specific responses to treatment

In an attempt to confirm and extend our analyses in Chapter 3 on the changes in TAA-specific cells during treatment to a larger number of patients and to different treatments, as well as to further characterise the phenotype of antigen-specific cells, we included tetramer staining for a pool of 22 TAA and a limited pool of microbial peptides (Flu, CMV, EBV) as part of the CyTOF staining for HLA-A*02:01 individuals.

The cohort analysed by mass cytometry included 13 HLA-A*02:01 positive individuals treated with PD-1 monotherapy (9 patients) or PD-1/CTLA-4 combination therapy (4 patients) totalling 41 samples over time. The median number of tetramer positive cells detectable across all patients and all timepoints was 63 for microbial (range 2-3652) and 8 (range 1-223) for tumour tetramer positive cells with median frequency as a percentage of total CD8⁺ cells of 0.21 (range 0-2.79%) and 0.02% (range 0-0.41%) respectively. Tetramer staining was considered evaluable in the presence of at least 5 tetramer positive events recorded and a tetramer positive frequency of at least 0.01% of the total CD8⁺ T cells detectable in at least one timepoint. Based on this, 11 patients had measurable microbial tetramer⁺ responses (4 combination treated, 7 PD-1 monotherapy) and 12 patients measurable tumour tetramer⁺ responses (3 combination treated, 9 PD-1 monotherapy). Due to the low number of HLA-A*02:01 patients in this cohort treated with combination therapy, we analysed the data from patients on both treatments together. A transient increase in the frequency of TAA specific tetramer⁺ cells was observed at cycle 2 (mean log₂ fold change 0.89. $p = 0.0486$ paired, two-sided t-test) (Figure 5-7 A). In contrast, a small but statistically significant (mean log₂ fold change -0.7. $p = 0.0219$ paired, two-sided t-test) reduction in the proportion of circulating microbial tetramer⁺ cells was observed at cycle 4.

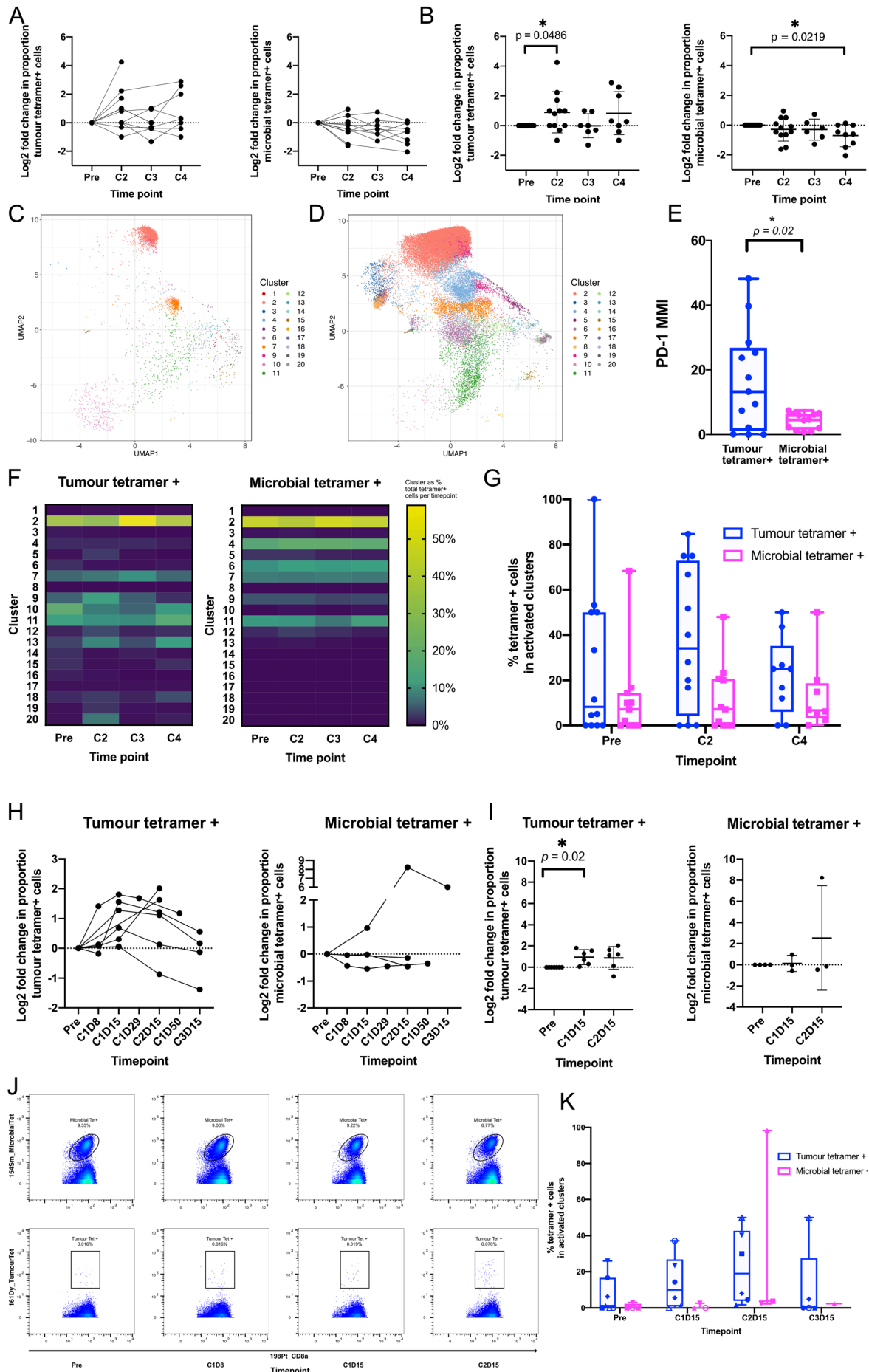


Figure 5-7. Antigen-specific responses to treatment. A) Log₂ FC in proportion of tumour (left) and microbial (right) tetramer+ cells over time in tetramer evaluable HLA*A2:01 patients in response to PD-1 or combination checkpoint inhibitor therapy. Each line represents a different patient (n=12 for tumour tetramer, 11 for microbial). B) Mean +/- SD for log₂ FC at each timepoint. Mean log₂ FC 0.89 for tumour tetramer+ cells at 3 weeks (p = 0.0486 paired, two-sided t-test). Mean log₂ FC -0.7 for microbial tetramer+ cells at 4 weeks (p= 0.0219 paired, two-sided t-test). C) CD8+ UMAP displaying only tumour tetramer+ cells for all patients. D) CD8+ UMAP displaying only microbial tetramer+ cells for all patients. E) Mean mass intensity (MMI) of PD-1 for tumour and microbial tetramer+ cells pre-treatment, p-value = 0.02 (two-tailed Wilcoxon test) F) Heatmap showing cells in each cluster at each timepoint for tumour tetramer+ (left) and microbial tetramer+ cells (right) as a percentage of the total number of tetramer+ cells at each timepoint for patients receiving combination ICB or PD-1 monotherapy. G) Percentage of tetramer+ cells in activated clusters (1, 5, 9, 12, 13, 18, 29 and 20) at different timepoints on therapy for tumour tetramer+ (blue) and microbial tetramer+ cells (red) for patients receiving combination ICB or PD-1 monotherapy. H) Log₂ FC in proportion of tumour (left) and microbial (right) tetramer+ cells over time in tetramer in response to treatment with IMCgp100 treatments. I) Mean +/- SD for log₂ FC at each timepoint for patients receiving IMCgp100 treatments. Mean log₂ FC 0.94 for tumour tetramer+ cells at 3 weeks (p = 0.02 paired, two-sided t-test). J) Serial biaxial plots for patient 4401115 of frequency of microbial tetramer+ cells (upper panel) and tumour tetramer+ cells (lower panel) over treatment with IMC-Treme-Durv. K) Percentage of tetramer+ cells in activated clusters (1, 5, 9, 12, 13, 18, 29 and 20) at different timepoints on therapy for tumour tetramer+ (blue) and microbial tetramer+ cells (red) for patients receiving combination IMCgp100 treatments.

In order to identify differences in the phenotype of tumour antigen-specific cells compared with microbial antigen-specific T cells, we overlaid the tetramer positive cells onto the CD8+ clustering to look at their phenotype (Figure 5-7 C & D). As expected given the inclusion of MART-1 in our tumour tetramer pool, we were able to detect both naïve (cluster 10) and memory cells amongst the tumour-specific population, whilst only memory cells within microbial-specific T cells. Unlike microbial tetramer+ cells, no tumour tetramer+ cells were detectable in cluster 8 and very few in cluster 3; both of which were characterised by expression of CD56 and CD57. Conversely, no microbial tetramer+ cells were detected in cluster 1 which contained tissue homing cells expressing CD103, Integrinβ7 and CD39. Notably, these markers have been described as markers of tumour specificity within tumours¹⁹⁷, however it is not known whether peripheral CD103+Integrinβ7+ and CD39+ cells are the circulating counterpart of these tumour infiltrating cells. Pre-treatment PD-1

expression was significantly higher in tumour tetramer+ than microbial tetramer+ cells (Figure 5-7 E).

Whilst the distribution of microbial tetramer positive cells across different clusters showed very little change during therapy, transient expansion of activated clusters such as cluster 20, cluster 13 and cluster 9 were observed at cycle 2 for TAA specific tetramer+ cells (Figure 5-7 F). To further assess this, we looked at the number of tetramer+ cells present in activated clusters (1,5,9,12,13,18,29 and 20) as a percentage of the total number of tetramer+ cells detected in each cluster at each timepoint and compared tumour and microbial-specific cells (Figure 5-7 G). This confirmed our previous observation that tumour but not microbial-specific T cells were activated by checkpoint inhibitor therapy.

Given that IMCgp100 has the potential to redirect T cells regardless of their specificity, we investigated whether this treatment could lead to changes in the phenotype and frequency of both tumour and microbial-specific T cells. All 7 patients receiving IMCgp100 were HLA-A*02:01 positive and had a measurable TAA-specific tetramer+ population (median tetramer positive count 31 and 0.02% total CD8+, ranges 1-1186 and 0.007-1.84%), whilst 4 had a measurable microbial-specific tetramer+ population (median microbial tetramer positive count 27 and 0.05% total CD8+, ranges 2-17434, 0.005-9.20%). With the exception of one individual who displayed a pronounced expansion of microbial tetramer positive cells at the time of C2D15, corresponding to a clinically documented upper respiratory tract infection, microbial tetramer frequencies were largely unchanged in response to IMCgp100 treatment (Figure 5-7 H & I). In contrast, the frequency of tumour tetramer+ cells increased in response to IMCgp100 with significantly increased percentages of tumour tetramer+ cells seen at day 15 following IMCgp100 monotherapy (Figure 5-7 H & I). One individual (patient 440115) had a large microbial tetramer+ population, most likely reactive to CMV based on the size of the population and the peptides we used (Figure 5-7 J), which did not show any change in frequency or activation status during the course of therapy. However, in this individual the frequency of tumour tetramer+ cells increased, although in contrast with the

other patients undergoing IMCgp100 treatment, not until after the introduction of the CTLA-4 and PD-1 inhibitor.

In contrast to the response to treatment with immune checkpoint inhibitors, the increased frequency of tumour tetramer+ cells detectable at day 15 was accompanied by a change in activation status in only one patient receiving IMCgp100. However, upon introduction of checkpoint inhibitors after two weeks, activation of tumour-specific cells was observed in 3 out of the 5 individuals, albeit to a lesser extent compared to patients who received combination checkpoint inhibitor therapy (Figure 5-7 K). Activation of microbial tetramer+ cells was not generally observed at this timepoint, except in the individual who had an increased frequency of tetramer positive cells, again potentially consistent with this being infection mediated rather than related to the treatment.

5.5 Correlations of immunological changes to checkpoint inhibitor treatment with outcome

One key question that follows on from our various observations is whether the extent and duration of T cell activation that we observed in response to checkpoint treatment is related to response to treatment. We therefore categorised patients into those experiencing clinical benefit (CB) from the treatment (defined as stable disease (SD) for at least 6 months, partial response (PR) or complete response (CR) as defined by the Response Evaluation Criteria in Solid Tumours (RECIST) v1.1) and those who had no clinical benefit (NCB) from the treatment (defined as progressive disease (PD) or stable disease lasting less than 6 months). Outcome data is presented in Table 5-2.

Table 5-2. Outcome data for all patients treated with checkpoint inhibitor therapy

	Ipilimumab + Nivolumab	Pembrolizumab monotherapy
Patients (n)	16	16
Best Response		
PD	2	6
SD	2	4
PR	8	6
CR	4	0
ORR (CR+PR %)	81.2%	37.5%
Clinical Benefit		
Yes	13	7
No	3	9
%	81.2%	43.8%
Duration on treatment (months)*		
Median	3.6	3.9
Range	0.7-24	1.4-52
Time to progression or censoring (months)*		
Median	19.1	5.5
Range	2.2-29	1.8-50
Overall survival (months)*		
Median	26.4	13.48
Range	11.6-39	3.7-50
Still alive	13	7

*Patients still on treatment, without progression or death censored at 9/9/2019 for analysis

We then analysed the data from patients treated with either PD-1 monotherapy or combination therapy and looked to see whether there were any significant differences in the clusters between responders and non-responders. Whilst no differences existed between the two groups in the absolute frequency of any of the clusters either before or during treatment, the magnitude of change from baseline was significantly different for 8 CD8+ clusters in those with clinical benefit compared to those without (Figure 5-8 A). These clusters included those with features of activation noted to increase in response to both anti-PD-1 and combination therapy (clusters 18, 20, 5, 12 and 13), where greater increases were observed for those with clinical benefit than those without. Greater increases were also observed for cluster 17 in those with clinical benefit, a cluster which is characterised by tissue homing memory phenotype cells expressing CLA and CCR4. Greater on-treatment reductions were observed for those with clinical benefit than without for cluster 8

(CD45RO+CD56+CD57+GranzymeB+). Increases in naïve cells (cluster 10) also appeared to be greater in those with clinical benefit than those without, although this appears less convincing on inspection of the data. No statistically significant differences were observed in either the absolute abundances of clusters on therapy or change in abundance of clusters during therapy between patients with and without clinical benefit for the CD4+ population.

To explore this further and to maximise statistical power, we combined the CD8+ populations from all 32 patients treated with PD-1 monotherapy or combination across both cohorts of patients we analysed and performed manual gating of the CD38+HLA-DR+ population. We found that whilst the percentage of CD38+HLA-DR+ cells present at any given timepoint was not correlated with clinical benefit, the extent of activation during treatment, as defined by the fold change in the percentage of CD38+HLA-DR+ cells at C2 compared to baseline, was significantly greater in patients who experienced clinical benefit from the treatment (median $\log_2$ fold change 2.05 vs 0.88 $p=0.0118$ two-tailed Mann-Whitney test). Whilst the median fold change at C4 was higher in patients with clinical benefit, this was not statistically significant (median $\log_2$ fold change 1.68 vs 0.74, $p=0.60$ two-tailed Mann-Whitney test).

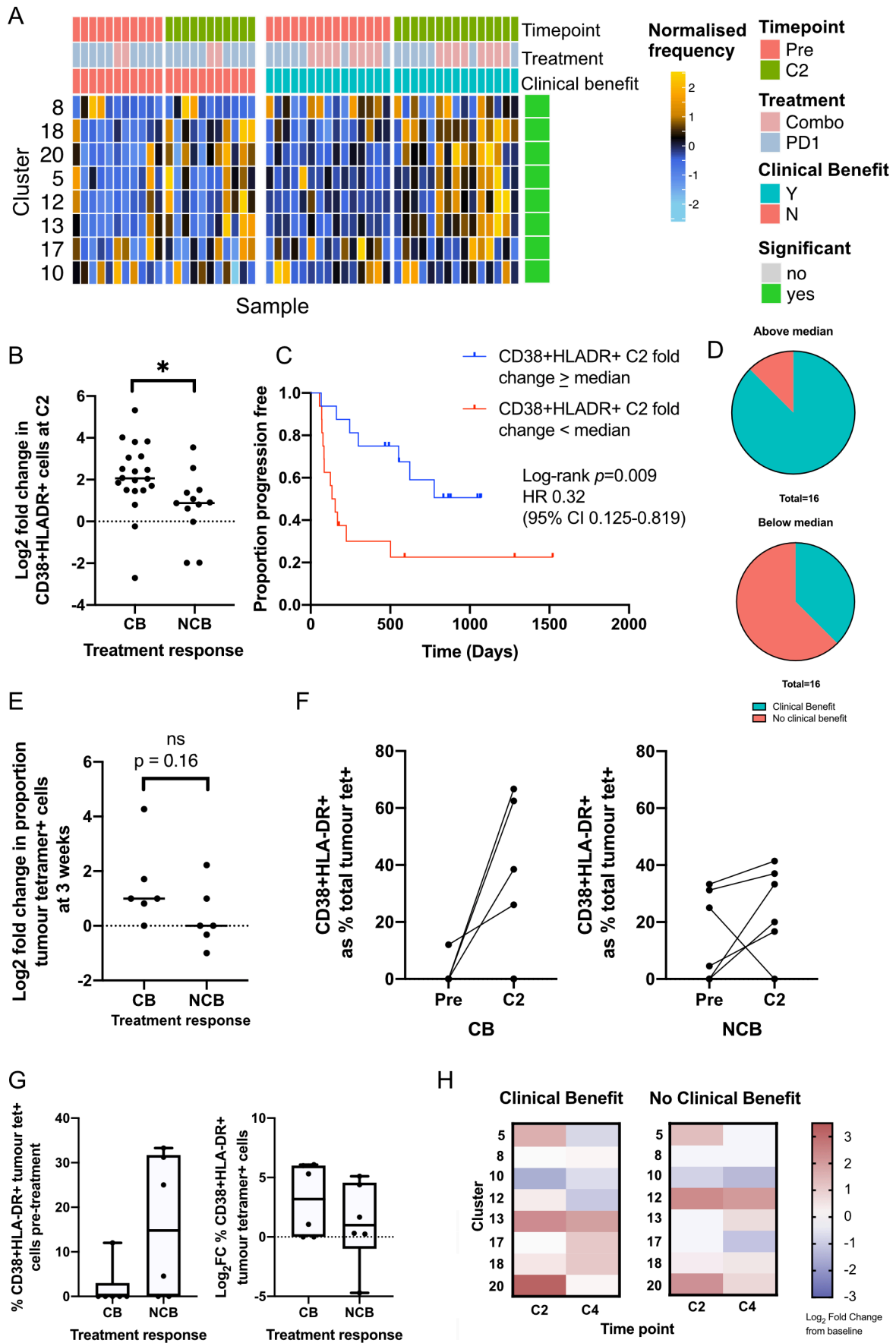


Figure 5-8. Correlations of immunological changes with clinical outcome. A) Clusters where change in frequency between C2 and baseline was significantly different for those with and without clinical benefit. B) Log₂ FC in CD38+HLA-DR+ cells at C2 compared to baseline for those with and without clinical benefit. C) Kaplan-Meier curve showing progression free survival in patients defined split by fold increase in CD38+HLA-DR+ cells above or below the median of 3.04 at C2. D) Pie chart showing proportion of patients experiencing clinical benefit with above and below fold increase in CD38+HLA-DR+. E) Log₂ FC in proportion of tumour tetramer+ cells at C2 for patients with and without clinical benefit. F) CD38+HLA-DR+ tumour tetramer+ cells as a % of total tumour tetramer+ cells pre and post treatment for patients with and without clinical benefit. No statistically significant changes by 2-tailed Wilcoxon test for difference in activated CD38+HLA-DR+ at C2 vs Pre for either patients with CB (p=0.125) or NCB (p=0.31). G) Percentage CD38+HLA-DR+ tumour tetramer+ cells as a percentage of total tumour tetramer+ cells pre-treatment (left) and fold change at C2 from baseline (right) by treatment response. No statistically significant differences are seen between those with CB and NCB for either comparison (p =0.10 and p= 0.45, two-tailed Mann-Whitney test). H) Log₂ FC in frequency of tumour tetramer+ cells from baseline for the different activated clusters in patients with and without clinical benefit.

We also looked at the median fold change in the percentage of CD38+HLA-DR+ cells at C2 and C4 from baseline for all patients regardless of response categorisation (3.04 and 2.62 respectively) and compared patients who were above or below the median for their progression free survival (defined as time from starting treatment to progressive disease as defined by RECIST 1.1). Patients with ongoing treatment response were censored at the date of their last imaging. We saw a statistically significant difference in progression-free survival (PFS) for patients with an increase in CD38+HLA-DR+ greater than the median compared to those where the increase was below the median at C2 (median PFS: not reached vs 144 days, $p=0.009$, hazard ratio 0.32 95% CI 0.125-0.819 log-rank test) but not at C4 (median PFS: 776 days vs 196 days, $p=0.09$, hazard ratio 0.432 95% CI 0.153-1.21). Of the 16 patients with above median increases in CD38+HLA-DR+ cells at C2, 14 experienced clinical benefit, whilst only 6/16 with below median increases experienced clinical benefit (Figure 5-8 D).

Given that activation appears to be related to outcome, and we have demonstrated that tumour-specific but not microbial-specific T cells are expanded and activated in response to therapy, we asked whether the change in either frequency or activation of tumour

tetramer+ cells was correlated with outcome. When comparing the change in abundance of tumour tetramer+ cells at 3 weeks between patients who experienced clinical benefit and those who did not (Figure 5-8 E), a trend towards a greater increase in tumour tetramer+ cells was seen for those experiencing clinical benefit, although this did not reach statistical significance (median log₂ fold change 1.0 vs 0, $p = 0.1619$, two-tailed Mann-Whitney test). Whilst there was a trend towards an increased proportion of activated tumour tetramer+ cells in response to treatment in patients with clinical benefit ($p = 0.125$, two-tailed Wilcoxon test), this was non-significant and changes in activated tumour tetramer+ cell frequencies could also be seen in patients without clinical benefit (Figure 5-8 F). Neither the magnitude of change in activation, nor the frequency of activated cells pre-treatment were significantly different between those with and without clinical benefit (Figure 5-8 G). We also looked to see whether the phenotype of activated cells as defined by the previously described clusters differed between those with and without clinical benefit (Figure 5-8 H). The distribution of tetramer+ cells across the different activated clusters was broadly similar for both groups over time, with the exception of increases in cells belonging to cluster 13 being seen in those with clinical benefit, whilst increases in cells belonging to cluster 12 were more dominant in those without clinical benefit. Cells in cluster 13 displayed higher levels of CD45RO and CXCR3 than those in cluster 12, which had more of an effector phenotype. Unfortunately, the low numbers of patients and relatively low frequencies of these cells make it difficult to draw any firm conclusions from this.

5.6 Discussion

In the analyses presented in this chapter, we have described characterisation by CyTOF of the peripheral immune response during immunotherapy treatment for a further 29 individuals treated with checkpoint inhibitor therapy. In addition to confirming the activation of CD8+ and CD4+ T cells in response to combination checkpoint inhibitor therapy that we observed in Chapter 3, we were able to demonstrate that whilst this activation was observed in patients treated with either anti-CTLA-4 or anti-PD-1 monotherapy, the magnitude of

activation in the combination therapy was far greater than for either treatment alone suggesting either an additive or synergistic effect of the two treatments. As well as activation, we observed proliferation of a small population of Ki67+CD8+ cells (cluster 20) in patients treated with treatments containing anti-PD-1 as either monotherapy or combination therapy. These proliferating cells, which expressed high levels of CD38, HLA-DR, CTLA-4 and PD-1 and were CD45RO^{intermediate}, CD27+, shared phenotypic similarities to populations of Ki67+ cells previously found to increase in the peripheral blood of patients being treated with anti-PD-1 containing treatments in melanoma or NSCLC^{118,132,129}. Interestingly, both our data and that of other investigators, show that changes in response to treatment are most pronounced early-on therapy with activation and proliferation typically not observed to be sustained on serial time-course sampling despite ongoing treatment administration. Together with the observation that responses are still seen in patients who have discontinued treatment due to toxicity and that the median duration of treatment in this study was less than 4 months for both anti-PD-1 monotherapy and combination therapy, this raises the question of whether ongoing treatment is necessary for efficacy. Whilst studies addressing the effect of duration of treatment upon efficacy are ongoing, such as the DANTE trial comparing 1 year of pembrolizumab monotherapy treatment with 2 years, it may be that much shorter durations of treatment could be sufficient to induce an adequate immunological response whilst also reducing the cost and risk of associated toxicities and further clinical trials in this area are therefore likely to be warranted.

In addition to looking at differences between various checkpoint inhibitor therapies, we also looked at the peripheral immune response to treatment with IMCgp100. Distinct immunological changes were observed between the two types of treatment. Early changes in response to IMCgp100 were characterised by changes in cells with a skin homing phenotype (CCR4+CLA+CD8+ T cells and CLA+CD4+Tregs). This expansion of skin homing cells is probably related to the pronounced skin rash in response to this treatment in nearly all individuals mediated by on-target, off-tumour attack of gp-100 expressing

melanocytes in the skin. The Treg response might either be a compensatory mechanism in response to the rash and inflammation observed, or the result of the local direct activation of skin resident Tregs by IMCgp100. Notably the expansion of these clusters was not observed in patients treated with anti-PD-1 or combination therapy, only a minority of whom experienced skin related toxicities that were very different in nature to those observed in patients treated with IMCgp100.

Conversely, the pronounced activation observed during checkpoint inhibitor therapy was not typically observed in patients during the IMCgp100 monotherapy treatment phase suggesting that this effect is specific to checkpoint blockade. Consistent with this, introduction of checkpoint inhibitors in these patients did provoke activation of CD8+ T cells, however this was less pronounced than seen in patients treated with checkpoint inhibitors alone and unlike the patients who were not receiving IMCgp100, no significant CD4+ activation was observed. This may be due to the fact that the checkpoint inhibitors used differed in the different settings (Ipilimumab compared with Tremelimumab for the anti-CTLA-4, and Pembrolizumab or Nivolumab (anti-PD-1) compared with the anti-PD-L1 inhibitor Durvalumab). Whilst Ipilimumab and Tremelimumab both inhibit anti-CTLA-4, Ipilimumab is an IgG1 isotype antibody and Tremelimumab is an IgG2 isotype. In murine models, IgG2a subclass CTLA-4 blocking antibodies (which corresponds to human IgG1) have been shown to induce more potent Fcγ-dependent Treg depletion¹²² than other subclasses. As human IgG1 has a higher binding affinity for Fcγ receptors than IgG2 there is a theoretical possibility of greater CTLA-4 antibody mediated Treg depletion resulting from Ipilimumab than Tremelimumab. However, evidence of Treg depletion has not been consistently observed in patients treated with Ipilimumab^{113,126} and indeed we saw no evidence of this in patients receiving either Ipilimumab monotherapy or combination therapy. PD-L1 antibodies have also been shown to differ from PD-1 antibodies, being superior in abrogating PD-1 signalling in functional assays²²², however unlike PD-1 inhibitors, they do not block the interaction between PD-1 and its other ligand PD-L2 which

is also thought to generate inhibitory signals which regulate the immune response. In addition, the doses of Tremelimumab and Durvalumab used in these patients, treated within the dose escalation phase of this clinical trial, were lower than have been used in other studies. Where Durvalumab was given in combination with IMCgp100, this was in most cases at a dose of 3mg/kg, and Tremelimumab in combination with Durvalumab and IMCgp100 was given at a dose of 0.5mg/kg. This compares with doses of 10-20mg/kg Durvalumab and 1mg/kg Tremelimumab in other clinical trial settings^{91,223,224}. This might also explain why less pronounced activation was observed than in patients receiving Ipilimumab and/or Pembrolizumab. Aside from the differences in the drugs and dosing, patients being treated with IMCgp100 were typically heavily pre-treated and many had been exposed to prior checkpoint inhibitor treatment. It is possible therefore that the effects of targeting the same pathway may diminish upon repeated exposure to checkpoint inhibitors.

In addition to our observations at the CD8+ and CD4+ total lymphocyte level, we were also able to extend our analyses of the effect of treatments upon antigen-specific cells. Tetramer based analyses in HLA-A*02:01 individuals confirmed our prior observation that a transient increase in frequency of tumour tetramer+ but not microbial tetramer+ cells was observed upon checkpoint inhibitor therapy, and that tumour but not microbial-specific cells are activated by therapy. The lack of changes in the microbial-specific populations is consistent with prior observations in both anti-CTLA-4¹³⁷ and anti-PD-1 monotherapy¹²⁹ and may be explained by the observation that microbial tetramer+ cells express lower levels of PD-1 pre-treatment than tumour tetramer+ cells. We were also able to show that IMCgp100 increased the frequency of tumour but not microbial tetramer+ cells, but did not by itself produce activation of these cells. Whilst we showed in Chapter 3 that combination checkpoint inhibitor treatment could induce both diversification and expansion of the anti-tumour response, it is not known whether IMCgp100 might induce epitope spreading, and the observation of increased tetramer+ cells in these patients suggests that this might be an important area for further research. The observation of increased frequency but limited

activation, also provides a potential rationale for combining IMCgp100 with checkpoint inhibitors. Together, these data suggest that the phenomenon of activation induced in response to checkpoint inhibitor therapy is relatively specific and does not simply reflect a generalised immunological response to immunotherapy.

One important consideration of the changes we observe is whether they relate to patient outcome. As the objective response rates of patients receiving combination therapy in our study was abnormally high (around 80% compared with 58% in published clinical trials⁹⁴), we combined patients receiving anti-PD-1 and combination therapy for the purposes of these analyses. From this we were able to demonstrate that the extent of activation (as defined by increase in CD38+HLA-DR+ cells on treatment) of CD8+ T cells was related to both clinical benefit and progression free survival. Whilst it is likely that this analysis is confounded by the fact that combination therapy produces higher response rates and greater activation, and indeed more patients treated with combination therapy were found in the clinical benefit group, we were able to see patients treated with combination therapy in the non-benefit group and conversely patients treated with monotherapy in the benefit group. Although we have shown previously that this activated population contains cells capable of recognising tumour antigens, we did not see a direct association between activation of tumour tetramer+ cells and response. This is perhaps unsurprising given that we were focusing on a single HLA locus and a relatively limited panel of peptides in a small number of patients. It is possible that taking into account other HLA class I alleles might indeed confirm the observations in the bulk CD8+ population.

As discussed previously, one of the clusters of activated cells we observed which showed evidence of proliferation was similar to those described by others previously in response to anti-PD-1 therapy^{132,129}. In both cases, associations between this population in the periphery and outcome were observed. Kamphorst *et al.*¹²⁹ found that whilst 80% of patients who benefited from treatment had expansion of the proliferating PD-1+CD8+ T cells, 70% of those who progressed on treatment had either a delayed or absent response. In the study

by Huang *et al.*¹³², whilst they found high pre-treatment levels of Ki67+ cells to be an indicator of poor prognosis (and to be correlated to the tumour burden) changes in the frequency of these cells alone during treatment did not predict outcome¹³². It was only when taking into consideration the ratio of tumour burden to exhausted cell reinvigoration that they were able to demonstrate an association with clinical benefit. Whilst they used CD38+HLA-DR+ cells as a proxy for PD-1+ proliferating cells for the purposes of cell sorting in a limited number of individuals, they did not report whether changes in CD38+HLA-DR+ cells correlated with outcome. Taken together, it is possible therefore that CD8+ activation might represent a unifying feature of a successful ICB response, regardless of the specific treatment and could be a potential biomarker of treatment efficacy.

Despite having extended our analyses to a greater number of patients, it is not possible to draw definitive conclusions about response from these data and to be able to determine whether increases in CD38+HLA-DR+ populations in response to treatment would hold as a biomarker for anti-PD-1 and combination therapies independently, or whether the higher number of responders in combination therapy were driving this association. Furthermore, due to the time at which this study commenced, and the rapid pace of change in the therapeutic landscape for melanoma since, a large number of the anti-PD-1 patients we studied had received prior Ipilimumab therapy. This would no longer be standard practice and it is hard to know therefore whether the changes we observe would be replicated in cohort of treatment naïve patients. The data does however raise interesting questions for further study and it would potentially be informative to see if we could validate these observations in a larger more balanced cohort of patients, more reflective of those currently receiving treatment in current practice.

Chapter 6. Summary and concluding remarks

Whilst checkpoint inhibitor therapies have dramatically improved outcomes for patients with metastatic melanoma, a thorough understanding of the determinants of response is still lacking. Previous analyses have typically focused on individual aspects of the immune response such as studying changes in cellular phenotype or TCR clonality without knowledge of antigen specificity, or changes in antigen specificity or T cell functionality without characterisation of the cellular phenotype *in vivo*. In addition, many prior studies have focused on intra-tumoural markers which may not fully reflect spatial or temporal heterogeneity and are challenging to obtain in routine clinical practice. In an attempt to provide a further understanding of the effect of immunotherapy treatment upon the tumour-specific T cell response, we performed immune monitoring using peripheral blood samples from melanoma patients at serial timepoints during treatment with either checkpoint inhibitors or the ImmTAC IMCgp100. By utilising various different single-cell techniques including FACS, CyTOF, and single-cell RNA sequencing we attempted to provide an integrated approach to studying various aspects of the tumour-specific response allowing us to study in great detail the phenotype, antigen specificity, functionality and clonality of antigen-specific T cells.

In the course of these analyses we have demonstrated that combination anti-CTLA-4 and anti-PD-1 checkpoint inhibitor therapy leads to a transient activation and expansion of CD8⁺ T cells capable of recognising melanoma tumour-associated antigens or tumour lines. Furthermore, this activation characterised by expression of CD38 and HLA-DR is selective for tumour antigen-specific T cells and is not observed in microbial-specific T cells. Whilst similar changes can be observed in patients receiving anti-CTLA-4 or anti-PD-1 monotherapy, the magnitude of this response is greater in combination treatment than either treatment alone. Activation of tumour-specific CD8⁺ T cells was not however observed in response to monotherapy treatment with IMCgp100, suggesting that this is resultant of the specific mechanism of action of checkpoint inhibitor blockade, and does not

simply represent a global immunotherapy induced immune response. As well as being characteristic of checkpoint inhibitor therapy, our CyTOF analysis has shown that the extent of activation of CD8⁺ T cells observed in response to PD-1 containing treatments is predictive of clinical benefit to treatment. This could potentially be explored as a possible biomarker for early detection and monitoring of responses to treatment in clinical practice. In addition, this enrichment of tumour-specific T cells found within the CD8⁺ population activated by checkpoint inhibitor therapy could potentially be harnessed as a source of tumour-specific T cells for other therapeutic approaches such as adoptive cell therapy. In the course of these experiments alone, using a combination of activation and tetramer guided cell sorting, we have isolated nearly 400 different tumour line or TAA reactive TCRs, many of which have not been previously described. This library of TCRs against public TAA might provide a valuable resource for the future development of off-the-shelf T cell therapeutic agents.

As well as activation, increases in both the breadth and depth of tumour-specific responses are seen with combination checkpoint inhibitor therapy treatment, both in terms of antigen specificity and of the TCR repertoire. Changes in functionality of MART-1-specific T cells isolated from different timepoints on therapy could also be observed and were independent of the TCR sequence. This might be explained by the phenotypic heterogeneity that could be observed amongst T cells bearing the same TCR over time upon single-cell sequencing. In addition, tracking clonally expanded tumour-specific T cells by scRNA-seq demonstrated that pre-existing anti-tumour immune responses showed limited plasticity compared to newly generated anti-tumour responses. Furthermore, whilst immunotherapy lead to the expansion of proliferating cells early on treatment, and to the differentiation of cytotoxic T_{EMRA} like and TCF7⁺ memory like T cells, activation of these differentiated cells was lost late on-treatment. Understanding whether this transition from an activated to a 'resting' phenotype is due to the transition from combination therapy to anti-PD-1 monotherapy is a reflection of a loss of efficacy of drug treatment, or is reflection of the natural course of an

antigen-specific immune response, warrants further investigation and could have profound implications for the regimen of drug administration in combination therapy. The substantial heterogeneity observed over time, between and within patients, and between different T cell clones of the same antigen specificity in the response to checkpoint inhibitor treatment highlights the importance of single-cell approaches, and may also be an important consideration when selecting T cells with optimal therapeutic potential for other treatment strategies.

Throughout these experiments, we have shown that it is possible to detect measurable effects upon tumour-specific T cells in the periphery of patients receiving immunotherapy treatment. We have also demonstrated that tumour-specific T cell clones detected in the blood could be found within tumour samples indicating that they are potentially relevant to and reflective of intra-tumoural responses. Taken together, this suggests that peripheral immune monitoring represents a valid approach for assessing immunotherapy induced immune responses, which has the advantage over tumour-based analyses of being minimally invasive and amenable to serial sampling over time. The in-depth characterisation of various aspects of tumour-specific T cell behaviour that we have performed has allowed us to identify a number of potential markers which could be explored further in future work. Validation of our findings in a larger cohort of patients might provide further insights into the characteristics of successful checkpoint inhibitor treatment and could help identify possible reasons for treatment failure and alternative approaches to overcome resistance.

Appendices

Appendix A – Clustering of CD45+ cells

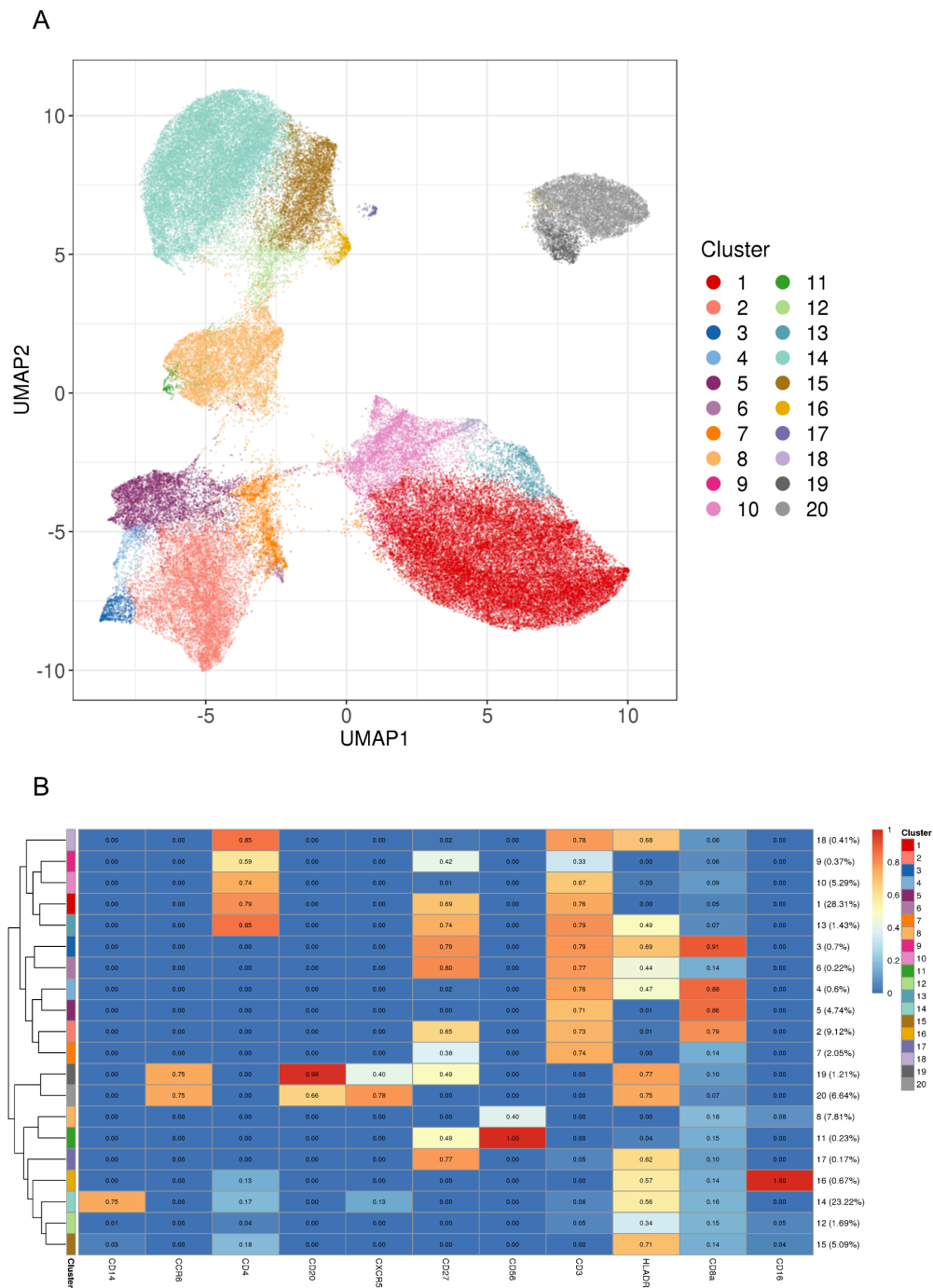


Figure A-1. Clustering of CD45+ cells based on CyTOF staining. A) UMAP based on arcsinh-transformed expression of 11 lineage markers in CD45+ cells for all patients (5000 cells randomly selected per patient). Cells coloured according to 20 metaclusters obtained from FlowSOM clustering. B) Heatmap of median arcsinh, 0-1 transformed marker expression.

Appendix B – Cloning efficiency of tetramer+ sorting experiments

Table B-1. Cloning efficiency of tumour tetramer+ cells by individual patient and timepoint

Timepoint	Pre		C2		C4		Late		Total No. sorted	Total No. expanded	Per patient cloning efficiency (%)
Patient	No. sorted	No. expanded	No. sorted	No. expanded	No. sorted	No. expanded	No. sorted	No. expanded			
NPB_03284	43	18	190	42	166	75	69	55	468	190	40.60
NPB_03640	40	16	30	10	13	4			83	30	36.14
NPB_03659	50	19	51	23	52	19	69	31	222	92	41.44
NPB_03661	119	28	157	34					276	62	22.46
NPB_03663	44	18	95	17	104	46	65	26	308	107	34.74
Total	296	99	523	126	335	144	203	112	1357	481	
Per timepoint cloning efficiency (%)	33.45		24.09		42.99		55.17		35.45		

Table B-2. Cloning efficiency of microbial tetramer+ cells by individual patient and timepoint

Timepoint	Pre		C2		C4		Late		Total No. sorted	Total No. expanded	Per patient cloning efficiency (%)
Patient	No. sorted	No. expanded	No. sorted	No. expanded	No. sorted	No. expanded	No. sorted	No. expanded			
NPB_03284	197	93	50	15	74	29	104	68	425	205	48.24
NPB_03661	42	20	23	6					65	26	40.00
NPB_03663	24	13	15	3					39	16	41.03
Total	263	126	88	24	74	29	104	68	529	247	
Per timepoint cloning efficiency (%)	47.91		27.27		39.19		65.38		46.69		

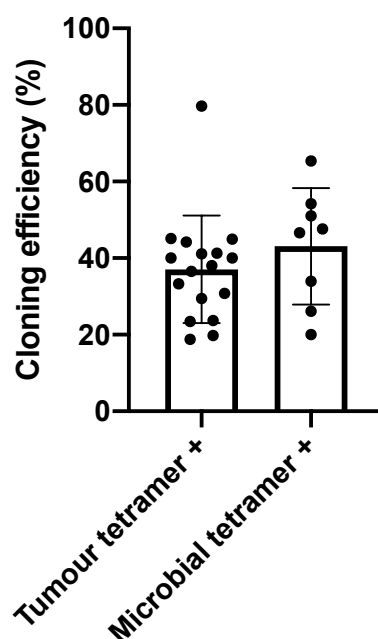


Figure B-1. Overall Cloning efficiency of tumour tetramer + and microbial tetramer + cells.

Mean + SD from all timepoints across all patients. $p = 0.33$ two-tailed unpaired t-test.

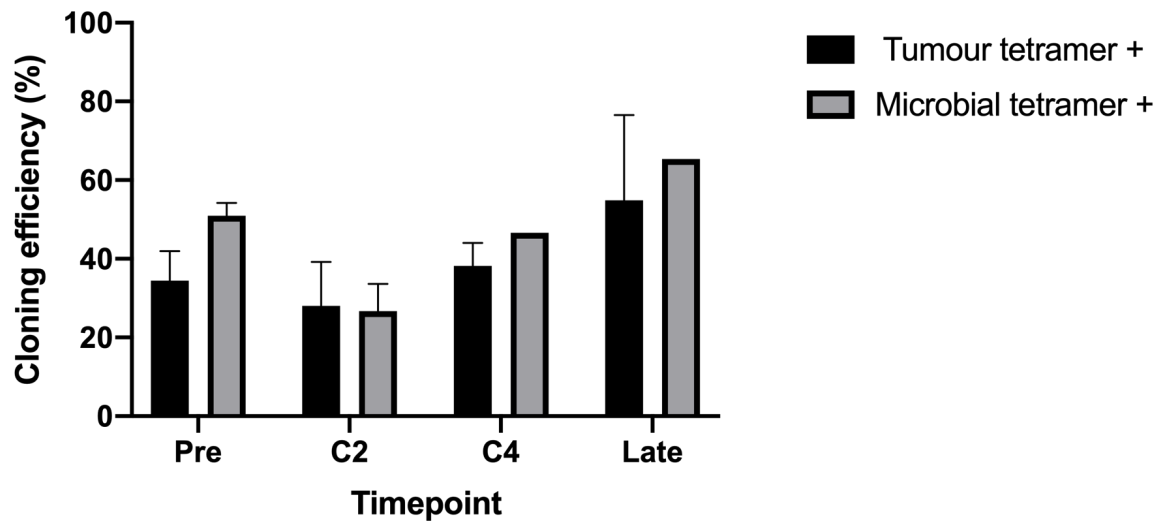


Figure B-2. Cloning efficiency of tumour tetramer+ vs microbial tetramer+ cells across all patients by individual timepoint. Cloning efficiency is shown as mean + SD. No significant differences were found between the cloning efficiency for tumour tetramer + and microbial tetramer + cells at any individual timepoint ($p > 0.05$ multiple unpaired t-tests).

Appendix C – Single-cell RNA-seq QC plots



Figure C-1. QC plots for the whole dataset sc-RNA-seq dataset including blank wells. Top panel – total counts vs total features(genes), bottom panel percent counts ERCC vs total features, coloured by type (blue = blank well, orange = single cell). Blank wells have low total counts, low number of expressed genes and high percentage ERCC.

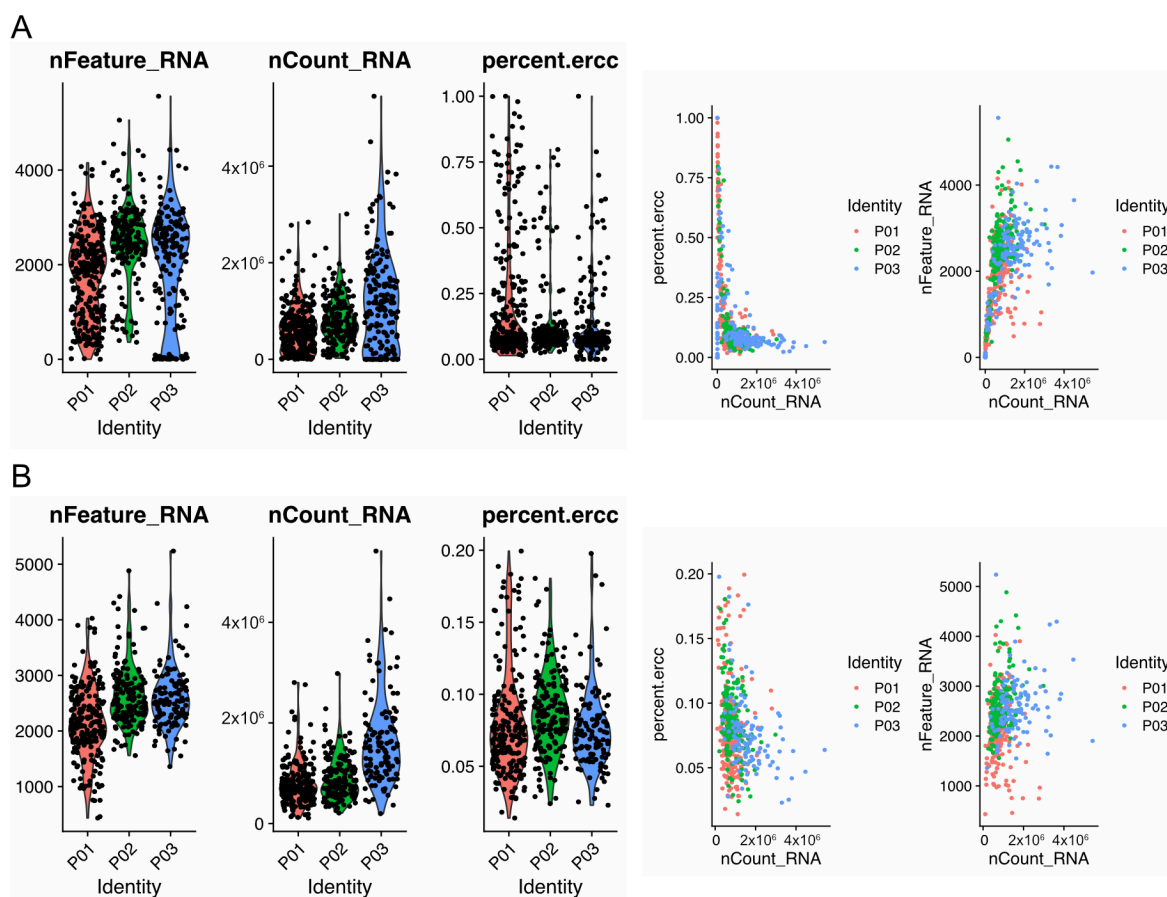


Figure C-2. QC plots for single-cells before and after application of filters. A) QC plots before application of filters and B) after application of filters. Data shown per patient for number of genes (nFeature_RNA), number of reads (nCount_RNA) and percentage reads mapping to ERCC (percent.ercc), and for the whole dataset coloured by patient ID for relationships between percentage of ERCC vs number of reads and number of genes vs number of reads.

Table C-1. Number of cells and genes in dataset before and after various QC filters

QC filter	Number of cells	Number of genes
Blank wells and ERCC genes removed	672	24569
Min 200 features/cell and gene expressed in min 20 cells	630	10372
Max 20 % ERCC	497	10372

Appendix D – GO terms for clusters in scRNA-seq data

Table D-1. Top 10 gene ontology terms per cluster based on top 50 upregulated genes

Cluster	Description	GeneRatio	qvalue	geneID
0	mitotic cell cycle arrest	4/48	3.99E-05	DUSP1/RGCC/CDKN1A/GADD45A
	response to glucocorticoid	7/48	5.15E-05	ZFP36L2/DUSP1/ZFP36/FOS/FOSB/DDIT4/CDKN1A
	response to corticosteroid	7/48	7.24E-05	ZFP36L2/DUSP1/ZFP36/FOS/FOSB/DDIT4/CDKN1A
	response to steroid hormone	9/48	0.00017477	ZFP36L2/NR4A2/DUSP1/ZFP36/FOS/FOSB/DDIT4/CDKN1A/NR4A1
	cellular response to calcium ion	5/48	0.00017477	JUNB/JUND/JUN/FOS/FOSB
	response to mechanical stimulus	7/48	0.00018527	JUN/FOS/NFKBIA/FOSB/CXCR4/BTG2/GADD45A
	response to calcium ion	6/48	0.00018527	JUNB/JUND/DUSP1/JUN/FOS/FOSB
	DNA damage response, signal transduction by p53 class mediator	5/48	0.00110553	RGCC/BTG2/CDKN1A/GADD45A/PMAIP1
	positive regulation of nuclear-transcribed mRNA catabolic process, deadenylation-dependent decay	3/48	0.00110553	ZFP36L2/ZFP36/BTG2
	response to ketone	6/48	0.00110553	DUSP1/FOS/FOSB/CCR7/DDIT4/CDKN1A
1	lymphocyte mediated immunity	8/41	0.00031839	GZMB/TBX21/PRF1/KLRD1/TNF/SLAMF7/SMAD7/CTSC
	positive regulation of immune effector process	6/41	0.00182237	SPON2/FGR/TBX21/TNF/CD300A/PTPRJ
	phagocytosis	7/41	0.00182237	SPON2/FGR/FCGR3A/TNF/CD300A/ITGAL/PTPRJ
	cellular defense response	4/41	0.00182237	CX3CR1/PRF1/KLRG1/GNLY
	regulation of leukocyte activation	8/41	0.00300977	FGR/CS7/TBX21/TNFSF9/TNF/CD300A/SMAD7/CTSC
	negative regulation of cytokine production involved in immune response	3/41	0.00446368	TBX21/TNF/SMAD7
	cell killing	5/41	0.00446368	GZMB/PRF1/GNLY/SLAMF7/CTSC
	regulation of cytokine production involved in immune response	4/41	0.00507026	SPON2/TBX21/TNF/SMAD7
	regulation of microglial cell activation	3/41	0.00507026	CS7/TNF/CTSC
	T cell mediated immunity	4/41	0.00507026	TBX21/PRF1/SMAD7/CTSC
2	antigen processing and presentation of exogenous peptide antigen via MHC class II	6/43	3.77E-05	CD74/HLA-DQB1/HLA-DMB/HLA-DRA/HLA-DMA/HLA-DRB1
	antigen processing and presentation of peptide antigen via MHC class II	6/43	3.77E-05	CD74/HLA-DQB1/HLA-DMB/HLA-DRA/HLA-DMA/HLA-DRB1
	antigen processing and presentation of peptide or polysaccharide antigen via MHC class II	6/43	3.77E-05	CD74/HLA-DQB1/HLA-DMB/HLA-DRA/HLA-DMA/HLA-DRB1
	antigen processing and presentation of exogenous peptide antigen	7/43	3.77E-05	CD74/HLA-DQB1/HLA-DMB/HLA-DRA/HLA-DMA/PSMCS/HLA-DRB1
	antigen processing and presentation of exogenous antigen	7/43	3.77E-05	CD74/HLA-DQB1/HLA-DMB/HLA-DRA/HLA-DMA/PSMCS/HLA-DRB1
	antigen processing and presentation of peptide antigen	7/43	4.92E-05	CD74/HLA-DQB1/HLA-DMB/HLA-DRA/HLA-DMA/PSMCS/HLA-DRB1
	antigen processing and presentation	7/43	0.00012177	CD74/HLA-DQB1/HLA-DMB/HLA-DRA/HLA-DMA/PSMCS/HLA-DRB1
	antigen receptor-mediated signaling pathway	5/43	0.02401653	HLA-DQB1/HLA-DRA/KLHL6/PSMCS/HLA-DRB1
	positive regulation of monocyte differentiation	2/43	0.02515326	CD74/JUN
	positive regulation of nuclear-transcribed mRNA poly(A) tail shortening	2/43	0.02515326	ZFP36/BTG2
3	negative regulation of immune system process	7/35	0.0067736	KLRK4-KLRK1/KLRK1/IL7R/CD96/CD84/N48P2L2/TIGIT
	regulation of leukocyte mediated immunity	5/35	0.0067736	KLRK4-KLRK1/KLRK1/IL7R/CD96/CD84
	regulation of natural killer cell mediated immunity	3/35	0.01196461	KLRK4-KLRK1/KLRK1/CD96
	negative regulation of leukocyte mediated immunity	3/35	0.01336896	IL7R/CD96/CD84
	regulation of lymphocyte mediated immunity	4/35	0.01336896	KLRK4-KLRK1/KLRK1/IL7R/CD96
	natural killer cell chemotaxis	2/35	0.01581125	KLRK4-KLRK1/KLRK1
	natural killer cell mediated immunity	3/35	0.01581125	KLRK4-KLRK1/KLRK1/CD96
	regulation of leukocyte mediated cytotoxicity	3/35	0.01581125	KLRK4-KLRK1/KLRK1/IL7R
	negative regulation of lymphocyte migration	2/35	0.02053	KLRK4-KLRK1/KLRK1
	lymph node development	2/35	0.02386116	IL7R/LTB
4	cellular response to interferon-gamma	8/48	7.38E-06	HLA-DRA/HLA-DQB1/ACTG1/HLA-DRB1/GAPDH/GBP1/GBP2/ACTR3
	response to interferon-gamma	8/48	9.68E-06	HLA-DRA/HLA-DQB1/ACTG1/HLA-DRB1/GAPDH/GBP1/GBP2/ACTR3
	interferon-gamma-mediated signaling pathway	5/48	0.00100945	HLA-DRA/HLA-DQB1/HLA-DRB1/GBP1/GBP2
	antigen processing and presentation of exogenous peptide antigen via MHC class II	5/48	0.00100945	HLA-DRA/CD74/HLA-DQB1/HLA-DRB1/CAPZA1
	antigen processing and presentation of exogenous peptide antigen	6/48	0.00100945	HLA-DRA/CD74/HLA-DQB1/HLA-DRB1/PSMA2/CAPZA1
	antigen processing and presentation of peptide antigen via MHC class II	5/48	0.00100945	HLA-DRA/CD74/HLA-DQB1/HLA-DRB1/CAPZA1
	antigen processing and presentation of exogenous antigen	6/48	0.00100945	HLA-DRA/CD74/HLA-DQB1/HLA-DRB1/PSMA2/CAPZA1
	antigen processing and presentation of peptide or polysaccharide antigen via MHC class II	5/48	0.00100945	HLA-DRA/CD74/HLA-DQB1/HLA-DRB1/CAPZA1
	immune response-activating cell surface receptor signaling pathway	8/48	0.0011635	HLA-DRA/HLA-DQB1/ACTG1/HLA-DRB1/ARPC5/PSMA2/GBP1/ACTR3
	antigen processing and presentation of peptide antigen	6/48	0.0011635	HLA-DRA/CD74/HLA-DQB1/HLA-DRB1/PSMA2/CAPZA1
5	alpha-beta T cell activation	6/47	0.00109183	SATB1/LEF1/FOXP1/TCF7/CD55/GPR183
	CD4-positive, alpha-beta T cell activation	5/47	0.00109183	SATB1/LEF1/FOXP1/CD55/GPR183
	alpha-beta T cell differentiation	5/47	0.00109183	SATB1/LEF1/FOXP1/TCF7/GPR183
	viral transcription	6/47	0.00109183	RPS14/RPL21/RPL13/LEF1/RPLP0/RPS4X
	SRP-dependent cotranslational protein targeting to membrane	5/47	0.00109183	RPS14/RPL21/RPL13/RPLP0/RPS4X
	cotranslational protein targeting to membrane	5/47	0.00109183	RPS14/RPL21/RPL13/RPLP0/RPS4X
	protein targeting to ER	5/47	0.00109183	RPS14/RPL21/RPL13/RPLP0/RPS4X
	viral gene expression	6/47	0.00109183	RPS14/RPL21/RPL13/LEF1/RPLP0/RPS4X
	establishment of protein localization to endoplasmic reticulum	5/47	0.00109183	RPS14/RPL21/RPL13/RPLP0/RPS4X
	nuclear-transcribed mRNA catabolic process, nonsense-mediated decay	5/47	0.00126663	RPS14/RPL21/RPL13/RPLP0/RPS4X
6	cellular defense response	4/41	0.00690017	KLRG1/CX3CR1/GNLY/PRF1
	positive regulation of neuroinflammatory response	3/41	0.01075786	TNF/CCL3/CTSC
	modification of morphology or physiology of other organism	5/41	0.01075786	CCL4/GNLY/CCL3/PRF1/CASP8
	regulation of microglial cell activation	3/41	0.01075786	TNF/CCL3/CTSC
	positive regulation of establishment of protein localization	7/41	0.01075786	GZMB/FGR/TNF/CCL3/PRR5L/CASP8/PAK1
	regulation of calcium-mediated signaling	4/41	0.01075786	CCL4/PLEK/TNF/CCL3
	positive regulation of protein complex disassembly	3/41	0.01075786	PLEK/TNF/ADRB2
	positive regulation of cellular protein localization	6/41	0.01075786	GZMB/TNF/PYHIN1/PRR5L/CASP8/PAK1
	cytolysis	3/41	0.01246517	GZMH/GZMB/PRF1
	positive regulation of cellular catabolic process	6/41	0.01262894	TNF/PYHIN1/ADRB2/PLEKHF1/PRR5L/CTSC

Table D-2. Top 10 gene ontology terms per cluster based on top 50 downregulated genes

Cluster	Description	GeneRatio	qvalue	geneID
0	cell killing	6/41	0.00088617	GZMB/PRF1/GNLY/CTSC/IFNG/SLAMF7
	modification of morphology or physiology of other organism	6/41	0.00088617	CCL4/PRF1/GNLY/IFNG/CASP8/ANXA2
	cellular defense response	4/41	0.00212886	PRF1/GNLY/CX3CR1/KLRG1
	response to virus	7/41	0.00212886	CCL4/PRF1/FGR/SPON2/APOBEC3G/IFNG/STMN1
	leukocyte mediated cytotoxicity	4/41	0.0136265	GZMB/PRF1/CTSC/SLAMF7
	neutrophil degranulation	7/41	0.0136265	ARPC5/FGR/PSMA5/CTSC/ITGAL/COMMD3/ANXA2
	cytolysis	3/41	0.0136265	GZMH/GZMB/PRF1
	neutrophil activation involved in immune response	7/41	0.0136265	ARPC5/FGR/PSMA5/CTSC/ITGAL/COMMD3/ANXA2
	neutrophil mediated immunity	7/41	0.0136265	ARPC5/FGR/PSMA5/CTSC/ITGAL/COMMD3/ANXA2
	neutrophil activation	7/41	0.0136265	ARPC5/FGR/PSMA5/CTSC/ITGAL/COMMD3/ANXA2
1	T cell activation	10/41	2.10E-05	TCF7/IL7R/LEF1/CCR7/CD27/CD28/SATB1/CD74/IL6ST/TNFSF8
	T cell differentiation	8/41	2.10E-05	TCF7/IL7R/LEF1/CCR7/CD27/CD28/SATB1/CD74
	lymphocyte differentiation	8/41	0.00017241	TCF7/IL7R/LEF1/CCR7/CD27/CD28/SATB1/CD74
	negative thymic T cell selection	3/41	0.00027944	CCR7/CD28/CD74
	negative T cell selection	3/41	0.0003069	CCR7/CD28/CD74
	negative regulation of leukocyte apoptotic process	4/41	0.00050707	IL7R/CCR7/CD27/CD74
	positive regulation of T cell activation	6/41	0.00058518	IL7R/CCR7/CD27/CD28/CD74/IL6ST
	regulation of T cell activation	7/41	0.00058518	IL7R/CCR7/CD27/CD28/CD74/IL6ST/TNFSF8
	positive regulation of translation	5/41	0.00063706	CD28/PABPC1/PASK/RPL5/VIM
	leukocyte cell-cell adhesion	7/41	0.00066141	SELL/IL7R/CCR7/CD27/CD28/CD74/IL6ST
2	response to virus	8/44	0.00113986	CCL4/SPON2/FGR/IFITM1/LAMTOR5/NPLOC4/TBX21/BST2
	negative regulation of immune effector process	4/44	0.09990567	ANXA1/NPLOC4/TBX21/BST2
	cellular defense response	3/44	0.09990567	CX3CR1/GNLY/TCIRG1
	negative regulation of type 2 immune response	2/44	0.09990567	ANXA1/TBX21
	regulation of immune effector process	6/44	0.09990567	SPON2/FGR/ANXA1/NPLOC4/TBX21/BST2
	negative regulation of T-helper cell differentiation	2/44	0.09990567	ANXA1/TBX21
	negative regulation of cytokine production	5/44	0.09990567	CX3CR1/ANXA1/NPLOC4/TBX21/BST2
	regulation of translational elongation	2/44	0.09990567	EIF5A/DPH2
	negative regulation of multi-organism process	4/44	0.09990567	CCL4/IFITM1/NPLOC4/BST2
	negative regulation of CD4-positive, alpha-beta T cell differentiation	2/44	0.09990567	ANXA1/TBX21
3	cellular response to calcium ion	5/47	0.00055341	JUNB/JUN/FOS/JUND/HSPA5
	response to calcium ion	6/47	0.00055341	DUSP1/JUNB/JUN/FOS/JUND/HSPA5
	negative regulation of endoplasmic reticulum unfolded protein response	3/47	0.00111449	PPP1R15A/HSPA5/DNAJB9
	cellular response to inorganic substance	6/47	0.00326461	JUNB/JUN/FOS/JUND/HSPA5/EIF4A3
	response to muscle stretch	3/47	0.00326461	NFKBIA/JUN/FOS
	neuron death	7/47	0.00630053	NR4A2/JUN/FOS/DDIT4/BTG2/HSPA5/CX3CR1
	regulation of endoplasmic reticulum unfolded protein response	3/47	0.00648846	PPP1R15A/HSPA5/DNAJB9
	response to endoplasmic reticulum stress	6/47	0.00687478	JUN/PPP1R15A/HSPA5/PMAIP1/LMNA/DNAJB9
	response to steroid hormone	7/47	0.00687478	DUSP1/NR4A2/ZFP36/FOS/DDIT4/NR4A1/NR1D2
	response to unfolded protein	5/47	0.00692718	PPP1R15A/HSPA5/DNAJB1/LMNA/DNAJB9
4	response to steroid hormone	9/46	0.00038757	TXNIP/DDX5/DUSP1/NR4A2/ZFP36L2/FOS/FOXP1/ZFP36/FO5B
	response to calcium ion	6/46	0.00039718	TXNIP/JUNB/JUND/DUSP1/FOS/FO5B
	negative regulation of leukocyte apoptotic process	4/46	0.00190082	TSC2D3/CCR7/IL7R/FOXP1
	positive regulation of nuclear-transcribed mRNA catabolic process, deadenylation-dependent decay	3/46	0.0020847	BTG2/ZFP36L2/ZFP36
	nuclear-transcribed mRNA catabolic process	6/46	0.0020847	RPL21/BTG2/DDX5/RPS27/ZFP36L2/ZFP36
	regulation of nuclear-transcribed mRNA catabolic process, deadenylation-dependent decay	3/46	0.0020847	BTG2/ZFP36L2/ZFP36
	cellular response to calcium ion	4/46	0.00288034	JUNB/JUND/FOS/FO5B
	response to glucocorticoid	5/46	0.00345087	DUSP1/ZFP36L2/FOS/ZFP36/FO5B
	regulation of tumor necrosis factor production	5/46	0.00379393	HLA-E/UBE2J1/SPON2/FOXP1/ZFP36
	response to corticosteroid	5/46	0.00379393	DUSP1/ZFP36L2/FOS/ZFP36/FO5B
5	regulation of leukocyte activation	12/46	3.27E-06	CCL5/CSF7/CD74/HLA-DPA1/ZNF683/TIGIT/PTPN22/ADAM8/CD84/FCRL3/IFNG/TNF
	leukocyte cell-cell adhesion	10/46	5.10E-06	CCL5/CD74/HLA-DPA1/ITGB1/TIGIT/PTPN22/ADAM8/IFNG/CX3CR1/TNF
	cellular response to interferon-gamma	7/46	4.06E-05	CCL5/HLA-DRB1/HLA-DRB5/HLA-DPA1/GBP5/HLA-DRA/IFNG
	cell killing	7/46	4.06E-05	LYST/PRF1/SH2D1A/GZMB/IFNG/SLAMF7/GNLY
	cellular defense response	5/46	5.48E-05	PRF1/ITGB1/SH2D1A/GNLY/CX3CR1
	response to interferon-gamma	7/46	5.48E-05	CCL5/HLA-DRB1/HLA-DRB5/HLA-DPA1/GBP5/HLA-DRA/IFNG
	natural killer cell mediated immunity	5/46	9.07E-05	LYST/SH2D1A/KLRD1/GZMB/SLAMF7
	regulation of lymphocyte activation	9/46	9.07E-05	CCL5/CD74/HLA-DPA1/ZNF683/TIGIT/PTPN22/ADAM8/FCRL3/IFNG
	lymphocyte mediated immunity	8/46	9.07E-05	CD74/LYST/PRF1/SH2D1A/KLRD1/GZMB/SLAMF7/TNF
	regulation of leukocyte cell-cell adhesion	8/46	9.07E-05	CCL5/CD74/HLA-DPA1/TIGIT/PTPN22/ADAM8/IFNG/TNF
6	leukocyte differentiation	12/49	6.87E-06	JUN/JUNB/FOS/ZFP36L2/TCF7/JMID6/CCR7/LEF1/ZBTB1/NFKBIZ/DNAJB9/KLF10
	myeloid cell differentiation	9/49	0.00086786	JUN/JUNB/FOS/NFKBIA/ZFP36/JMID6/CCR7/LEF1/KLF10
	positive regulation of leukocyte differentiation	6/49	0.00086786	JUN/FOS/LEF1/ZBTB1/NFKBIZ/KLF10
	lymphocyte differentiation	8/49	0.00086786	ZFP36L2/TCF7/JMID6/CCR7/LEF1/ZBTB1/NFKBIZ/DNAJB9
	T cell differentiation	7/49	0.00087265	ZFP36L2/TCF7/JMID6/CCR7/LEF1/ZBTB1/NFKBIZ
	regulation of hemopoiesis	9/49	0.00097007	JUN/FOS/NFKBIA/ZFP36/ZFP36L2/LEF1/ZBTB1/NFKBIZ/KLF10
	regulation of leukocyte differentiation	7/49	0.00148296	JUN/FOS/ZFP36L2/LEF1/ZBTB1/NFKBIZ/KLF10
	positive regulation of hemopoiesis	6/49	0.00155229	JUN/FOS/LEF1/ZBTB1/NFKBIZ/KLF10
	positive regulation of nuclear-transcribed mRNA catabolic process, deadenylation-dependent decay	3/49	0.00155229	ZFP36/ZFP36L2/BTG2
	positive regulation of myeloid leukocyte differentiation	4/49	0.00155229	JUN/FOS/LEF1/KLF10

Appendix E – PD-1 expression of CD8+ T cell clusters pre-treatment

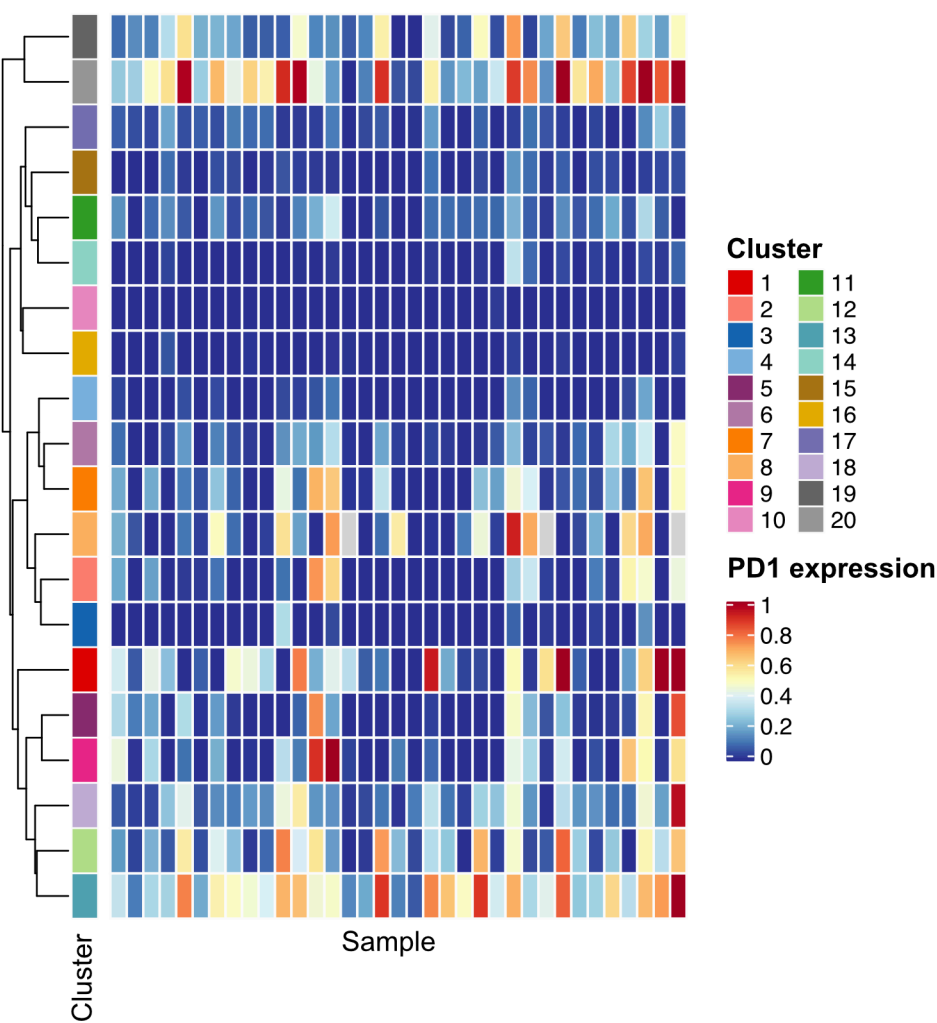


Figure E-1. Pre-treatment PD-1 expression of CD8+ clusters.

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