

Investigating factors affecting functionality of virus-specific CD8⁺ T cells



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Abstract

CD8⁺ T cells play a fundamental role in protection against viral infections, including SARS-CoV-2 and influenza. Although CD8⁺ T cells are well studied, many questions regarding their function remain. As T cells play a key role in determining disease outcome during respiratory viral infection, understanding the factors that influence their function is of paramount importance. Focussing on the T cell response to influenza and SARS-CoV-2, we address two key research questions in this thesis: how does antigen-specificity shape the ageing trajectory of CD8⁺ T cells, and which features of CD8⁺ T cells contribute to their association with severe disease.

The first part of this study (Chapter 3) addresses the question of how antigen specificity shapes the ageing trajectory of CD8⁺ T cells, focussing on the immunodominant influenza M1₅₈₋₆₆-specific T cell response. We perform single-cell RNA sequencing of longitudinal samples from three individuals over a 19-year period to determine how M1₅₈₋₆₆-specific T cells change with age and how these changes compare to those in the broader CD8⁺ T cell population. We observed reduced diversity of the M1₅₈₋₆₆-specific TCR repertoire as the donors aged and identified transcriptomic changes, including altered expression of genes associated with metabolism and cytotoxicity. Several of the transcriptomic changes are unique to M1₅₈₋₆₆-specific T cells, highlighting that T cells with different specificities undergo distinct age-related changes.

Although CD8⁺ T cells protect against viral infections, they can also be associated with driving immunopathology but the factors that cause them to do this are not well understood. In Chapter 4 we focus on the SARS-CoV-2 ORF3a₂₀₇₋₂₁₅-specific T cell response which has been associated with severe disease in COVID-19. We identify pairs of ORF3a₂₀₇₋₂₁₅-specific T cell clones with identical TCR sequences but differences in their functionality and use the highly functional clones as an example of T cells that can contribute to immunopathology. Through omic analysis we identify high expression of integrins on the highly functional T cells and thus explore how integrin molecules influence T cell function.

In Chapter 5, we utilise *in vitro* functional assays and immune synapse imaging to investigate how the integrins $\alpha 4\beta 1$ and $\alpha 4\beta 7$ influence T cell function and whether the expression of these integrins could be related to the association of ORF3a₂₀₇₋₂₁₅-specific T cells with disease severity. We found that the integrins $\alpha 4\beta 1$ and $\alpha 4\beta 7$ increased T cells function, and immune synapse imaging revealed localisation of $\alpha 4$, $\beta 7$ and $\beta 1$ in the immune synapse. Thus, these integrins may function as fine tuners of T cell activity and enhance T cell functionality through stabilisation of the immune synapse.

Declaration

I hereby declare that I am the sole author of this thesis titled “Investigating factors affecting functionality of virus-specific CD8+ T cells”. This thesis has not been submitted in whole or in part for any other degree or qualification at this, or any other university. The work in this thesis is entirely my own and contains nothing which is the outcome of work done in collaboration with others, except when specified in the text and outlined in the following:

- T cell clones used throughout this thesis were isolated and established by Dr Yanchun Peng. Subsequent functional assays with these clones were performed by me.
- TCR sequencing of isolated T cell clones was performed by Dr Yanchun Peng.
- Technical help and advice in single cell RNA sequencing library generation was provided by Dr Elie Antoun.
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Abbreviations list

ADP	Adenosine diphosphate
ADT	Antibody-derived tag
APC	Antigen presenting cell
ATP	Adenosine triphosphate
BCL	B cell line
BSLB	Bead supported lipid bilayer
CITE-seq	Cellular indexing of transcriptomes and epitopes by sequencing
CD	Cluster of differentiation, as in CD4 CD8 etc
CD	Crohn's disease
CDR	Complementarity determining region
CLR	Centered log ratio
CMV	Cytomegalovirus
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
cSMAC	Central supramolecular activation complex
cTECs	Cortical thymic epithelial cells
D1-3	Donor 1-3
DC	Dendritic cell
Dex	Dextramer
DISC	Death inducing signalling complex
DN	Double negative
DNA	Deoxyribonucleic acid
DOPC	1,2-Dioleoyl-sn-glycero-3-phosphocholine
DP	Double positive
dSMAC	Distal supramolecular activation complex
EBV	Epstein-Barr virus

ECAR	Extracellular acidification rate
ECM	Extracellular matrix
FACS	Fluorescence activated cell sorted
FAO	Fatty acid oxidation
FasL	Fas Ligand
FCS	Foetal calf serum
FDR	False discovery rate
HA	Haemagglutinin
HBS	Hepes buffered saline
HBV	Hepatitis B virus
HIV	Human immunodeficiency virus
HSCs	Haematopoietic stem cells
HUVEC	Human umbilical vein endothelial cells
IAV	Influenza A virus
ICAM-1	Intracellular adhesion molecule 1
ICS	Intracellular cytokine staining
IFN	Interferon
IFN γ	Interferon gamma
IL	Interleukin
IL-2	Interleukin-2
ITAMS	Immunoreceptor tyrosine based activation motif
J	Joining
KO	Knockout
LAD-I	Leukocyte adhesion deficiency
LCMV	Lymphocytic choriomeningitis virus,
LFA-1	Lymphocyte function-associated antigen 1

LFC	Log2 fold change
LUAD	Lung adenocarcinoma
mAb	Monoclonal antibody
MAIT	Mucosal associated invariant T cells
MAdCAM-1	Mucosal addressin cellular adhesion molecule 1
MESF	Molecules of Equivalent Soluble Fluorochrome
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MS	Multiple Sclerosis
mTECs	medullary thymic epithelial cells
NA	Neuraminidase
NFAT	Nuclear factor of differentiation
OCR	Oxygen consumption rate
OXPHOS	Oxidative phosphorylation
PAMPs	Pathogen associated molecular patterns
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCA	Principal component analysis
PHA	Phytohemagglutinin
PLC γ 1	phospholipase C gamma 1
pMHC	Peptide-major histocompatibility complex
PRR	Pattern recognition receptor
pSMAC	Peripheral supramolecular activation complex
RNA	Ribonucleic acid
RNP	Ribonucleoprotein
RPCA	Reciprocal PCA

RT	Room temperature
S1PR1	Sphingosine-1-Phosphate Receptor 1
scRNAseq	Single cell RNA sequencing
SFC	Spot forming cell
SKCM	Skin cutaneous melanoma
SLB	Supported lipid bilayer
SMAC	Supramolecular activation cluster
SP	Single positive
T1-T5	Timepoint 1-5
Tcm	Central memory T cell
TCR	T cell receptor
Tem	Effector memory T cell
Temra	Terminally differentiated effector memory T cells re-expressing CD45RA
TFs	Transcription factors
TIRFM	Total internal reflection fluorescence microscopy
TN	Triple negative
TNF	Tumour necrosis factor
TP	Triple positive
TRAs	Tissue restricted antigens
Trm	Tissue resident memory T cell
TSPs	thymic seeding progenitors
UMAP	Uniform manifold approximation and projection
V	Variable
VCAM-1	Vascular cellular adhesion molecule 1

1. Introduction

1.1 Overview of the immune system

1.1.1 Purpose of the immune system

Throughout our lives we are continuously exposed to foreign bodies, some of which are harmless, but others are pathogenic. Our immune system functions to differentiate between the two and protect us from foreign bodies capable of causing harm. Physical and chemical barriers, including our skin and mucus secretions function as the first hurdle for invading microorganisms to cross¹. Beyond these barriers the immune system is divided into two: the innate immune system and the adaptive immune system. The innate immune system is the first line of defence against invading pathogens, kicking into action within minutes to hours². The adaptive immune system is slower, with days required before it can mount an effective response. The adaptive system is antigen specific, and the receptors required to recognise antigens undergo gene rearrangement. Together the cells of the innate and adaptive immune system, along with the soluble factors they generate, mount a coordinated response which protects us from the onslaught of invading pathogens that we are exposed to throughout our lifetime².

1.1.2 Innate Immune System

The innate immune system is evolutionarily very old, with the first known examples of innate immunity starting with the occurrence of multicellular organisms, around 1 billion years ago³. Although the innate immune system that humans have today is significantly advanced from its earliest renditions, it retains the some of the same fundamental principles.

In humans, the cells of the innate immune system include macrophages, dendritic cells (DCs), neutrophils and mast cells, most of which derive from the common myeloid progenitor. These cells deploy a range of specialised mechanisms to combat invading pathogens, including granule release and the formation of neutrophil extracellular traps. A key function among several innate cell types is phagocytosis, which enables them to engulf and destroy microbes^{2, 3}. Among innate immune cells, DCs play a unique role by

acting as a bridge between the innate and adaptive immune system, where they are fundamental in the activation of T cells.

It was initially unclear how innate immune cells could recognise invading pathogens and detect that they were not self. In 1989, Charles Janeway hypothesised that pattern recognition receptors (PRRs) which recognise pathogen associated molecular patterns (PAMPs), must exist and allow innate immune cells to detect foreign bodies^{4, 5}. Further decades of research confirmed Janeway's hypothesis and nowadays we know there are several classes of PRRs, including toll-like receptors and NOD-like receptors, which recognise various microbial products, from lipopolysaccharide to viral nucleic acids. These PRRs act as the warning signal to alert the cell that a pathogen is present as their recognition of their ligand triggers a signalling cascade that induces expression of pro inflammatory cytokines and type I interferons (IFNs). PRRs are germline encoded and do not undergo recombination like the receptors of the adaptive immune system⁵.

In addition to this cellular aspect of the innate immune system, there is also a humoral component, known as complement. The complement system consists of around 50 proteins that are found both in circulation and in tissues. During an infection, they can be activated via three different pathways: classical, alternative and lectin. Activation of the complement system can result in the direct lysis of extracellular pathogens through generation of the membrane attack complex, as well as production of further inflammatory mediators³.

1.1.3 Adaptive Immune System

The adaptive immune system evolved much more recently than the innate one, around 500 million years ago in jawed fish⁶. The key distinction between the innate and adaptive immune system is that adaptive immune cells express antigen receptors that are not germline encoded and undergo somatic recombination. This process allows the cellular components of the adaptive immune system, T and B lymphocytes, to generate and express a diverse range of antigen receptors. In the case of B cells these receptors are also released as a soluble form known as antibodies which can opsonise and neutralise pathogens in the blood.

Another key feature of the adaptive immune system is its ability to “remember”. This immune memory allows a faster, more effective response the second time that we are exposed to a particular pathogen. This is partly due to increased frequency of antigen-specific cells after the initial exposure but also due to enhanced effector function of the memory T and B cells.

1.1.4 Historical perspective on T cells

Despite their fundamental role in the adaptive immune system, T cells were not recognised as a distinct cell type until long after antibodies were discovered⁷. In the 1960s, while doing work on the onset of leukaemia, Jacques Miller performed neonatal thymectomies on mice. Miller discovered that an early neonatal thymectomy caused reduction of lymphocyte counts and shortened life span. When kept in pathogen free conditions this reduction in life span was abrogated⁸. These fundamental experiments provided the first evidence that the thymus was involved in the generation of cells that carry out immune functions. Further experiments in the 1970s demonstrated that these thymus derived cells were capable of killing^{9, 10}. Another breakthrough occurred when Rolf Zinkernagel and Peter Doeherty demonstrated major histocompatibility complex (MHC) restriction through experiments with T cells from Lymphocytic Choriomeningitis Virus (LCMV) infected mice^{7, 11, 12}. Work throughout the 1970s and 80s led the way for identification of helper (CD4⁺) and killer (CD8⁺) T cells as distinct subsets of T cells with separate but complementary roles. Research on these fascinating immune cells has intensified over the past 50 years, with much of current research focusses on their role in cancer elimination and protection from viral infections. However, many questions remain unanswered.

Several distinct lineages of T cells have now been identified, including Mucosal-Associated Invariant T (MAIT) cells and $\gamma\delta$ T cells, in addition to conventional $\alpha\beta$ T cells. While these non-conventional T cells play important immunological roles, the term *T cell* in this thesis is referring to $\alpha\beta$ T cells and unless otherwise specified, predominantly to CD8⁺ T cells.

1.2 Development of T cells

1.2.1 Commitment to the T cell lineage

Miller's thymectomy experiments in the early 1960s led the way to the understanding that T cell development occurs in the thymus^{13, 14}. Although this largely holds true, it is now understood that the earliest stages of T cell development take place in the bone marrow. Haematopoietic stem cells (HSCs) are multipotent stem cells that can give rise to all cell types in the blood. In the bone marrow HSCs undergo initial stages of commitment and enter the blood stream as multipotent progenitor cells¹⁵. Not all circulating progenitor cells are destined to settle in the thymus; those that do express the chemokine receptors CCR7 and CCR9, allowing them to migrate to and enter the thymus. These thymus homing progenitors are known as thymus seeding progenitors (TSPs). Definitive identification of TSPs remains challenging because their expression profiles change rapidly, and recent single-cell studies indicate three distinct TSP populations with differing marker expression and kinetics of T-cell lineage commitment¹⁶.

TSPs enter the thymus via veins adjacent to the corticomedullary junction¹⁷ (Figure 1.1), and upon entry receive signals that drive their commitment to the T cell lineage. One of the most crucial pathways in this process is Notch signalling, triggered by ligands expressed on thymic stromal cells. Notch signalling induces expression of the transcription factor (TF) TCF-1 (encoded by *TCF7*), which in turn upregulates expression of the T cell TFs GATA-3 and BCL11B¹⁸. These TFs restrict the ability of the TSPs to become B cells or cells of the myeloid lineage. The fundamental role of Notch signalling has been demonstrated by several studies. Overexpression of *Notch1* in haematopoietic precursors drives T cell differentiation in the bone marrow¹³, and in a simplified coculture assay Jaleco and colleagues showed that the Notch ligand Delta-1 completely blocked B cell development¹⁹. Additionally, *Notch1*-deficient mice show arrest in development towards the T cell lineage and an increase in B cells in thymus²⁰. The cytokines stem cell factor and Flt3L and the Wnt signalling pathway also play key roles in the initial stages of T cell lineage commitment¹⁷.

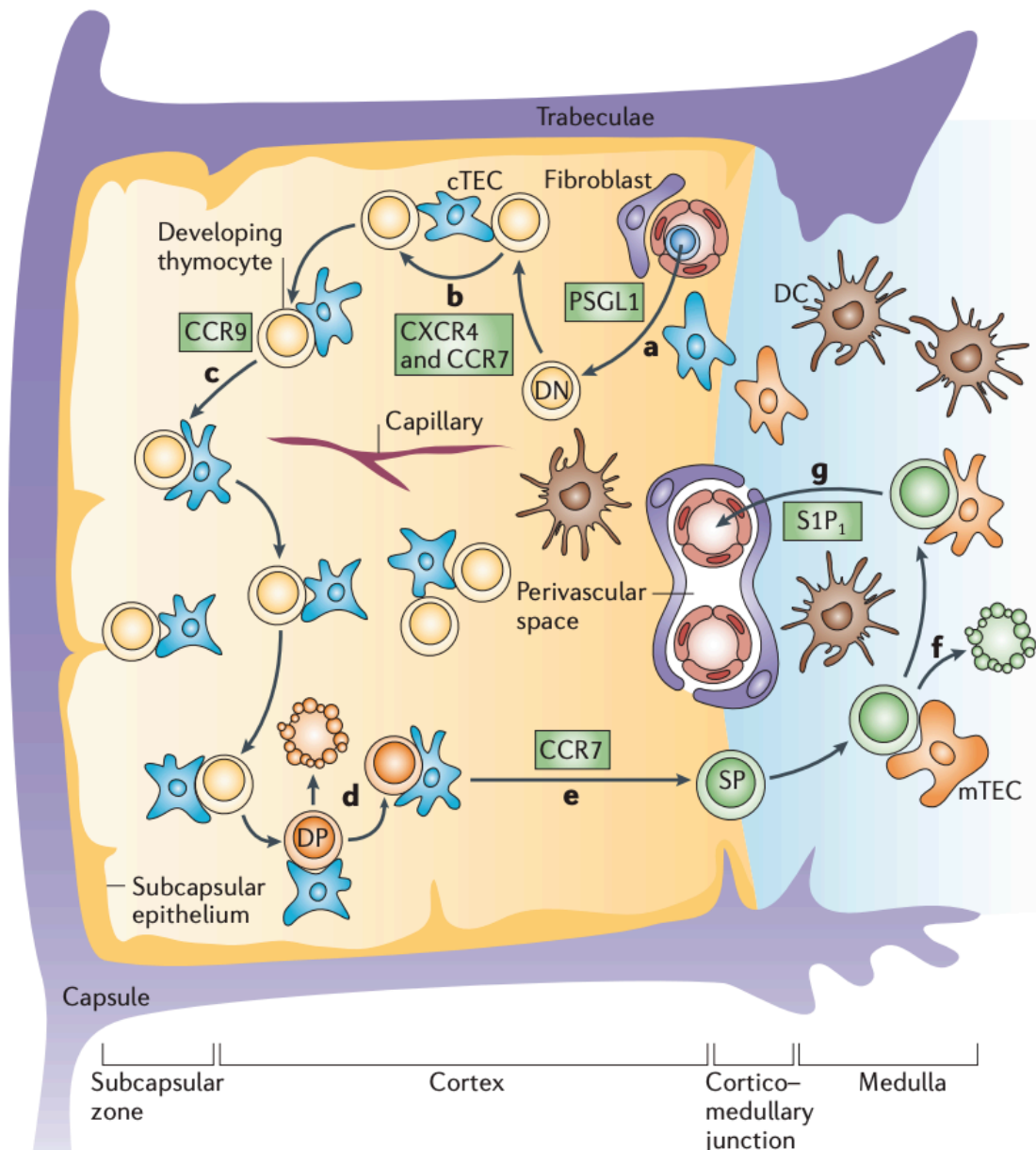


Figure 1.1: Migration of thymocytes through the thymus for T cell development and selection. (A) Circulating TSPs migrate into thymus through veins adjacent to the cortico-medullary junction **(B)** The migration of CD4-CD8-DN thymocytes to the cortex is regulated by chemokine signals through the chemokine receptors CXCR4 and CCR7. **(C)** Further migration of the DN thymocytes is mediated by CCR9 signals as the thymocytes progress through the DN stages. **(D)** CD4+CD8+ DP thymocytes generated in cortex interact with cTECs to undergo positive selection and negative selection. **(E)** Positively selected DP thymocytes can then differentiate into CD4 or CD8 SP thymocytes, which show an increase in the surface expression of CCR7, through which the cells are attracted to the medulla, which expresses CCR7 ligands. **(F)** In the medulla, further negative selection of SP thymocytes involves the deletion of tissue-specific-antigen-reactive T cells, through their interaction with mTECs. **(G)** Mature SP thymocytes express S1P₁, through which the cells are attracted back to the circulation by cells surrounding the blood vessels at the cortico-medullary junction that express high concentration of sphingosine-1-phosphate. Reproduced from Takahama, 2006, with permission from Springer Nature. Abbreviations: TSP, thymic seeding progenitor; DN, double negative; cTEC, cortical thymic epithelial cell; SP, single positive; mTEC, medullary thymic epithelial cells; S1P₁ express sphingosine-1-phosphate receptor 1.

1.2.2 Thymocyte development and TCR gene rearrangement

After entering the thymus and committing to the T-cell lineage, these cells must progress through several developmental stages before emerging as mature T cells²¹. These stages of commitment are characterised by their surface expression of key markers including CD4 and CD8. Thymocyte development initially occurs through double negative (CD4-CD8-, DN), which are further subdivided into four stages (DN1 to DN4) based on expression of CD25 and CD44 in mice. In humans, immature thymocytes do not express the same surface markers but the corresponding populations can be identified based on differential expression of the markers CD34, CD38 and CD1a²². After progressing through the DN stages thymocytes express both CD4 and CD8 and are referred to as double positive (DP). Finally, they lose expression of one of the coreceptors and become either CD4+CD8-CD3+ or CD8+CD4-CD3+ and are referred to as single positive (SP). As thymocytes progress through these stages of development they move through several anatomical zones of the thymus (Figure 1.1) which provide them with different signals, aiding their development¹⁷.

As thymocytes reach the subcapsular zone they upregulate several genes necessary for V(D)J gene rearrangement, including the recombinase activating genes (*RAG1* and *RAG2*) which initiate TCR gene assembly. The RAG proteins function by creating double stranded breaks at sites flanking TCR variable (V), diversity (D) and joining (J) gene segments^{23, 24} (Figure 1.2). These breaks are repaired by nonhomologous end joining, during which random nucleotides are inserted at the junctions between the gene segments, contributing to the diversity of the TCR repertoire. Rearrangement of the *TRB* gene, as well as the *TRG* and *TRD* genes, occurs during DN2 and DN3 stages. The successful rearrangement of *TRB* permits pairing of the TCR β chain with pre-TCR α to form the pre-TCR. If the pre-TCR cannot signal another round of recombination of the *TRB* gene can occur. If the pre-TCR successfully signals, then the RAG proteins are downregulated and the thymocytes undergo several rounds of proliferation and further differentiation. At the DP stage the RAG proteins are re-expressed and *TRA* recombination takes place.

When combined, the multiple sources of TCR diversity, such as gene segment selection, combinatorial and junctional diversification, and the pairing of the α and β chains, are estimated to generate a theoretical repertoire of up to 10^{61} unique TCR $\alpha\beta$ receptors²⁵. However this number is massively larger than the actual predicted diversity of the TCR repertoire with estimations²⁶ of this as low as 3.8×10^8 .

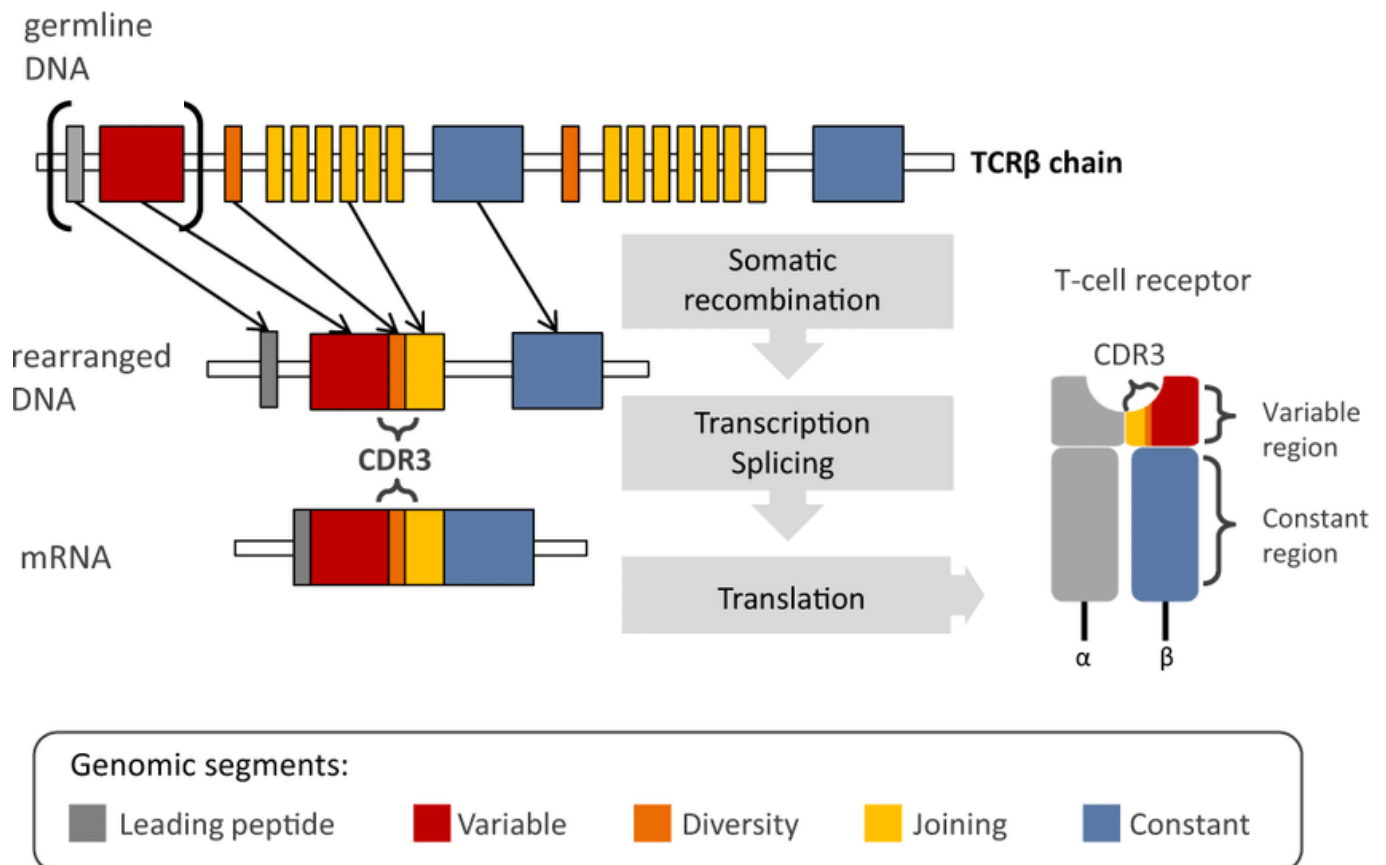


Figure 1.2 TCR β gene recombination. The antigen-binding domain of a TCR β is formed by the variable region of each chain, encoded by recombined V, D and J segments. The RAG proteins function by creating double stranded breaks at sites flanking the V, D, and J gene segments. The breaks are repaired by nonhomologous end joining, during which random nucleotides are inserted into the junctions between the genes. Coding TCR β mRNA is composed of the following segments: leading region, V, D, J, and C. A similar process occurs for TCR α gene recombination, although no D segments are involved. Figure adapted from Migalska et al., 2018, under CC-BY license. Abbreviations: TCR, T cell receptor; RAG, recombinase activating genes; V, variable; D, diversity; J, joining; C, constant.

1.2.3 Positive and negative selection

As the diversity of the TCR repertoire is generated with some degree of randomness it is of paramount importance that checks take place before the T cells are released into the periphery. A rigorous process of “thymic education”, which consist of positive and negative selection, occurs before thymocytes exit the thymus²⁵. At the DP stage thymocytes test out their TCR by attempting to interact with MHC molecules expressed by cortical thymic epithelial cells (cTECs). cTECs have specialised antigen presenting machinery that allows them to express a wide range of peptides on both MHC class I and MHC class II. The vast majority (90%) of thymocytes fail to interact with MHC at this stage and thus die due to death by neglect²⁷. Successful interaction of the TCR with MHC provides a survival signal to the developing thymocyte. At this stage the thymocytes will downregulate one of the coreceptors and become either CD8⁺ or CD4⁺ SP cells. This stage of thymic education in which only T cells that can recognise self-MHC molecules are able to survive is known as positive selection.

Negative selection, in which thymocytes that recognise self too strongly are removed from the repertoire, is now understood to take place in both the cortex and medulla. Expression of the chemokine receptor CCR7 attracts SP thymocytes to the medulla as CCL21 and CCL19 are highly expressed by medullary thymic epithelial cells (mTECs)²⁸. TCRs must be tested against antigens originating from diverse tissues. To enable this, mTECs express the transcriptional regulators AIRE and FEZF2, which drive the ectopic expression of tissue-restricted antigens (TRAs) that developing thymocytes would not otherwise encounter. The importance of these transcriptional regulators is highlighted by the severe autoimmunity observed in patients with *AIRE* mutations²⁸. Some TRAs are transferred from cTECs to dendritic cells in the medulla. Thymocytes that strongly recognise self-antigen MHC complexes on DCs or TECs are deleted, removing clones that would theoretically go on to cause autoimmunity in the periphery. Although most self-reactive TCRs are deleted at this stage, some instead develop into FOXP3⁺ regulatory T cells.

It is important to note that most of our understanding around these processes of thymocyte development and selection comes from mouse studies, and that these processes may differ in humans. Insights into human T cell development have been gained from rare cases of thymic transplantation surgery which is performed on infants that have DiGeorge Syndrome and completely lack a functional thymus^{29, 30}. The implantation of allogenic thymus tissue allows affected individuals to develop T cells that are both self-tolerant and able to react to invading pathogens.

1.2.4 Egress to the periphery

Once T cells have undergone selection, they must then exit the thymus and enter the periphery. This process is controlled by the expression of the G protein coupled receptor, sphingosine-1-phosphate receptor 1 (S1PR1)^{31, 32}. Following selection, SP mature thymocytes upregulate S1PR1, allowing them to migrate towards an S1P gradient, formed by cells surrounding the blood vessels in the corticomedullary junction (Figure 1.1). Mature thymocytes can then cross the basement membrane to the perivascular space and finally undergo reverse transendothelial migration, allowing them to enter the blood stream as recent thymic emigrants³¹. In addition to S1PR1, several chemokine receptors, including CCR7 and CXCR4 have been implicated the egress of mature thymocytes^{31, 33}.

1.3 The TCR and activation of T cells

1.3.1 Structure of the TCR

The complex gene rearrangement events that occur in the thymus affect only two of the eight chains that comprise the TCR-CD3 complex. This octameric complex is composed of four membrane embedded dimers: TCR $\alpha\beta$, CD3 $\epsilon\gamma$, CD3 $\epsilon\delta$ and CD3 $\zeta\zeta$ ³⁴ (Figure 1.3A). These dimers interact with each other through a mixture of non-covalent and disulphide bonds. The exact structure of the TCR complex remained unclear for many years, with progress made throughout the 1980s and 1990s^{35, 36}. In 2019, Dong and colleagues used single particle cryo-electron microscopy to determine the structure of the TCR-CD3 complex³⁷. This was followed a few years later with the determination of a TCR complex bound to peptide MHC (pMHC) (Figure 1.3B)³⁸. CD4 and CD8 act as coreceptors in the

TCR-pMHC interaction. Although they operate through distinct mechanisms, both have been shown to stabilise TCR binding to pMHC^{39, 40}. In CD8⁺ T cells, the CD8 coreceptor has been demonstrated to be essential in enhancing low to medium affinity TCR-pMHC interactions⁴¹.

Despite being fundamental in pMHC recognition the TCR $\alpha\beta$ heterodimer does not contain signalling motifs in its relatively short intracellular domain. However, the three other dimers collectively have 10 immunoreceptor tyrosine-based activation motifs (ITAMs) which allow for the signalling of the TCR-CD3 complex. The ζ chains contain three ITAMs each, with the ε , γ and δ chains each bearing one⁴².

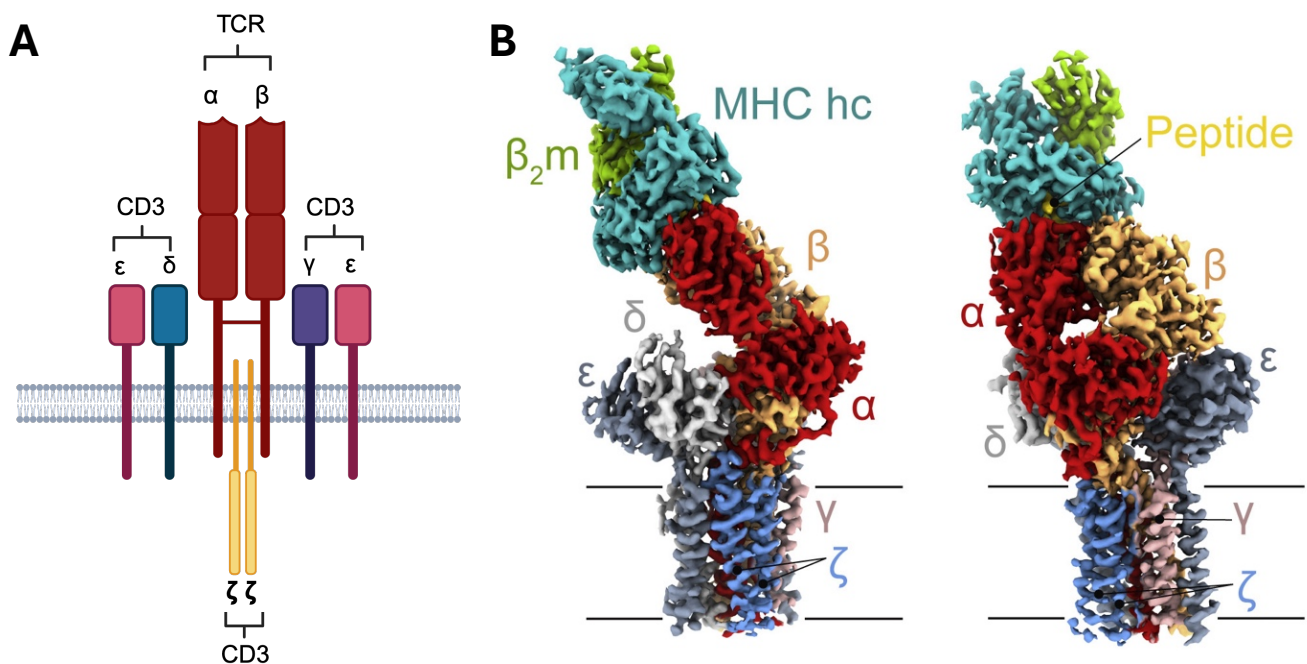


Figure 1.3: Structure of the TCR-CD3 complex.

(A) Schematic of TCR-CD3 complex. The octameric complex is composed of four membrane embedded dimers: TCR $\alpha\beta$, CD3 $\varepsilon\gamma$, CD3 $\varepsilon\delta$ and CD3 $\zeta\zeta$. The dimers interact with each other through a mixture of non-covalent and disulphide bonds. The TCR $\alpha\beta$ heterodimer does not contain signalling motifs in its intracellular domain. Figure made using Biorender. **(B)** Cryo-EM structure of the TCR-CD3 complex bound to peptide-MHC. The TCR $\alpha\beta$ dimer binds to the peptide-MHC via CDR loops. Figure adapted from Sušac et al., 2022, under CC-BY license. Abbreviations: TCR, T cell receptor; MHC, major histocompatibility complex; EM, electron microscopy.

1.3.2 Three signals of T cell activation

The interaction between TCR and pMHC provides for one of the three signals required for T cell activation. Costimulatory interactions and cytokines released from a nearby cell are also required for a T cell to become activated. This important interaction usually occurs in lymph nodes where naïve T cells encounter DCs. DCs migrate from infection sites, carrying antigenic peptide presented on both MHC I and MHC II molecules⁴³. The T cells in the lymph node can scan these MHC molecules for their cognate antigen and if they find it, TCR activation can be triggered.

Upon TCR recognition of pMHC, tyrosine residues within the ITAMs of the ϵ , γ , δ and ζ chains become phosphorylated by the kinase Lck, which initiates downstream TCR signalling. Phosphorylation of the ITAMs in the TCR-CD3 complex generates docking sites for ZAP70 kinase, which is then recruited and phosphorylated by Lck. ZAP70 in turn phosphorylates the adaptor proteins LAT and SLP76⁴⁴. This creates docking sites for GADS which allows for generation of a complex containing LAT, GADS and SLP76, which in turn facilitates several downstream signalling pathways.

One of these key pathways is the calcium-calcineurin-NFAT pathway⁴². To initiate this pathway phospholipase $C\gamma 1$ ($PLC\gamma 1$) is recruited to LAT-GAD-SLP76 complex and activated. $PLC\gamma 1$ can then split membrane bound- PIP_2 into the secondary messengers DAG, which itself initiates a variety of distal signalling cascades important for T cell activation, and IP_3 . IP_3 binds to calcium channels on the endoplasmic reticulum, facilitating the release of calcium into the cytoplasm. Increased calcium levels activate the phosphatase calcineurin which dephosphorylates the transcription factor nuclear factor of activated T cells (NFAT), enabling its nuclear translocation. NFAT then forms a complex with other transcription factors that induce the transcription of several genes involved in T cell activation, including IL-2⁴².

Another important outcome triggered by TCR signalling is remodelling of the cytoskeleton. This is triggered by the recruitment of Vav1 to SLP76 which activates GTPases which indirectly activate Arp2/3, stimulating the growth of branched actin filaments⁴⁵. This also triggers the recruitment of talin which can activate integrins (see Section 1.7.4). These TCR induced signalling cascades are known as signal 1, but alone,

they are not sufficient to fully activate T cells. T cells that only receive signal 1 can become anergic or hyporesponsive.

Signal 2 is provided by interactions between costimulatory receptors and their ligands. The most well studied of these interactions is the interaction between CD28 on T cells and B7 family members (CD80 and CD86) on DCs or other antigen presenting cells. CD28 binding to its ligands triggers phosphorylation of its intracellular tail which results in downstream signalling involving PI3K, Grb2, Vav and ITK. This results in activation of the transcription factor NF κ B which promotes proliferation, metabolic shifts and cytokine production⁴⁴. Some integrin molecules including LFA-1 (α L β 2) have also been shown to have costimulatory effects^{46, 47}.

Inflammatory cytokines are required for a productive CD8⁺ T cell response and thus are classified as signal 3 of T cell activation⁴⁸. Cytokines capable of providing this third signal include interleukin-12 and type I interferons. If a T cell receives all 3 of these signals it will go on to proliferate, producing many copies of themselves, which can then travel to infection sites and carryout effector functions.

1.3.3 The Immune Synapse

In the late 1990s further advances in our understanding of the physical interaction between T cells and antigen presenting cells were made through the discovery of the immune synapse^{49, 50}. The immune synapse, defined as the contact that a T cell makes with an antigen presenting cell or target cell, is composed of concentric ring structures known as supramolecular activation clusters (SMAC). The synapse is subdivided into 3 regions known as the central SMAC (cSMAC), peripheral SMAC (pSMAC) and distal SMAC (dSMAC), which are each enriched in different receptors⁵¹ (Fig 1.4).

Initial studies determined that the cSMAC contains receptors including the TCR, CD4, CD8 and the costimulatory receptor CD28^{49, 51}. In recent years, high resolution imaging has found that the location of TCRs in the immune synapse is more complex than initially thought. The active TCR-pMHC interactions that cause signalling are initially found in small microclusters in the dSMAC which then move centripetally to the cSMAC. Further research has also found that the cSMAC can be subdivided into two zones, the endo-

cSMAC, in which CD28 and TCR signal within the synapse and the exo-cSMAC which contains TCR-enriched extracellular vesicles. The exo-cSMAC is a short-lived compartment which is usually internalised by APCs⁵¹.

The pSMAC is dominated by the integrin molecule LFA-1(α L β 2)⁴⁹, interacting with ICAM-1⁴⁶. LFA-1 is a heterodimer composed of the subunits α L and β 2, the primary ligand for which is ICAM-1. LFA-1 is of paramount importance in immune synapse organisation as negatively charged glycocalyx of cell membranes prevents stable interactions between T cells and APCs. LFA-1 can however interact with ICAM-1 (and other ligands) at ~40nm distances, thus allowing T cells close access to APCs. Additionally, the signalling from LFA-1 synergises with TCR signalling and can increase sensitivity of T cells for their cognate peptide^{52, 53}. The adhesion molecules CD2 and CD58 were also identified in the cSMAC but recent research has identified further accumulation of CD2/CD58 in the

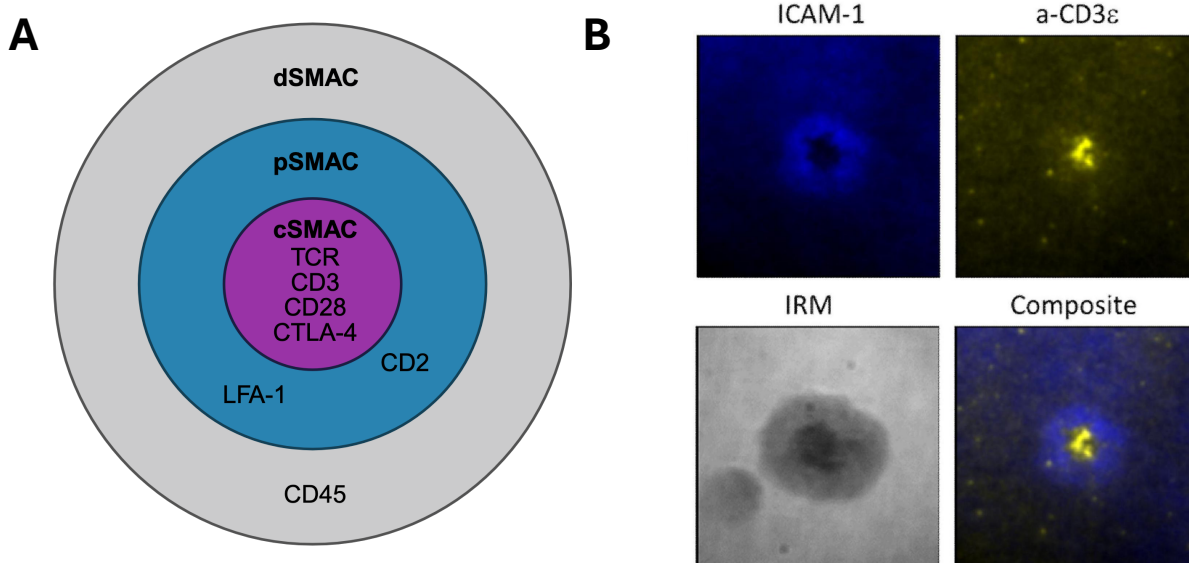


Figure 1.4: Organisation of the immune synapse.

(A) Schematic of immune synapse with central supra-molecular activation complex, the peripheral ring surrounding the cSMAC, and the region distal to the synapse outside the pSMAC, with the molecules enriched in each of these zones. Figure made using Biorender. **(B)** Representative TIRFM images of the immune synapse of Jurkat cells interacting with ICAM1-AF405 + anti-CD3 SLBs for 15 min. Figure adapted from Cassioli et al, 2021., under CC-BY license. Abbreviations: cSMAC, central supramolecular activation clusters; pSMAC, peripheral supramolecular activation clusters; dSMAC, distal supramolecular activation clusters; TIRFM, total internal reflection microscopy; ICAM-1, intracellular adhesion molecule 1.

outer part of the dSMAC region in mature synapses which the authors name CD2-corollas⁵⁴.

The transmembrane phosphatase molecule CD45 is primarily found in the dSMAC. However, some CD45 molecules have been shown to translocate to the cSMAC where it is hypothesised, they can play a role in halting TCR signalling, due to their phosphatase activity^{55, 56}.

The immune synapse is still an active area of research and as imaging techniques continue to advance so too shall our understanding of the organisation of the protein interactions required for the activation of T cells. The role of the immune synapse in directing the release of cytotoxic proteins has been a focus of recent research. Wang and colleagues showed that integrins within the synapse, predominantly LFA-1, act as spatial cues that guide perforin and granzyme containing granules to the plasma membrane for secretion⁵⁷.

1.4 Effector Functions of CD8⁺ T cells

1.4.1 Cytotoxic functions

One of the key functions of CD8⁺ cells is their ability to kill target cells. This effect was noted following the observation of lesions in target cell membranes after they had been incubated with cytotoxic T cells or granules released from them^{58, 59}. This was later discovered to be due to the protein called perforin, which is released from cytotoxic cells in granules and can then polymerise to form a membrane attack complex that can perforate target cell membranes. Through these perforations, cytotoxic T cells can deliver granzymes, a family of serine proteases. Granzymes are then able to induce apoptosis through several pathways, for example, granzyme B can initiate apoptosis through direct activation of caspase 3 or indirectly through cleavage of the caspase substrate BH3- interacting domain death agonist⁶⁰.

Although perforins and granzymes are understood to be the primary mechanism for inducing cell death, cytotoxic T cells can also trigger target cell death through the Fas-Fas Ligand (FasL) pathway. Following activation FasL is upregulated by CD8⁺ T cells,

allowing it to interact with the death receptor Fas expressed by target cells. This interaction triggers assembly of an intracellular death-inducing signalling complex (DISC) in the target cell^{61, 62}. Formation of a DISC triggers activation of a caspase cascade which leads to cell death via apoptosis⁶³.

1.4.2 Cytokine and chemokine release

CD8⁺ T cells, along with many other cells of the immune system, can release cytokines, including interferon- γ (IFN γ), Interleukin-2 (IL-2) and tumour necrosis factor (TNF). IFN γ can interfere directly with the replication of some viruses, including influenza, but can also promote a general anti-viral state by inducing the switch to the immunoproteasome and upregulating and stabilising the expression of MHC-I⁶⁴. Additionally IFN γ can increase susceptibility of target cells to apoptosis by causing upregulation of Fas⁶³.

TNF, previously known as TNF α ⁶⁵, was first identified and named due to its ability to cause necrosis of tumours but has since been shown to have anti-viral effects. TNF is initially expressed by T cells in a transmembrane form which can then be cleaved by the matrix metalloproteinase ADAM17 and released in its soluble form^{66, 67}. Soluble TNF can bind to the receptor TNF-RI, signalling from which can initiate a signalling cascade that causes apoptosis in infected cells. TNF can also increase expression of MHC-I and the cytokines IL-12 and IL-18 which in turn upregulate production of IFN γ from CD8⁺ T cells⁶³.

Upon activation CD8⁺ T cells can also release chemokines which can contribute to inflammatory responses by recruiting other immune cells to the site of infection. In response to costimulation T cells can release the chemokine CXCL10 which recruits other CD8⁺ T cells to the site and to facilitate their participation in the ongoing immune response⁶⁸.

1.5 Memory CD8⁺ T cell responses

1.5.1 Generation and features of memory responses

Generation of effective memory responses is one of the key features of the adaptive immune system. Following a primary infection and pathogen clearance, the majority of

expanded T cells die during the contraction phase of the immune response⁶⁹. However, specialised memory T cells that are generated during the primary response can persist in the absence of antigen and are poised to provide protection against subsequent infections.

The factors that drive a T cell to become a memory T cell and the kinetics of this process are still debated. Mouse studies have elucidated several survival factors that promote memory cell generation, including IL-7R α , which allows cells to respond to the survival cytokine IL-7, and expression of the anti-apoptotic protein BCL2⁶⁹. A recent study found that most memory T cells are generated at late timepoints during primary infections, arising from T cells that have already undergone extensive division⁷⁰.

A key feature of memory CD8⁺ T cells is their increased frequency in circulation compared to naïve virus-specific T cells. It was estimated that within the CD8⁺ T cell pool, naïve virus specific cells are found at a frequency of between 1 in 10⁷ and 1 in 10⁶. Whereas virus-specific memory T cells⁷¹ are estimated to be at frequencies between 1 in 10⁴ to 1 in 10². This drastic increase in frequency increases the likelihood of a T cell finding its cognate antigen quickly during a secondary infection, contributing to increased speed of memory responses.

Beyond differences in cell numbers, memory T cells have molecular profiles that differ significantly from those of naïve T cells. Initial studies using microarray analysis found that antigen-specific memory CD4⁺ and CD8⁺ T cell had distinct transcriptional profiles compared to naïve T cells^{72, 73} and that these differences became more pronounced following activation⁷³. Memory T cells also have a distinct epigenetic profile, including changes in DNA methylation, histone acetylation and methylation. These epigenetic modifications cause changes in the accessibility of genes thereby shaping the level of expression. Memory T cells have increased chromatin accessibility at promoters for the genes *PRF1* and *GZMB* when compared to naïve T cells, allowing for increased and rapid production of these effector molecules^{74, 75}.

In addition to these changes at the DNA level, memory T cells exhibit changes at the protein level, both on the cell surface and intracellularly. On the cell surface, memory T cells have increased expression of adhesion molecules, including integrins which

promote their interactions with APCs. These changes reduce the dependence of memory T cells on CD28 costimulation for activation, allowing memory T cells to be activated by a wider range of APCs^{76, 77}. During inflammatory responses, memory T cells can also be activated in response to stimulation from cytokines such as IL-12 and IL-18⁷⁸. Analysis of downstream signalling pathways has revealed more efficient TCR induced signal transduction in memory T cells, meaning they require fewer signalling events to induce the equivalent functional output⁷⁹.

Memory T cells also exhibit notable metabolic differences, being more metabolically flexible than naïve T cells. They have increased mitochondrial mass and spare respiratory capacity and rely more heavily on fatty acid oxidation (FAO) for energy^{80, 81 82, 83}. (see Section 1.7.3) These extensive molecular changes enable memory T cells to respond faster and with greater potency than naïve T cells.

1.5.2 Heterogeneity of memory subsets

Although memory T cells share many of the features mentioned above, advances in techniques such as flow cytometry and single-cell RNA sequencing (scRNAseq), have revealed significant heterogeneity within memory T cell populations.

Differences in lymph node homing receptors (CCR7 in humans and CD62L in mice) allowed for differentiation between the first two memory subsets identified. CD45RA⁺CCR7⁺ cells were designated as central memory (T_{cm}) due to their lymph node homing potential⁸⁴. The CD45RA⁺CCR7⁻ subset was designated as effector memory (T_{em}), which circulate through the blood and non-lymphoid tissues in search for sites of infection⁸³. At a functional level T_{cm} have relatively low cytotoxic activity, high proliferative capacity, produce IL-2 and IFN γ upon stimulation⁸⁵. T_{em} have lower proliferative capacity but greater immediate effector function, being able to produce several cytokines and the cytolytic molecules granzyme and perforin⁸⁶. Studies over the past 15 years have identified the existence of a memory subset localised to largely to tissues, which were designated as tissue resident memory (T_{rm}) cells⁸⁷. They are typically classified as CD69⁺CD103^{+/+}CD45RA⁻CCR7⁻ and can improve protection from secondary infections

at barrier sites and increase the rate of clearance of viral, bacterial and parasitic infections of mucosal tissues and the skin^{87, 88}.

Temra are a subset of memory CD8⁺ T cells, named due to their reexpression of CD45RA⁸⁹ in the absence of CD27. Temras express high levels of cytotoxic molecules but are terminally differentiated and have the shortest telomeres of all human T cell subsets⁹⁰. Markers of senescence such as KLRG1 and CD57 can be used to differentiate these cells from others^{91,92, 93}. Functionally, Temras have decreased proliferation, defective mitochondrial function but increased secretion of TNF and IFN γ , high cytotoxic activity, and elevated p38 MAPK signaling^{94, 95}. Temras increase in frequency in response to repeated stimulation in situations of chronic viral infection. Stem cell memory T cells are a long-lived population, reported to have a stem-cell like ability to self-renew and differentiate into several memory and effector populations^{96,97}. This rare population can be characterised as CD45RA⁺CCR7⁺CD27⁺ CD28⁺CD62L⁺IL7R⁺ and distinguished from naive T cells by expression of CD95, CD122, CXCR3, and/or LFA-1.

As research continues and single cell datasets grow in size and granularity, more subsets are being identified, with recent findings including virtual memory T cells⁹⁸ and a NKG2C⁺GZMB⁺CD8⁺ subset⁹⁹ that declines with age. While research in this area continues it is important to note that many of the qualities that we use to separate T cells into these subsets, actually exist on a continuum⁸³ and the further subdivision of T cells into progressively smaller subsets may not provide new biologically meaningful information.

1.6 T cell responses to viruses

1.6.1 Importance of T cells in viral infections

Early studies demonstrating the importance of T cells in viral infection largely focussed on mouse models such as LCMV infection and found that CD8⁺ cytotoxic T cells are required for the elimination of virus from acutely infected mice¹⁰⁰. Kyburz and colleagues demonstrated cytolytic activity of CD8⁺ T cells *in vivo* as when mice were treated with anti-CD8 mAbs cell internal viral proteins were not released into the serum as infected cells were not lysed in the absence of CD8 T cells.¹⁰¹ Additionally, Butz and colleagues

demonstrated expansion of LCMV-specific CD8⁺ T cells that secrete IFN γ during infection¹⁰² and likely play a role in viral clearance.

In humans, directly studying the importance of T cells in viral infections, while accounting for the effect of antibodies, has been challenging. However, a recent study elegantly demonstrated the importance of T cells in orthoflavivirus infection in a human context¹⁰³. Using the live attenuated yellow fever vaccine (YF17D) which has previously been shown to induce low/no levels of cross reactive antibodies to the YF17D and chimeric Japanese encephalitis–YF17D (JE/YF17D) vaccine^{104, 105}, Kalimuddin and colleagues investigated the capacity of cross reactive T cells to protect against JE/YF17D challenge. They found that despite not inducing neutralizing antibodies, YF17D vaccination was able to control JE/YF17D viral replication, suggesting the importance of T cells in protection. Additionally they found that the vaccine-induced T cell response to the capsid protein was associated with viral control¹⁰³. Rare human studies such as this, combined with extensive animal work have demonstrated the fundamental role that T cells can play during viral infections.

1.6.2 Influenza virus and influenza-specific T cell responses

Influenza viruses are enveloped negative-sense single stranded RNA viruses that have a segmented genome and belong to the *Orthomyxoviridae* family. There are three genera of influenza virus (A, B and C) that cause human disease, with influenza A and B being the most clinically important¹⁰⁶. Influenza A and B viruses have eight RNA segments encoding for their proteins including the surface proteins haemagglutinin (HA) and neuraminidase (NA) and the internal proteins matrix protein M1 and viral nucleoprotein (Fig 1.5A). Influenza A viruses (IAV) are classified into subtypes based on the antigenicity of their HA and NA. Symptoms associated with infection include fever, runny nose, sore throat, headache and muscle pain with severity of influenza infection ranging from mild to fatal. Annual epidemics of influenza can result in as many as 1 billion infections globally, which leads to between 3-5 million severe cases and 300,000 to 500,000 deaths¹⁰⁷.

The major targets for antibody responses, induced both by natural infection and vaccination are HA and NA¹⁰⁷. However, they are subject to both antigenic drift and antigenic shift. Antibodies targeting the head domain of HA, which is the immunodominant B cell epitope, are often neutralising, as they block receptor binding and thereby prevent infection of host cells. Although these antibodies are long lasting,

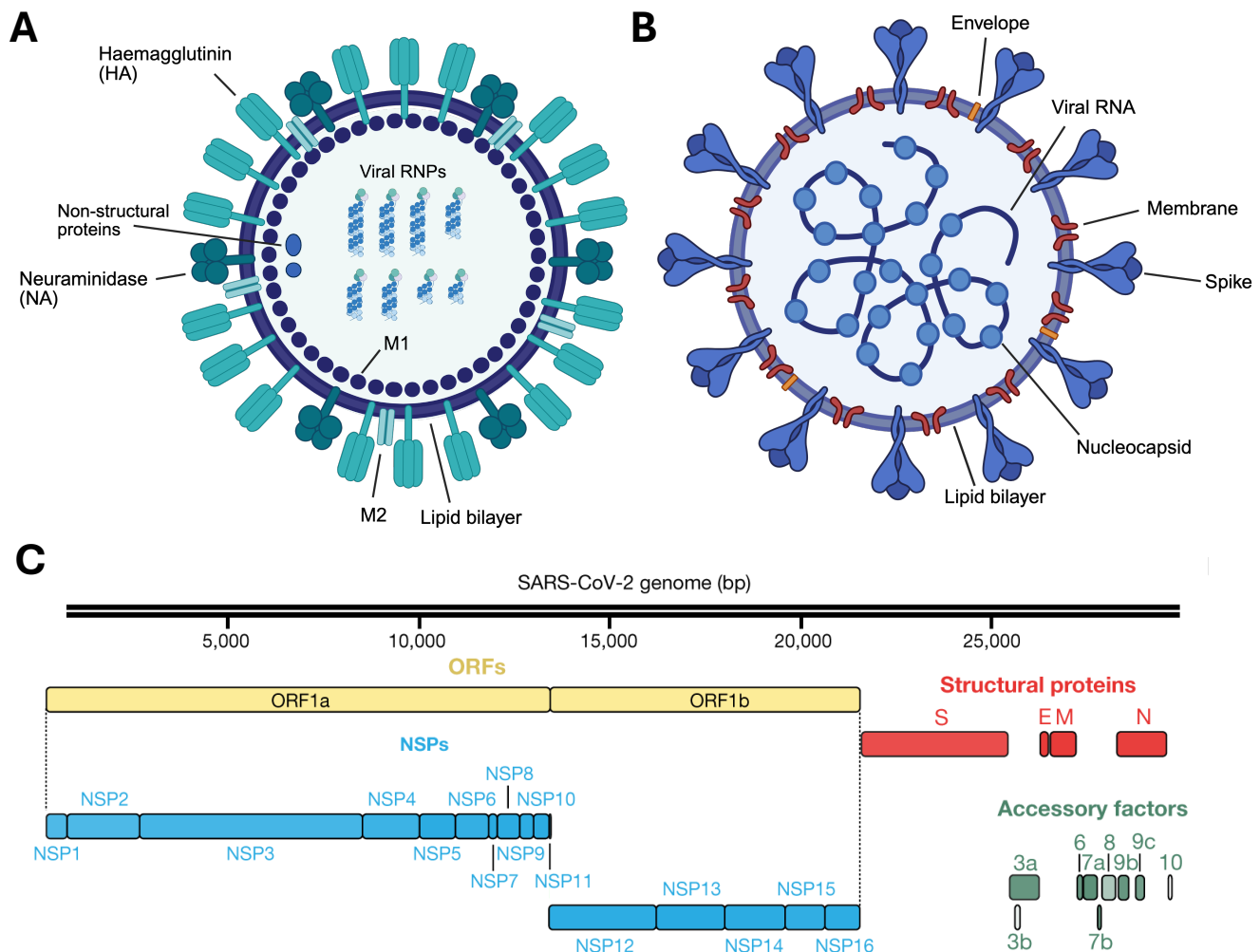


Figure 1.5: Structure of influenza and SARS-CoV-2 viruses.

(A) Influenza virus structure. The IAV genome is divided into eight segments that encode 11 viral proteins. Viral RNPs include RNA, nucleoprotein and the RNA polymerase complex (PB1, PB2, PA). Figure made using Biorender. **(B)** Structural elements of the SARS-CoV-2 virus, including the spike protein, envelope, membrane, and internal components including the viral single-stranded RNA and nucleocapsid proteins. Figure made using Biorender. **(C)** SARS-CoV-2 genome components including genes encoding NSPs, structural proteins and accessory factors. Adapted from Gordon et al., 2020 with permission from Springer Nature. Abbreviations: RNP, ribonucleoprotein; NSP, non-structural protein; S, spike; E, envelope; M, membrane; N, nucleoprotein.

the high mutational rate of the HA head domain^{108, 109} means that these antibodies are usually not cross-reactive to other strains of influenza. In addition to neutralising strain-specific antibodies, natural influenza infection can also induce antibodies against conserved epitopes of viral proteins, including the membrane proximal stalk of HA^{110, 111} and the extracellular domain of the M2 protein¹¹², but levels of these antibodies are low, limiting their potential to provide complete protection.

The limited ability of influenza-specific antibodies to provide heterosubtypic protection highlights the importance of T cell mediated immunity against influenza. In an early study, Schulman and Kilborne infected mice with H1N1 strain and then challenged with a lethal H2N2 virus and observed a reduction in viral titres in the lung, lung pathology and mortality in mice that had been previously exposed to the H1N1 strain¹¹³. Despite a lack of antibodies, the authors observed an early reduction in viral titres, which we now know is likely due to T cell mediated immunity¹¹⁴. This finding has been reproduced in other animal models, including non-human primates, where reductions in viral load, lung pathology, and mortality have been observed, although infection rates remain unchanged^{115, 116}. Throughout the 1990s several studies used mice deficient in B cells found that memory CD8⁺ T cells, established by an initial infection, mediated accelerated viral clearance and reduced pathology during a challenge^{117, 118}.

Although animal studies provide us with evidence of T cell involvement in influenza protection, obtaining this same evidence in the human setting is logistically challenging. Nonetheless, opportunistic epidemiological observations suggest that heterosubtypic protection also occurs in humans. Using historical data from the 1957 H2N2 pandemic, a study found that adults with lab-confirmed H1N1 influenza in 1950-1957 were approximately 3 times less likely to have symptomatic laboratory-confirmed pandemic H2N2 influenza compared to those not previously infected^{114, 119}, while not having any detectable neutralizing antibodies to the H2N2 virus, suggesting T cells were mediating protection. Cytotoxic T cells from PBMCs stimulated *in vitro* with IAV can lyse target cells infected with different IAV subtypes, due to their targeting of cross-reactive antigens^{120, 121}. As research progressed, the field aimed to identify which parts of the influenza virus T cells could recognise. Several studies found that M1, NP and the PB proteins are the most common targets for T cells^{122, 123}. To date 335 CD8⁺ T cell epitopes from IAV have

been identified (Immune epitope database). Of these identified IAV-specific responses, the HLA-A*02:01-restricted M1₅₈₋₆₆-specific CD8⁺ T cell response, is one of the most immunodominant, well-studied and is of particular interest to this thesis¹²⁴. These M1₅₈₋₆₆ specific cells have been found in the lungs of IAV-infected individuals¹²⁴ and were shown to be protective against influenza in an HLA-A2/K(b) transgenic mouse model¹²⁵.

The 2009 H1N1 pandemic provided a unique opportunity to explore the association of T cells with clinical outcomes in a naturally exposed study population lacking protective antibody responses¹²⁶. Following a cohort of seronegative H1N1 individuals during the pandemic, Sridhar and colleagues found that people with higher frequencies of preexisting CD8⁺IFN γ ⁺IL-2⁻ T cells specific for conserved epitopes from PB1, M1 and NP has milder symptoms and reduced viral shedding following infection with pandemic H1N1¹²⁷. A second study showed that the presence of IFN γ ⁺ NP-specific T cells correlated with a decreased risk of viral shedding¹²⁸.

CD4⁺ T cells also play an important role in the immune response against influenza virus^{129, 130}. A human challenge study in individuals without detectable humoral immunity to the challenge strains found that preexisting CD4⁺ T cells specific for internal influenza proteins were associated with lower virus shedding and less severe illness¹³¹. Together, these data from animal models, *in vitro* studies and rare epidemiological observations highlight the importance of T cells in heterosubtypic immunity against influenza virus.

1.6.3 SARS-CoV-2-specific T cell responses

SARS-CoV-2 is a single-stranded, positive-sense RNA virus of the coronavirus family¹³². The genomic RNA of SARS-CoV-2 encodes for four structural proteins (spike, envelope, membrane and nucleoprotein) and several non-structural and accessory proteins (Fig 1.5B-C)^{132, 133}. The virus spread rapidly around the world in early 2020¹³⁴ and was declared a pandemic by the World Health Organisation (WHO) in March 2020. As of December 2025, there have been 779 million confirmed infections, and 7.1 million deaths attributed to SARS-CoV-2 (WHO, COVID-19 Dashboard).

Upon the emergence of SARS-CoV-2, the scientific community sprung to action to determine features of the virus and host response, which successfully lead to the

development of vaccines that allowed the world to re-open following lockdowns. Many groups studied the T cell response to the virus and identified regions of the virus, and subsequently individual epitopes that were targeted by CD4⁺ and CD8⁺ T cells^{135, 136, 137, 138, 139}. These studies identified epitopes targeted by T cells within the structural proteins spike and nucleoprotein, as well as within the ORF proteins, particularly ORF3a and ORF1^{136, 137, 138}. Researchers also investigated the association of T cells with disease severity in these patient cohorts^{136, 140}; Peng and colleagues found that patients with mild disease had higher proportions of SARS-CoV-2 specific CD8⁺ T cells. Additionally, a longitudinal study with sampling points throughout infection, found that patients with mild disease and rapid viral clearance had early induction of functional SARS-CoV-2-specific T cells¹⁴¹.

Further investigation also identified that T cells specific for individual epitopes were associated with infection outcome; a study demonstrated that the immunodominant NP_{105–113}-specific CD8⁺ T cell response is associated with mild COVID-19 disease¹⁴². However, higher frequencies of T cells responding to ORF3a-derived peptides were found in patients with severe disease compared to those with mild disease¹³⁶. In particular, the ORF3a_{207–215}-specific CD8⁺ T cell response has been associated with severe disease (Dong lab, unpublished). Questions remain about what features of these ORF3a_{207–215}-specific CD8⁺ T cells may contribute to their association with severe disease.

1.6.4 Immunopathology caused by T cells in viral infections

Although T cells play a fundamental role in helping the host recover from viral infections, they can sometimes cause problems themselves. Immunopathology, caused by the overproduction of cytokines and cytotoxic molecules, can contribute to sustained inflammation, tissue injury, remodelling and fibrosis. In some non-cytopathic virus infections, including hepatitis B virus (HBV), a significant portion of the damage is caused by CD8⁺ T cells killing liver cells rather than via direct viral effects^{143, 144}. Immunopathology during LCMV infection in mice includes pulmonary oedema, increased bronchus-associated tissue, interstitial lung infiltrates and liver damage. Using T cell deficient

mice, Cornberg and colleagues demonstrated that this pathology is largely caused by T cell activity rather than the virus itself¹⁴⁵.

Memory T cells can be activated by ‘bystander activation’, a TCR independent mechanism of activation, requiring proinflammatory signals provided by cytokines such as IL-12, IL-15 and type I IFN^{146, 147}. A recent study found that the transcriptional signature of bystander-activated CD8⁺ T cells was enriched in patients with mild disease, early on in SARS-CoV-2 infection but that patients with severe disease had delayed and sustained bystander activated signatures¹⁴⁸, suggesting that this excessive non-specific T cell cytotoxicity could be contributing to immunopathology.

Studies using spatial transcriptomics looking at immune infiltration in the lungs in patients who died of severe COVID-19 have also shed light on the role of T cells in immunopathology^{149, 150}. One study found enriched TCR signalling and cytotoxicity pathways in areas of pathology in the lung. This led to the hypothesis that in severe COVID-19 the T cell activation in the lung may be spatially removed from the virus and that these activated CD8⁺ T cells may remain and cause damage after viral clearance¹⁵⁰. This thinking is supported by another study which concluded that in fatal COVID-19 “virus-independent immunopathology, rather than direct viral cytotoxicity” was a primary pathogenic mechanism in the lung¹⁵¹. However, further work is required to identify the factors that differentiate protective T cell responses that help to clear viral infection from those that contribute to immunopathology.

1.7. Factors affecting T cell functionality

1.7.1 TCR affinity, avidity and T cell functional avidity

Of the many factors that affect T cell functionality the biophysical properties of the TCR are considered to be some of the most important. TCR affinity is generally defined as the interaction between a single TCR molecule and peptide-MHC¹⁵². The strength of this interaction is largely determined by the electrostatic forces between amino acid residues of the TCR molecule and pMHC. TCR affinity is measured by surface plasmon resonance and is represented by the equilibrium dissociation constant. TCR avidity takes into consideration some of the other factors that influence the interaction between T cell and

pMHC; it is a measure of the strength of multiple TCR-pMHC interactions and the effect of molecules such as the CD8 coreceptor¹⁵². The CD8 coreceptor stabilises the interaction between TCR and pMHC, is required for T cells with low affinity TCRs to function¹⁵³ and can modulate the functional sensitivity of antigen recognition.

Unlike the biophysical measurements, T cell functional avidity is a biological measure of T cell activity that defined by the T cell response to a cognate peptide at a range of concentrations¹⁵⁴. This is usually determined using an ELISpot assay and provides additional information to affinity and avidity as it gives insight into how a T cell actually functions in response to its antigen. Factors thought to affect functional avidity include TCR affinity, co-receptor expression, balance of costimulatory/coinhibitory receptors and metabolism of the T cell.

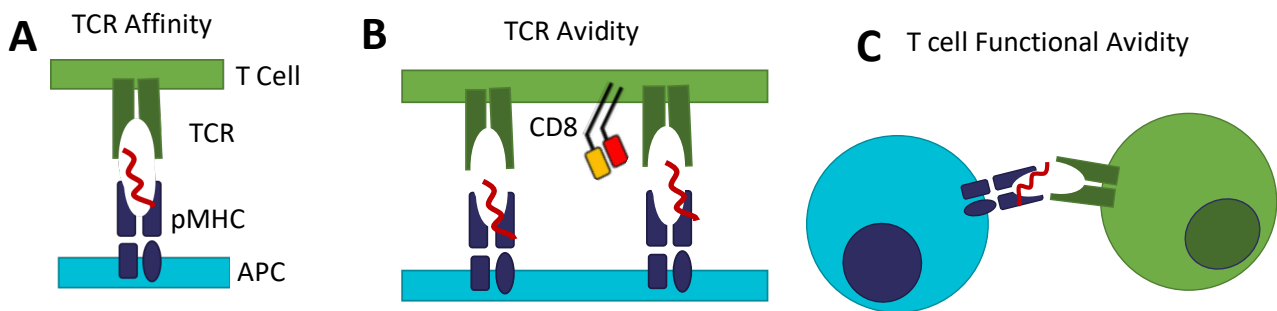


Figure 1.6: TCR affinity, TCR avidity and T cell functional avidity.

(A) TCR affinity is defined as the interaction between a single TCR molecule and pMHC. (B) TCR avidity is a measure of the strength of multiple TCR-pMHC engagements and considers the effects the CD8 co-receptors. (C) T cell functional avidity is a biological measure that defines the extent to which a T cell responds to its peptide epitope at a range of concentrations and is usually determined by measuring IFN γ (or other cytokine) secretion. Abbreviations: TCR, T cell receptor; pMHC, peptide major histocompatibility complex; IFN, interferon.

1.7.2 Balance of costimulatory and coinhibitory receptors

T cell co-signalling receptors are defined as cell-surface receptors that regulate TCR derived signals, either positively (costimulatory receptors) or negatively (coinhibitory receptors), and thus influence T cell activation and function^{155, 156}. These receptors are members of several different molecular families, including the immunoglobulin superfamily and tumour necrosis factor receptor superfamily¹⁵⁶. Initially T cells were

thought to express either co-stimulatory receptors or co-inhibitory receptors, acting like an on/off switch to determine whether TCR signalling would have a functional effect. T cells are now understood to express both families of co-signalling molecules at once¹⁵⁷, with the balance of signals received from each, determining the outcome of TCR signalling. Costimulatory receptors, such as CD28, provide signal 2 required for T cell activation (see section 1.3.2). The development of checkpoint inhibitors, targeting the coinhibitory molecules CTLA-4 and PD-1^{158, 159}, and their revolution of cancer treatment, highlights the importance of understanding exactly how the plethora of cell surface molecules impact T cell functionality.

1.7.3 Metabolism

The importance of T cell metabolism has long been appreciated with research into it beginning as early as the 1960s¹⁶⁰ when Hedeskov and colleagues looked into the metabolism of T cells in the resting state. It is now understood that T cells are an extremely metabolically flexible immune cell, utilising different metabolic pathways depending on their differentiation status (Fig 1.7)^{161, 162}.

Naïve T cells have relatively low metabolic demands and primarily utilise oxidative phosphorylation (OXPHOS) for their energy production. OXPHOS is a highly efficient metabolic pathway which occurs in the mitochondria and couples the oxidation of nutrients such as glucose to the phosphorylation of ADP to ATP⁸². Upon activation, naïve T cells undergo a significant metabolic shift^{163, 164, 165} to primarily utilise glycolysis, which takes place in the cytoplasm and breaks glucose down into pyruvate. Activated T cells make this switch to glycolysis, as they require rapid production of ATP from glucose to fuel their increased energy needed to proliferate and produce effector molecules⁸². Signals from the costimulatory molecule CD28 and the cytokine IL-2 promote^{166, 167} this switch through AKT driven activation of the mTOR pathway^{168, 169}. TCR stimulation increases the trafficking of Glut1, a key glucose transporter, to the cell surface¹⁶⁷, allowing activated cells to take up more glucose for glycolytic metabolism. In addition to supporting T cell metabolism, several glycolytic enzymes directly influence effector function. Lactate dehydrogenase promotes effector gene expression via histone acetylation, and its loss prevents acetylation at promoters for genes such as *IFNG*^{161, 170}.

¹⁷¹. However, glucose alone cannot meet the energetic demands of effector T cells. Glutaminolysis is also essential, and in the absence of glutamine, T cells fail to proliferate^{172 173, 174}.

Instead of glycolysis, memory CD8+ T cells use long-chain FAO as a primary mechanism of energy generation. This dependence on FAO in memory cells is thought to be mediated by TRAF6⁸⁰ and IL-15 signalling¹⁷⁵. IL-15 signalling increases expression of the enzyme CPT1A¹⁷⁶ which transports long chain fatty acids into the mitochondria, allowing for FAO to occur.

The interplay between metabolism and T cell functionality has been studied extensively in cancer, where functional and metabolically flexible tumour-infiltrating T cells are

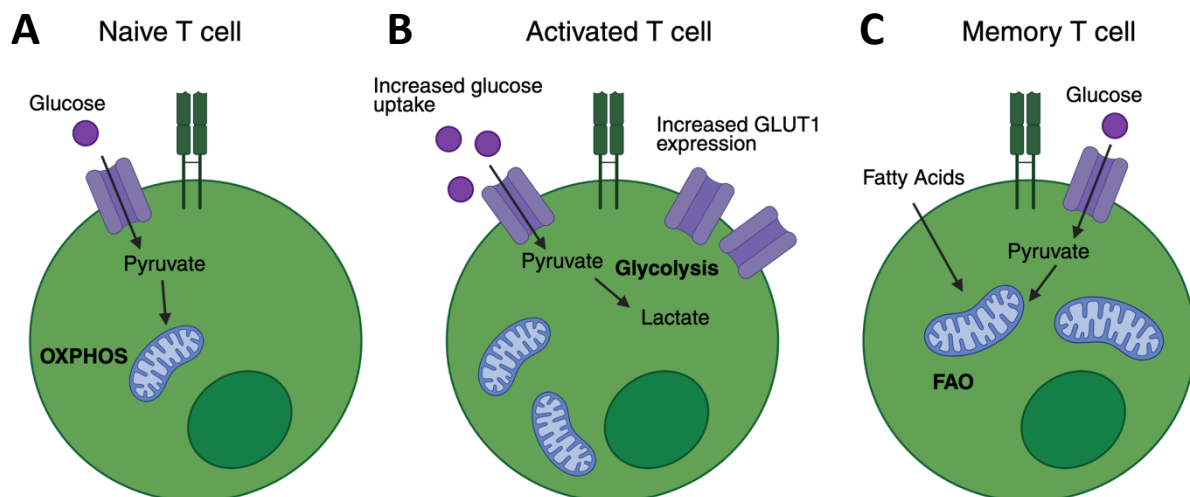


Figure 1.7: T cell metabolism.

(A) Naive T cells generally have low metabolic demand and largely rely on OXPHOS to fulfil their metabolic demands. OXPHOS takes place in the mitochondria where electrons are transferred from NADH and FADH₂ to proteins involved in the electron transport chain. At the end of this process ADP is phosphorylated, allowing for the generation of ATP **(B)** When they are activated T cells undergo a rapid shift in their metabolic pathway usage. They switch to predominantly using aerobic glycolysis, which occurs in the cytoplasm and involves the conversion of glucose to pyruvate. Although glycolysis generates less ATP than OXPHOS, it can generate it very quickly which allows activated T cells to execute their effector function. Activation via the TCR increases surface expression of the glucose transporter GLUT1 which facilitates increased glucose uptake. **(C)** Memory T cells predominantly utilise by β -oxidation of fatty acids (FAO) to fuel their relatively low energy demands. Memory T cells have increased mitochondrial mass. Figure made in Biorender. Adapted from Montironi et al., 2021, under CC-BY license. Abbreviations: OXPHOS, oxidative phosphorylation; NADH, nicotinamide adenine dinucleotide; FAD, flavin adenine dinucleotide; ATP, adenosine triphosphate; ADP, adenosine diphosphate; TCR, T cell receptor; FAO, fatty acid oxidation, GLUT1, glucose transporter type 1.

advantageous and similar findings have been observed in the context of viral infection^{177, 178, 179}. In a study of CD8⁺ T cells from HIV (human immunodeficiency virus) patients, Angin and colleagues found that HIV-specific T cells from HIV non-controllers were largely dependent on glucose, while those from HIV controllers utilised more diverse metabolic pathways that enhanced their effector function and survival¹⁸⁰. Additionally functionally exhausted HBV-specific T cells have impaired ability to utilise OXPHOS, relying more heavily on glycolysis¹⁸¹. These data suggest that full effector function of virus-specific T cells relies on the flexibility to utilise energy from both OXPHOS and glycolysis.

1.7.4 Integrins

The pioneering work of Richard Hynes, Erkki Ruoslahti and Timothy Springer in the 1980s led to the discovery of the cell adhesion machinery of cells – integrins^{182, 183}. Integrins exist as heterodimers of α and β subunits, of which humans express 18 and 8 subunits, respectively, which combine to form 24 different integrin heterodimers (Fig 1.8)^{184, 185, 186}. These subunits are type I transmembrane proteins with large extracellular domains, single pass transmembrane domains and short cytoplasmic tails^{185, 187}. The extracellular regions of integrins differ between the α and β subunits. The α subunits have four extracellular domains: a seven-bladed β -propeller, a thigh, and two calf domains, with half of the α subunits having an additional 200 amino acid domain inserted into the β -propeller¹⁸⁸. The β subunits consist of seven extracellular domains, which can be divided into a head region and a leg/stalk region¹⁸⁹. Integrin heterodimers have overlapping but non-redundant functions, with ligand specificity and signalling determined by their pairing. Ligands for these integrins vary, from extracellular matrix proteins (ECM) such as fibronectin and collagen, to cell surface receptors such as intracellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1)¹⁸⁵ which are members of the immunoglobulin superfamily.

Integrins play fundamental roles in adhesion of many cell types in our bodies, but are particularly important in immune cells, mediating their ability to move throughout the body, crossing tissue boundaries to protect us from pathogens. The importance of integrins in immune cell migration and function is demonstrated by the fact that

mutations in the $\beta 2$ subunit cause leukocyte adhesion deficiency type 1, in which patients suffer from recurrent bacterial infections and impaired wound healing^{184, 190}.

Much of the initial research into the function of integrins on T cells focussed on their role in mediating extravasation from blood vessels to tissues. Integrins can exist in inactive, primed and active conformations. While T cells are in circulation, their integrins exist in the low affinity conformation. Chemokines or other signals on the endothelial surface of blood vessels, can convert LFA-1 (and other integrins) into its primed conformation, allowing interaction with ICAM-1 on the endothelial cells^{184, 191}. This supports T cell arrest, followed by extravasation through blood vessel walls and into tissues. Through this mechanism, distinct integrin heterodimers help to dictate tissue specificity: $\alpha 4\beta 1$ and $\alpha 4\beta 7$ can bind VCAM-1 to support entry into inflamed peripheral tissues¹⁹² while $\alpha 4\beta 7$ can interact with MAdCAM-1 (Mucosal Vascular Addressin Cell Adhesion Molecule 1) on gut-associated endothelium to direct lymphocyte homing to the intestinal mucosa¹⁹³.

The signalling of integrins is tightly intertwined with that of other receptors. Stimuli received from the TCR, or chemokine receptors can generate intracellular signals via small GTPases, and adaptor molecules, such as talin-1 and kindlin-3 that bind to the cytoplasmic tails of β integrins and induce conformational changes in the integrin molecules. This converts integrins from their low affinity, bent conformation to the extended or “primed” conformation^{194, 195, 196}. This is known as ‘inside-out’ signalling. With this extended conformation, the integrins have increased affinity for their ligands. Upon ligand binding, ‘outside in’ signalling is triggered, in which adaptor proteins are recruited to the cytoplasmic tails of the integrins. This results in actin remodelling and downstream signalling that can modulate T cell adhesion, motility and function¹⁹⁴.

As research into integrins has continued, an appreciation of their effects on T cell biology, aside from migration and adhesion, has grown. As discussed in section 1.3.3, LFA-1 also functions in stabilising the immune synapse, acting as a costimulatory receptor and more recently has been shown to function as a spatial cue to attract granules containing the cytotoxic proteins perforin and granzyme to the plasma membrane where they can be released¹⁹⁷. There have also been studies suggesting that $\alpha 4\beta 1$ and $\alpha 4\beta 7$ can influence T cell function^{198, 199} and these integrins have been implicated in the

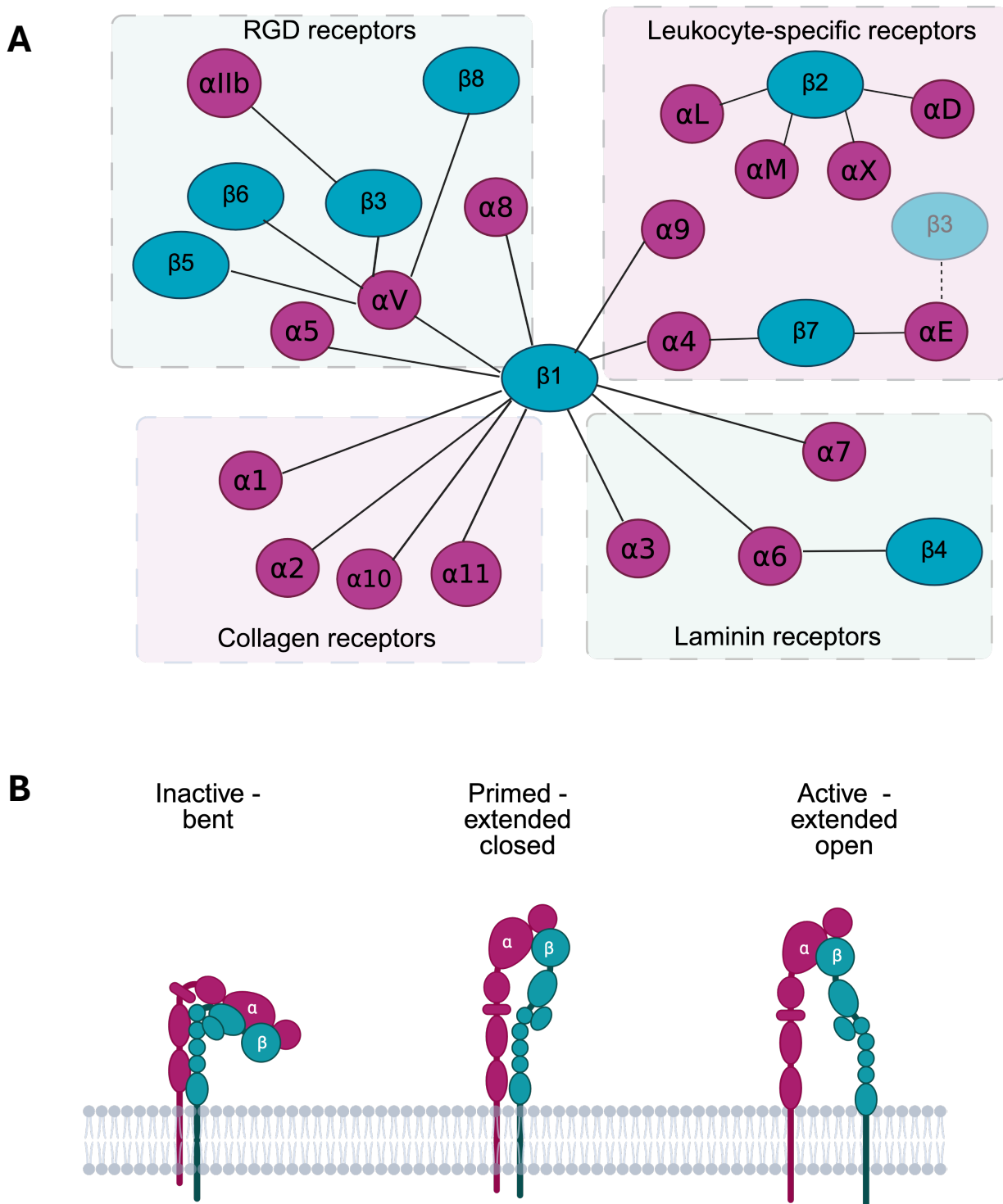


Figure 1.8: Integrin pairings and structure.

(A) Integrin α and β pairings and their categories of ligands. There are 24/25 integrin heterodimers in humans, which can be divided into 4 categories according to their receptors. Categories are RGD receptors, leukocyte-specific receptors, collagen receptors, and laminin receptors. $\alpha\text{E}\beta 3$ is a recently identified unconventional pairing with the ligand E-Cadherin. Figure made in Biorender. Adapted from Zhang et al., 2023, under CC-BY license. **(B)** Structural studies have shown that integrin heterodimers exist in several distinct conformations: a bent conformation which has low affinity for ligands, an extended conformation with closed headpiece which has intermediate affinity for ligands and a high affinity conformation in which the heterodimer is extended and has an open headpiece. Figure made in Biorender. Abbreviations: RGD, Arg- Gly-Asp.

pathogenesis of inflammatory diseases. However, despite clinical targeting^{200, 201}, questions remain about the functional effects of other integrins, such as $\alpha 4\beta 1$ and $\alpha 4\beta 7$, on T cell biology.

1.7.5 Ageing

Research into the impact of ageing on T cells began in the 1960s. Early *in vitro* studies showed stimulation of human lymphocytes from aged individuals stimulated with phytohemagglutinin (PHA), resulted in reduced T cell proliferation^{202, 203}. Research using mouse models showed numerical and functional decline in T cells from aged mice^{204, 205}. Investigating disease specific contexts, studies found decreased magnitude and delayed kinetics of the T cell response in response to influenza and *Mycobacterium tuberculosis* in aged mice^{206, 207}.

Since these initial studies research into the effects of ageing on T cells has progressed and a recent review summarised the effects into 10 hallmarks of T cell ageing⁹⁵ (Fig 1.9). Thymic involution, the age-related shrinkage of the thymus, begins in childhood, peaks during puberty and continues throughout life²⁰⁸. Thymic involution, marked by changes in tissue architecture, increased adipocytes²⁰⁹, and reduced thymic mass, result in fewer thymocytes and a consequent reduction in naïve T cell output⁹⁵. Together with a compensatory clonal expansion of memory T cells, this leads to reduced diversity of the TCR repertoire. Detailed analysis of TCR sequences from naïve T cells has revealed age-related changes in the TCRs generated in the thymus, with Egorov and colleagues demonstrating a gradual reduction in TCR β CDR3 length and non-templated N nucleotide additions, along with significant alterations in the physicochemical properties of the central CDR3 loop²¹⁰.

Mitochondrial dysfunction is a common feature of many ageing cells. Although T cells from older individuals contain higher levels of mitochondrial proteins they exhibit altered mitochondrial morphology^{211, 212} and impaired oxidative phosphorylation, indicating an accumulation of dysfunctional mitochondria²¹³. This mitochondrial dysfunction in aged T cells, not only affects their metabolic capacity but also contributes to an acquisition of a proinflammatory phenotype. The accumulation of inflammatory metabolites and release of mitochondrial DNA into the cytoplasm which can activate cGAS-STING

pathway, resulting in activation of the inflammasome and activation of genes for several proinflammatory cytokines contributes to the functional alteration of aged T cells^{95, 214}.

T cells, like most other cells in the body, undergo an age-related reduction in telomere length²¹⁵. This telomeric attrition is usually accompanied with cellular senescence²¹⁶ which in the context of T cells largely refers to low proliferative activity. Senescent T cells are generally characterised by cell surface expression pattern CD27⁻CD28⁻CD45RA⁺ KLRG1⁺ CD57⁺^{216, 217}. The frequency of senescent T cells increases with age, with chronic CMV infection further enhancing accumulation of senescent T cells²¹⁸. Senescent T cells lose their metabolic flexibility due to profound mitochondrial dysfunction which causes them to rely solely on glycolysis for their effector function, meaning there is little energy left for proliferative capacity²¹⁶. Sestrin-induced stress sensing can cause senescent T cells to lose signalling capacity through their TCR and instead kill using a protein complex containing the NK receptor NKG2D and the NK adapter molecule DAP12²¹⁹. Overall, with age, senescent T cells that have reduced immune function but heightened proinflammatory activity accumulate, thereby accelerating inflammaging⁹⁵.

In recent years, much of the research on immune system ageing has focused on generating large, multi-donor single-cell RNA-sequencing datasets to profile transcriptomic changes in immune cell subsets across the human lifespan^{99, 220, 221}. These large studies have identified age-associated changes in T cell populations, such as the accumulation of GZMK⁺CD8⁺ T cells and the loss of NKG2C⁺GZMB⁻CD8⁺ memory T cells with age⁹⁹. Although these datasets are useful in furthering our understanding of T cell ageing, they largely do not contain information on the antigen-specificity of the T cells so cannot be used to address the question of how antigen-specificity affects the ageing trajectory of T cells. The increased susceptibility of older individuals to severe disease from respiratory viral infections including influenza and SARS-CoV-2 highlights the need to be able to address these questions in an antigen-specific manner.

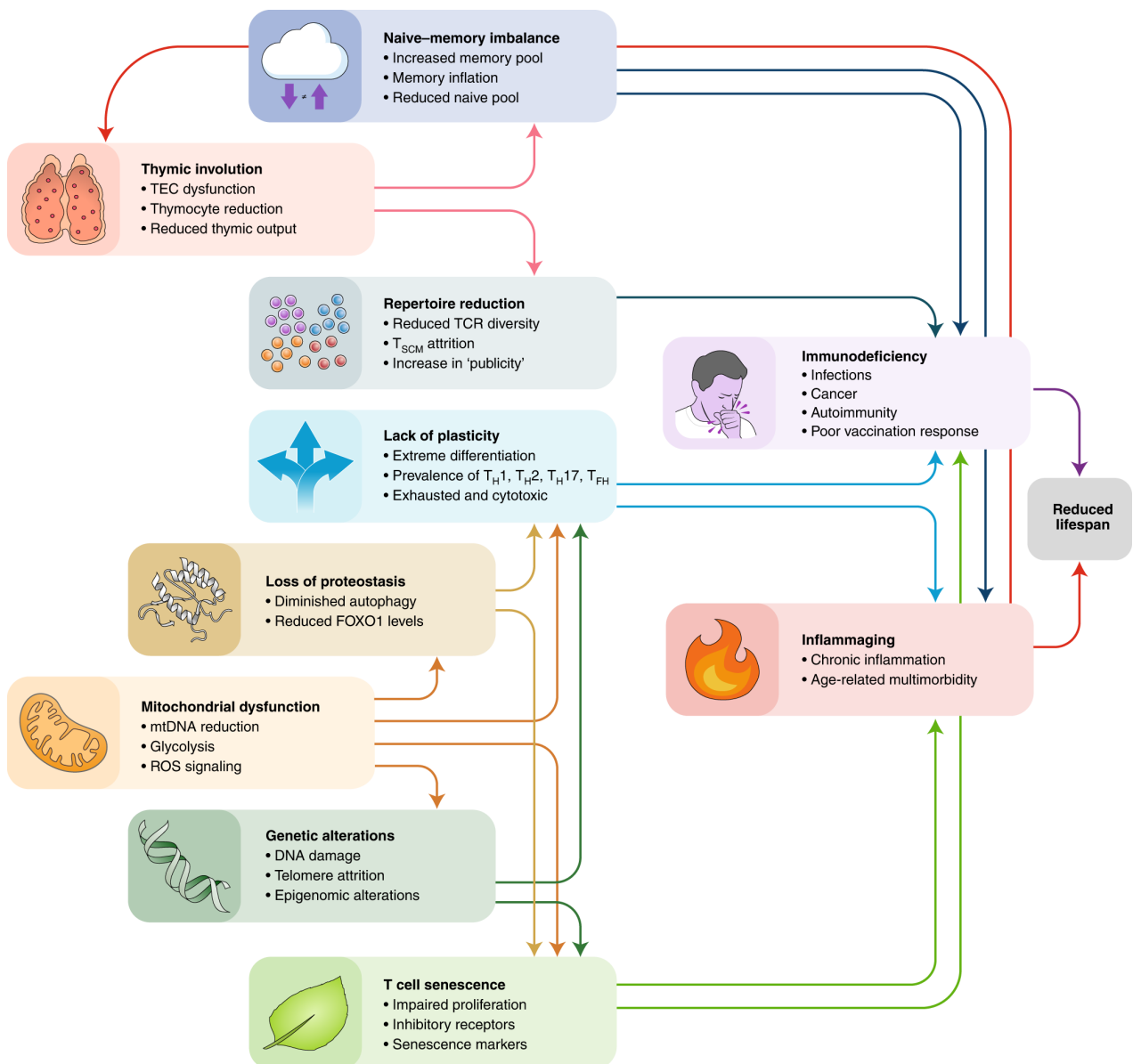


Figure 1.9: Hallmarks of T cell ageing.

The 10 hallmarks of T cell ageing are divided up into four primary hallmarks (thymic involution, genetic alterations, loss of proteostasis and mitochondrial dysfunction) which are the causes of the damages; four secondary hallmarks (repertoire reduction, naïve-memory imbalance, T cell senescence and lack of plasticity) which are the consequences of the age-related changes and two integrative hallmarks of inflammaging and immunodeficiency. Characteristics of each hallmark are listed, and the most important connections between the hallmarks are indicated by arrows. Figure reprinted from Mittelbrun et al., 2021, with permission from Springer, Nature. Abbreviations: TEC, thymic epithelial cells, TCR; T cell receptor; ROS, reactive oxygen species; Tscm, stem cell memory T cell.

1.8 Aims of this thesis

With the overall aim of investigating factors affecting virus-specific CD8⁺ T cell function, this thesis focuses on T cell responses against the respiratory viruses, influenza A virus and SARS-CoV-2. The research in this thesis stemmed from two distinct questions:

- How does antigen specificity shape the ageing trajectory of CD8⁺ T cells? For this question we focus on the immunodominant influenza M1₅₈₋₆₆-specific T cell response.
- Which features of CD8⁺ T cells contribute to their association with severe disease and immunopathology? To address this, we focus on SARS-CoV-2 ORF3a₂₀₇₋₂₁₅-specific T cell response which has previously been associated with severe disease in COVID-19.

To address these questions and our overall aim, this thesis is divided into three results chapters:

Chapter 3: Profiling age-related changes in influenza M1₅₈₋₆₆-specific CD8⁺ T cells

In this chapter, we perform single-cell RNA sequencing of samples from 3 individuals over a 19-year period with the aim of investigating age-associated changes in both transcriptomic profiles and TCR gene usage in M1₅₈₋₆₆ specific T cells. To determine how antigen-specificity affects the ageing trajectory of these cells, we compare M1₅₈₋₆₆-specific T cells to the broader CD8⁺ T cell population and to T cells specific for epitopes from the chronic viruses CMV and EBV.

Chapter 4: Functional and molecular profiling of SARS-CoV-2 ORF3a₂₀₇₋₂₁₅-specific CD8⁺ T cells associated with severe disease

In this chapter, we identify pairs of ORF3a₂₀₇₋₂₁₅-specific T cell clones with identical TCR sequences but differences in their functionality. We use the highly functional clones as an extreme example of these ORF3a₂₀₇₋₂₁₅-specific T cells that potentially contribute to association with severe disease. To investigate features of these T cells we perform proteomics and RNA-sequencing of the clones with and without stimulation.

Chapter 5: Functional effects of α 4 integrins on CD8⁺ T cells

Having identified higher expression of integrins on the highly functional ORF3a₂₀₇₋₂₁₅-specific T cells in Chapter 4, we utilise *in vitro* functional assays and immune synapse imaging to investigate how the integrins $\alpha 4\beta 1$ and $\alpha 4\beta 7$ influence T cell functionality and whether the expression of these integrins could be related to the association of ORF3a₂₀₇₋₂₁₅-specific T cells with disease severity.

2. Materials and methods

2.1 Antibodies and reagents

Target	Clone/Tetramer specificity	Fluorophore/D NA Barcode	Supplier	Catalogue number	Used for
CD107a	H4A3	PE	BD	555801	ICS
IFN γ	B27	AF488	BD	557718	ICS
TNF	MAB11	APC	Invitrogen, eBioscience	17-7349-82	ICS
CD8	SK1	BV421	Biolegend	344748	Flow cytometry
CD8	SK1	APC	BD	345775	Flow cytometry
CD8	SK1	FITC	Biolegend	344704	Flow cytometry
VCAM-1	STA	PE	Invitrogen, Thermo Fischer Scientific	12-1069-42	Flow cytometry
MAdCAM-1	683715	AF405	Bio-technie, R&D systems	FAB6056V	Flow cytometry
MAdCAM-1	683715	APC	Bio-technie, R&D systems	FAB6056A	Flow cytometry
MAdCAM-1	MECA-367	PE	Santa-Cruz Biotechnology	sc-19604 PE	Flow cytometry
CD3	SK7	FITC	BD	345764	Flow cytometry/sorting
Propidium iodide	N/A	Propidium iodide	Invitrogen, eBioscience	BMS500PI	Flow cytometry
7-AAD	N/A	7-AAD	Invitrogen, eBioscience	00-6993-50	Flow cytometry
CellTrace-CFSE	N/A	CFSE	Invitrogen, Thermo Fischer Scientific	C34554A	Flow cytometry
Live/dead-aqua	N/A	Aqua (405)	Invitrogen, Thermo Fischer Scientific	L34957	Flow cytometry
CD19	HIB19	BV421	Biolegend	302234	Flow cytometry/sorting
CD19	HIB19	BV510	Biolegend	302242	Flow cytometry/sorting
CD14	M5E2	BV510	Biolegend	301842	Flow cytometry/sorting
Integrin α 4/CD49d	9F10	PE	Biolegend	304304	Flow cytometry/imaging
Integrin α 4/CD49d	9F10	BB515	BD	564593	Flow cytometry
Integrin α 4/CD49d	9F10	Biotin	Biolegend	304334	Imaging
Integrin α 4/CD49d	9F10	AF647	Biolegend	304336	Imaging
Integrin β 1/CD29	TS2/16	AF488	Biolegend	303016	Flow cytometry/imaging
Integrin β 1/CD29	TS2/16	PE	Biolegend	303004	Flow cytometry/imaging
Integrin β 7	FIB504	APC	Biolegend	321208	Flow cytometry

Integrin β 7	FIB504	Biotin	Biolegend	321209	Flow cytometry/imaging
Integrin β 7	FIB504	PE	Biolegend	321204	Flow cytometry/imaging
Integrin β 7	FIB27	PE	Biolegend	121006	Flow cytometry/imaging
CD103	Ber-ACT8	FITC	Biolegend	984806	Flow cytometry
CD45RA	HI100	TCAATCCTTCC GCTT	Biolegend	304163	CITE-Seq for 10X
CD27	O323	GCACTCCTGC ATGTA	Biolegend	302853	CITE-Seq for 10X
CD57	QA17A04	AACTCCCTATG GAGG	Biolegend	393321	CITE-Seq for 10X
CD28	CD28.2	TGAGAACGAC CCTAA	Biolegend	302963	CITE-Seq for 10X
CCR7	G043F11	TCCCTTGTAGA GTAT	Biolegend	387203	CITE-Seq for 10X
CXC3R1	K0124E1	AGTATCGTCTC TGGG	Biolegend	355705	CITE-Seq for 10X
ORF3a Tetramer	HLA-A*01:01 FTSDYYQLY	APC	Proimmune	Batch: UP/7908-22	Flow cytometry/sorting
ORF3a Tetramer	HLA-A*01:01 FTSDYYQLY	PE	Proimmune	Batch: UP/7908-21	Flow cytometry/sorting
M1 tetramer	HLA-A*02:01 GILGFVFTL	PE	in-house conjugation	N/A	Flow cytometry/sorting
EBV tetramer	HLA-A*02:01 GLCTLVAML	PE	in-house conjugation	N/A	Flow cytometry/sorting
Flu Dextramer	HLA-A*02:01 GILGFVFTL	PE/TCAAGCTTA GATAGA	Immudex	WB02161DXG PE 25 FBC0872	Sorting and barcoding for 10X
EBV Dextramer	HLA-A*02:01 GLCTLVAML	PE/GACGCCAC AGCTAGG	Immudex	WB02130DXG PE 25 FBC0873	Sorting and barcoding for 10X
CMV Dextramer	HLA-A*02:01 NLVPMVATV	PE/TACGGCCG TTCTACT	Immudex	WB02132DXG PE 25 FBC0871	Sorting and barcoding for 10X
Negative control Dextramer	HLA-A*02:01 Neg. control	PE/CTACGTTGA CGAATT	Immudex	WB02666CXG PE 25 FBC0870	Sorting and barcoding for 10X
Rat IgG2a λ isotype control	G013C12	APC	Biolegend	402305	Flow cytometry
Mouse IgG1 K isotype control	MOPC-21	AF488	Biolegend	400132	Flow cytometry
Mouse IgG1 K isotype control	MOPC-21	PE	Biolegend	400111	Flow cytometry
IgG1 k isotype control	MOPC-21	GCCGGACGAC ATTAA	Biolegend	400187	CITE-Seq for 10X
IgG2a isotype control	MOPC-173	CTCCTACCTAA ACTG	Biolegend	400293	CITE-Seq for 10X
IgG2b isotype control	MPC-11	ATATGTATCAC GCGA	Biolegend	400381	CITE-Seq for 10X

Table 2.1: List of antibodies and tetramers/dextramers used for staining for flow cytometry, CITE-seq and imaging.

Protein/Antibody Target	Clone	Supplier	Catalogue number
anti-CD3/CD28	Immunocult	Stemcell technologies	10971
anti-CD3	UCHT1 Fab, His tagged	Made in house	Made in house
anti-CD3	OKT3	Invitrogen	16-0037-81
anti-CD28	CD28.2	Invitrogen	13-0289-82
Natalizumab (anti-α4β1 and anti-α4β7)	Hu144	R&D systems	MAB10603
Abrilumab (anti-α4β7)	Abrilumab	Invitrogen, Thermo Fischer Scientific	MA5-42028
Vedolizumab (anti-α4β7)	LDP-02	MedChemExpress	HY-P9911
IgG4 isotype	S108P	Acro biosystems	DNP-C17P1-100UG
Human IgG2 Isotype Ctrl	QA16A13	Biolegend	403602
Human IgG4 Isotype	QA16A15	Biolegend	403702
VCAM-1 protein	N/A	R&D systems	862-VC
VCAM-1 protein (biotinylated)	N/A	Sino Biological	10113-H02H-B
MAdCAM-1 protein	N/A	R&D systems	VI11276
ICAM-1 (His-tagged)	N/A	Made in house	Made in house

Table 2.2: List of antibodies and proteins used in T cell functional assays and supported lipid bilayers.

2.2 Cell Culture

2.2.1 Freezing and thawing of cryopreserved cells

Vials of cryopreserved cells were held in a water bath at 37°C until almost thawed. Cells were then transferred to R10 media, pelleted and counted before seeding in flasks/plates in their pre-warmed media.

For freezing cells were counted, resuspended in 500-1000 μ l of freezing media (Foetal calf serum (FCS) with 10% DMSO) and transferred to cryovials. T cell clones were frozen at approx. 2×10^6 /ml and B cell lines (BCLs) at 5×10^6 /ml. Cryovials were stored at -80°C for 24-48 hours before transfer to liquid nitrogen (-180°C) for long term storage.

2.2.2 *In vitro* culture of T cell clones

Established primary T cell clones were cultured in H10 media (RPMI 1640 supplemented with 10% heat inactivated human serum, 2mM L-glutamine, 100 U/ml penicillin, 0.1 U/ml streptomycin) in 24-well or 48-well plates in 5% CO_2 at 37°C . They were stimulated with feeder cells (irradiated mixed PBMCs from 4-6 donors) with 50 $\mu\text{g}/\text{ml}$ phytohemagglutinin (PHA) and cultured for 10 days before functional assays were performed. After three days stimulation with feeder cells their media was supplemented with 200 U/ml IL-2, and then with 100 U/ml IL-2 every 3 days thereafter.

2.2.3 *In vitro* culture of B cell lines

Epstein-Barr virus (EBV) B cell lines (BCLs) were cultured in T-25 flasks in R10 media (RPMI 1640 supplemented with 10% FCS, 2mM L-glutamine, 100 U/ml penicillin, 0.1 U/ml streptomycin) in 5% CO_2 at 37°C . 50% of the media was changed every 3-4 days. BCLs were cultured for a minimum of 7 days before use in functional assays.

2.2.4 Isolation and cryopreservation of PBMCs

Fresh blood from donors was diluted with RPMI media and layered on Lymphoprep (Stemcell technologies) for density gradient centrifugation. Following centrifugation PBMCs were isolated from the thin white film. PBMCs were washed, incubated in red blood cell lysis buffer for 3 minutes, washed and counted. PBMCs were then cryopreserved in freezing media (FCS with 10% DMSO) and stored at -80°C for 24-48 hours before transfer to liquid nitrogen (-180°C) for long term storage.

2.3 Flow cytometry staining

2.3.1 Tetramer Staining

To confirm that cultured T cells were expressing their TCR and remained specific for their epitope, tetramer staining was performed 10 days after the T cell clones were stimulated with feeder cells. Briefly, the T cells were stained with APC/PE-conjugated tetramer for 15 minutes at 37°C, washed twice, stained with anti-CD8-FITC for 20 minutes at 4°C and washed again. Cells were stained with propidium iodide prior to running on the flow cytometer. Samples were acquired on the Attune NxT Flow Cytometer (ThermoFisher) and analysed using FlowJo (v10.8.1).

2.3.2 Staining of surface proteins

T cell clones/BCLs were seeded in the wells of a 96 well plate at approx. $0.5-1 \times 10^5$ cells/well. Cells were washed with PBS and stained with Live/Dead Fixable Aqua (Invitrogen) for 15 minutes at room temperature. Cells were washed with FACS wash buffer (PBS with 1% BSA and 0.4% EDTA) and then stained with fluorescently conjugated antibodies targeted cell surface proteins (e.g. integrins, VCAM-1, CD8, CD19) for 30 minutes at 4°C. Cells were washed twice, resuspended in FACS wash buffer and run on the Attune NxT Flow Cytometer (ThermoFisher) or LSR Fortessa X20 (BD). Analysis was performed using FlowJo (v10.8.1).

2.4 Functional Assays and Metabolic Analysis

2.4.1 IFN γ ELISpot

IFN γ ELISpot were performed with *in vitro* cultured T cell clones. EBV-transformed BCLs expressing the necessary HLA molecules (HLA- A*01:01 for ORF3a₂₀₇₋₂₁₅ specific T cells) were preloaded with peptides at titrated concentrations (starting at 2 μ g/ml) for 1 hour. T cell clones and peptide loaded BCLs were then cocultured together at an E:T ratio of 1:50 for a minimum of 6 hours in precoated 96-well MultiScreen filter ELISpot plates (Merck Millipore) After the plate was developed the number of spot forming cells (SFCs) were counted using an AID ELISpot 7.0.

2.4.2 Luminex

BCLs were pre-loaded with peptide at fourfold titrated concentrations (starting at 2 µg/ml) and then cocultured with T cell clones at an E:T ratio of 5:1 for 48 hours. Supernatants from the coculture were collected and stored at -80°C until needed. The Luminex assay (Bio-Rad) was then performed. Briefly, once thawed and spun down, 50 µl of supernatant was added to Luminex plates and incubated with assay beads for 30 minutes at room temperature with shaking. After three washes, detection antibodies were then added to the samples and incubated for 30 minutes at room temperature with shaking. After 3 washes, Streptavidin-PE was added to the samples and incubated for 10 minutes at room temperature. After 3 washes, the samples were run on the Luminex machine.

2.4.3 Proliferation assay

BCLs were preloaded with peptides at fourfold titrated concentrations (starting at 2 µg/ml) for 1 hour, washed three times and then irradiated with 2000 rads for 20 mins. T cell clones were stained with CFSE and cocultured with irradiated BCLs for 72 hours at an E:T ratio of 10:1. Cells were then stained with anti-CD8-APC for 20 minutes at 4°C, washed twice and stained with propidium iodide. Samples were run on the Attune NxT Flow Cytometer and analysed using FlowJo software (v10.8.1). Proliferation was measured by CFSE dilution, and each T cell clone was normalised to the background levels of proliferation from the coculture with BCLs that were not peptide-loaded.

2.4.4 Intracellular Cytokine Staining – Coculture assay

BCLs were preloaded with peptides at a range of concentrations for 1 hour and then washed three times prior to coculture with T cell clones for 4-6 hours. GolgiPlug (BD Bioscience), GolgiStop (BD Bioscience) and anti-CD107a-PE (3ul/test) were added at the start of the coculture. Negative controls with BCLs that had not been peptide-pulsed

were included. Following the co-culture, cells were stained with Live/Dead Fixable Aqua (Invitrogen) for 15 minutes at room temperature. Cells were then fixed and permeabilised using Cytofix/Cytoperm (BD Bioscience) for 15 minutes at 4°C and subsequently stained with anti-IFN γ -AF488 (1.5ul/test), anti-TNF α -APC (0.2ul/test) and anti-CD8-BV421 (1.5ul/test) for 20 minutes at 4°C and then washed twice. Samples were acquired on the Attune NxT Flow Cytometer and analysed using FlowJo software (v10.8.1). When blocking antibodies (Vedolizumab, Natalizumab, Abirilumab) or isotype controls were used the T cell clones were pretreated with these at 10 μ g/ml for 1 hour at 37°C prior to coculture with BCLs.

2.4.5 Intracellular cytokine staining – plate bound assay

Flat bottomed 96-well cell culture plates were coated with 50ng/well of anti-CD3 (clone OKT3) for 2 hours at 4°C. Plates were then coated overnight at 4°C with 200 ng/well of Neutravidin in PBS. Plates were rinsed and 200 ng/well of costimulatory ligand (anti-CD28, biotinylated VCAM-1 and biotinylated MAdCAM-1) were added for 1 hour at 37°C. T cell clones were counted and plated at 0.5-1x10⁵/well. When blocking antibodies (Vedolizumab, Natalizumab, Abirilumab) were used the T cell clones were pretreated for 1 hour at 37°C. T cell clones were transferred to the coated flat-bottom plate and GolgiPlug (BD Bioscience), GolgiStop (BD Bioscience) and PE-anti-CD107a (3ul/test) were added. After a 4-hour incubation, the cells were stained with Live/Dead Fixable Aqua (Invitrogen) for 15 minutes at room temperature. Cells were then fixed and permeabilised using Cytofix/Cytoperm (BD Bioscience) for 15 minutes at 4°C and subsequently stained with anti- IFN γ -AF488 (BD Bioscience, 1.5ul/test), anti-TNF α -APC (Invitrogen, 0.2ul/test) and anti-CD8-BV421 (Biolegend, 1.5ul/test) for 20 minutes at 4°C and then washed twice. Samples were acquired on the Attune NxT flow cytometer and analysed using FlowJo software (v10.8.1).

2.4.6 Killing assay

BCLs were stained with CFSE and staining was quenched with R20 media. After washing BCLs were loaded with peptide for 1 hour (2 μ g/ml). BCLs were then co-cultured with T

cell clones for 6 hours at several Effector:Target ratios (4:1, 2:1 and 1:1). BCLs that had not been loaded with peptide were also co-cultured with T cell clones at each of the E:T ratios. Following the coculture the cells were stained with anti-CD19-BV421 for 20 minutes at 4°C, washed twice and stained with 7-AAD (Invitrogen). Samples were acquired on the Attune NxT Flow Cytometer and analysed using FlowJo software (v10.8.1). To calculate the killing capacity of the T cell clones at each of the E:T ratios the percentage of live cells among CFSE⁺CD19⁺ cells was determined, and the following calculation was performed:

$$\text{Killing capacity} = \frac{\% \text{ live cells at E:T ratio (no peptide)} - \% \text{ live cells at E:T ratio (peptide)}}{\% \text{ live cells at E:T ratio (no peptide)}} \times 100$$

When blocking antibodies (Vedolizumab, Natalizumab, Abirilumab) or isotype controls were used the T cell clones were pretreated with these at 10 µg/ml for 1 hour at 37°C prior to coculture with BCLs.

2.4.7 Seahorse assays

2.4.7.1 Peptide stimulation assay

T cell clones were counted and resuspended in Seahorse XF RPMI medium (Agilent), supplemented with glutamine (2 mM) and glucose (10 mM) and plated in a XFe96 PDL microplate (200,000 cells/well). The plate was centrifuged to ensure the cells formed a monolayer on the bottom of the wells. M1₅₈₋₆₆ (GIL) peptide (final concentration of 10 µg/ml) was diluted in XF RPMI media and added to port A in the Seahorse Cartridge. After calibration, the seahorse assay was run using an XFe96 Extracellular Flux Analyzer. Peptide was injected after 20 minutes, and the oxidative consumption rate (OCR) and extracellular acidification rate (ECAR) were recorded over a period of 4 hours.

2.4.7.2 Metabolic profiling assay

T cell clones were counted and resuspended in Seahorse XF RPMI medium (Agilent), supplemented with glutamine (2 mM) and glucose (10 mM) and plated in a XFe96 PDL microplate (200,000 cells/well). The plate was centrifuged to ensure the cells formed a monolayer on the bottom of the wells. 25 µl of Oligomycin (13.5 µM), BAM15 (25 µM) and Rotenone/antimycin A (5.5 µM) were loaded into ports A, B and C respectively. Following

calibration, the seahorse assay was run using an XFe96 Extracellular Flux Analyzer and the drug compounds were injected into the cells every 20 minutes. The the oxidative OCR and ECAR were measured over a period of 4 hours.

2.5 Generation of integrin knockout T cell clones

2.5.1 CRISPR RNP production

CRISPR sgRNAs were synthesised by IDT. For each reaction (for approx. 1M cells) CRISPR RNP annealing was performed with 30 pmol Alt-R S.p.Cas9 Nuclease V3 protein and 90 pmol of sgRNA for 20 minutes at room temperature prior to electroporation (giving a 1:3 Cas9 protein to gRNA molar ratio).

2.5.2 Electroporation of primary T cell clone

T cells were activated for 24-48 hours with 25 µl/ml anti-CD3/CD28 Immunocult activator. Following activation T cells were washed with PBS, counted and aliquoted to give 0.5M-1M/reaction. P3 Primary Cell Nucleofector buffer was prepared by adding 16.4 µl solution and 3.6 µl supplement per reaction. T cells were resuspended in 20 µl P3 buffer and plated in a 16-well Nucleocuvette strip (Lonza). 2 µl of RNP reaction was added and cells were electroporated (pulse code EH115) with previously generated CRISPR RNPs using a 4D Nucleofector X unit (Lonza). Following electroporation, T cells were transferred to a 48-well plate with 900 µl of pre-warmed H10 culture media and rested at 37°C for 4 hours prior to stimulation with feeder cells and 50 µg/ml phytohemagglutinin.

Integrin	Guide name (IDT)	Guide sequence	Predicted on target score	Predicted off target score	Location of binding
ITGA4	Hs.Cas9.ITGA4.1.AC	CGCTTCAGTGATCAATCCCG	50	87	Exon 2
ITGB1	Hs.Cas9.ITGB1.1.AB	AATGTAACCAACCGTAGCAA	60	65	Exon 4
ITGB7	Hs.Cas9.ITGB7.1.AE	ACGCCAAGATCCCATCCACA	77	60	Exon 3

Table 2.3: Integrin sgRNAs for CRISPR.

2.5.3 Sorting and expansion of integrin knockout T cell clones

After 14 days of feeder cell stimulation integrin KO T cell clones were stained with Live/Dead Fixable aqua, CD8-BV421, $\alpha 4$ -PE, $\beta 1$ -AF488 and $\beta 7$ -APC as per section 2.2.2. From the generated KOs, cells were sorted on a FACS Aria Fusion cell sorter (BD), to obtain five populations: triple positive (TP, $\alpha 4+\beta 1+\beta 7+$), $\beta 7$ KO ($\alpha 4+\beta 1+\beta 7-$), $\beta 1$ KO ($\alpha 4+\beta 1-\beta 7+$), ($\alpha 4-\beta 1+\beta 7-$), and triple negative (TN, $\alpha 4-\beta 1-\beta 7-$). These sorted KO T cell clones were restimulated with feeder cells and 50 $\mu\text{g/ml}$ PHA. After 11 days of expansion the KO cells were stained with Live/Dead Fixable aqua, CD8-BV421, $\alpha 4$ -PE, $\beta 1$ -AF488 and $\beta 7$ -APC again to assess their purity.

2.6 Bulk RNA and TCR sequencing

2.6.1 Bulk RNA extraction and sequencing

RNA extraction was performed using RNeasy Micro Kit (Qiagen). T cell clones were activated for 2-4 hours with 25 μl of anti-CD3/CD28 (Immunocult, Stem cell) at 37°C. Unactivated T cell clones were also included. Each condition was performed in triplicate with 0.5 million cells/sample. After activation cells were washed with PBS and resuspended in 350 μl of RLT plus buffer and the lysate was homogenised. The homogenised lysate was transferred to a gDNA eliminator column in a 2ml collection tube and centrifuged at 13000 x g for 1 minute. The column was discarded and 350 μl of ethanol was added to the flow-through. The samples were transferred to a RNeasy MinElute spin column in 2 ml collection tubes. The samples were centrifuged at 13000 x g for 1 minute and the flow through discarded. 700 μl of Buffer RW1 was added to the RNeasy MinElute spin column, samples were centrifuged at 13000 x g for 1 minute and flow-through discarded. The previous step was repeated with 500 μl of Buffer RPE and then again with 500 μl of 80% ethanol. The RNeasy MinElute spin column was then transferred to a new 2 ml collection tube at full speed for 5 minutes. The RNeasy MinElute spin column was then transferred to a 1.5 ml collection tube and 20 μl of RNAase-free water was added before spinning at full speed for 1 min to elute the RNA. The RNA

concentrations were determined by Nanodrop before sending samples for sequencing with Azenta.

2.6.2 TCR sequencing of T cell clones

This was performed by Dr Yanchun Peng. From each T cell clone, 0.5 million cells were harvested and washed with PBS. Total RNA was extracted using RNeasy Plus Micro Kit (QIAGEN) as described above, and 100 ng of total RNA from each T cell clone was used to generate full-length TCR repertoire libraries for Illumina Sequencing using a SMARTer Human TCR a/b Profiling Kit (Takara) following the supplier's instructions. The cDNA sequences corresponding to variable regions of TCR α and TCR β transcripts were amplified with primers, including Illumina indices, allowing for sample barcoding. PCR products were then purified using AMPure beads (Beckman Coulter). The quantity and quality of cDNA libraries were checked on an Agilent 2100 Bioanalyzer system. Sequencing was performed using MiSeq reagent Kit v.3 (600 cycles) on MiSeq (Illumina) with MiSeq Control Software v.2.6.2.1.

2.7 Proteomics

2.7.1 Preparation of samples

T cell clones were grown to required numbers by feeder stimulation and tetramer staining was performed to confirm tetramer positivity. Paired T cell clones were activated for 2-4 hours with 25 μ l of anti-CD3/CD28 (Immunocult, Stem cell) at 37°C. Unactivated T cell clones were also included. Each condition was performed in triplicate with 1 million cells/sample. To prevent secretion of proteins from cells 0.7 μ g/ml Monensin and 1 μ g/ml Brefeldin were added prior to activation. After activation cells were then washed with PBS and then resuspended with 100 μ l 1% NP-40 complete lysis buffer containing 1X protease inhibitor (Sigma Aldrich) and 1mM phenylmethylsulfonyl fluoride (Thermo Fisher) on ice for 1 hour. During the 1 hour ice incubation the cells were vortexed every 10 minutes. The samples were centrifuged at 13000 x g for 10 min at 4 °C and supernatants were transferred to new tubes and frozen at -80°C.

A BCA assay was performed according to manufacturer's instructions to determine protein concentrations of the samples. Samples were diluted to 100 µg/ml of protein prior to LC-MS. A comassie blue gel was run to ensure proteins of a range of molecular weights were contained in each sample. The gel was run for 45 minutes at 180V. The gel was then washed and stained overnight with instant comassie blue stain.

2.7.2 Liquid Chromatography-Mass Spectrometry

This was performed by Iolanda Vendrell and Sarah Flannery. Samples were thawed and proteins were denatured in 8 M urea for 30 min. Protein reduction was performed with 10 mM tris(2-carboxyethyl(phosphine) for 30 min at RT. Alkylation with 50 mM iodoacetamide for another 30 min at RT in the dark was then performed. Samples were diluted with 1.5 mM urea and 50 mM triethylammonium bicarbonate before digestion with 1.5 µg trypsin and incubation overnight at 37 °C. Digested samples were cleaned on a SOLA HRP C18 and evaporated to dryness using a vacuum centrifuge. Samples were then reconstituted in 5% DMSO and 5% formic acid. Peptides were analysed on the NeoVanquish-Orbitrap Ascend system, with a 1-hour liquid chromatography gradient. The mass spec was operated in DIA mode. Peptides were loaded onto a trap column and separated on a 50-cm-long EasySpray column (ES803, Thermo Fisher) with a gradient of 2–35% acetonitrile in 5% dimethylsulfoxide and 0.1% formic acid at a flow rate of 250 nl min⁻¹ over 60 min. Eluted peptides were then analysed on an Orbitrap ascend system. Survey scans were acquired in the Orbitrap at a resolution of 120,000 over a m/z range of 400–1500, AGC target of 4e5 and S-lens RF of 30. Fragment ion spectra (MS/MS) were obtained in the Ion trap (rapid scan mode) with a Quad isolation window of 1.6, 40% AGC target and a maximum injection time of 35 ms, with HCD activation and 28% collision energy. Raw mass spectrometry files were label-free quantified using DIA-NN (version 1.8) in library-free mode using the Uniprot proteome UP000005640 as a FASTA file. Data were further processed in Perseus (version 1.6.2.3).

2.8 Single Cell RNA sequencing

2.8.1 Staining and sorting of PBMCs

Cryopreserved PBMCs were thawed, counted, filtered and rested for one hour at 37°C prior to staining. PBMCs were stained with Live/Dead Fixable Aqua for 10 mins at RT. Cells were stained with Flu, CMV, EBV and negative control dextramers in stain buffer (2% FBS PBS with 0.1 g/L herring sperm DNA) for 10 mins at RT. D-biotin (0.2ul/Dextramer) was added to the dextramer pool prior to staining. After 10 min incubation CITE-Seq antibodies (anti-CD45RA, anti-CXC3R1, anti-CCR7, anti-CD28, anti-CD27, anti-CD57 and isotype controls) and fluorescently conjugated antibodies (anti-CD3-FITC, anti-CD8-APC, anti-CD14-BV510, anti-CD19-BV510) were added to the cells for a further 30 minutes at 4°C. For some samples Hashtag (Biolegend) antibodies were also added at this stage. Cells were washed and filtered prior to sorting.

Samples were sorted using a BD FACS Aria Fusion Flow Cytometer. Live CD19-CD14-CD3⁺CD8⁺Dex⁺ and Live CD19-CD14-CD3⁺CD8⁺Dex⁻ cells were sorted into 1.5ml Eppendorf tubes. Eppendorf tubes were prepared in advance to contain 0.02% BSA after sorting. Between 280-3000 CD8⁺Dex⁺ T cells were sorted, depending on the frequency of them in each sample. 20000 CD8⁺Dex⁻ T cells were sorted per sample. CD8⁺Dex⁻ T cells were added to the CD8⁺Dex⁺ T cells to bring the total estimated number of cells to 10000. These cells were then loaded onto the 10x Genomics Chromium instrument according to the manufacturer's instructions.

2.8.2 10X single cell library preparation

The scRNAseq libraries were prepared using Chromium Next GEM Single Cell 5' V.2 kit (10x Genomics), the Chromium Single Cell Human TCR Amplification kit (10x Genomics) and Chromium 5' Feature Barcode Kit (10x Genomics). Libraries were generated according to the manufacturer's instructions. Briefly, cells underwent in-drop lysis and reverse transcription, generating cDNA from mRNA in each cell and from the oligonucleotide-tagged antibodies, bearing bead-specific sequences, used as a barcode to identify the cell of origin. Feature barcodes were also captured from the CITE-Seq antibodies and dextramers. cDNA was then amplified and cleaned using SPRIselect

beads (Beckman Coulter). 2 µl of amplified cDNA was used to enrich for the TCR cDNA for the VDJ libraries. Gene expression and enriched VDJ libraries were enzymatically fragmented and subjected to A-tailing, ligation to adaptors sample indexing by PCR. Feature barcode libraries were amplified and indexed via PCR. At several steps of throughout the library preparation the concentration and quality of the cDNA libraries were checked on an Agilent 2100 Bioanalyzer system, using high sensitivity D5000 tapes. cDNA libraries were diluted prior to running on the Agilent 2100 Bioanalyzer system when recommended by the 10X protocol. Following library generation, libraries were pooled and sequenced on the Illumina NextSeq sequencing platform with Azenta or NovaSeq X with Novogene.

2.9 Supported Lipid Bilayers and TIRF Microscopy

2.9.1 MAdCAM-1 Protein Conjugation

Filtered sodium bicarbonate (1 M) was used to reconstitute AF488 dye. 22 µl of AF488 dye was added to 200 µl of 100 µg/ml MAdCAM-1 protein. The protein was vortexed gently and incubated for 2 hours at RT without light. The protein was transferred to 4 degrees for overnight incubation. The protein was filtered 4 times using a Zeba column. The F/P ratio of the conjugated protein was determined using a nanodrop.

2.9.2 Bead Supported Lipid Bilayers

5.00 ±0.05 µm diameter non-functionalised silica beads were washed three times with PBS. 50 µl of 12.5 mol% of 0.4 mM DGS-NTA(Ni) (1,2-dioleoyl-sn-glycero-3-[(N-(5-amino-1-carboxypentyl) iminodiacetic acid) succinyl]) was added to the silica beads with gentle pipetting. The BSLBs were mixed on a vertical mixer for 30 min at RT using orbital mixing at 20 rpm. The BSLBs were washed three times using supplemented (7 mM Na₂HPO₄, 1400 mM NaCl, 50 mM KCl, 60 mM glucose, 10 mM CaCl₂, and 20 mM MgCl₂) HEPES-buffered saline containing 1% bovine serum albumin (HBS/BSA). Starting concentrations of proteins of interest (VCAM-1: 0.5 µg/ml and MAdCAM-1: 1 µg/ml) were prepared in 100 µl of HBS/BSA. A 12 point 2-fold serial of the proteins was performed with the final concentration left as a blank reference. The BSLBs were split into 12 aliquots of 100 µl

and spun down. 100 μ l of the protein titration was added to the BSLBs with pipette mixing. The BSLBs were incubated for 30 minutes at RT on a plate shaker at 1200 rpm. BSLBs were washed 3 times with HBS/BSA. MAdCAM-1 was conjugated to AF488 so sample acquisition was performed after this step. VCAM-1 BSLBs were stained with anti-VCAM-1-PE antibody (3 μ l/test) for 30 minutes at RT using a plate shaker. BSLBs were washed twice and resuspended in PBS for sample acquisition. AF488 or PE MESF standards were acquired on an LSR Fortessa X50 flow cytometer, with the brightest peak in the detection range. BSLB samples were acquired and analysed in FlowJo (v10.8.1). Fluorescence levels were used to determine the density of molecules on each BSLB population as described previously²²².

2.9.3 Preparation of Supported Lipid Bilayers

An acid and plasma-cleaned glass coverslip (D 0.17 ± 0.005 mm, Schott Nexterion) was attached to Sticky-Slide VI0.4 chambers (Ibidi, 80608) to create six-well chambers. To form glass-supported lipid bilayers (SLBs) a liposome mix containing 25% 0.4 mM DGS-NTA(Ni) in a 0.4 mM 1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC) matrix was added to the chambers and incubated for 30 minutes at RT. The SLB were washed three times with HBS/BSA (1%). SLBs were reconstituted with proteins to form an artificial antigen presenting cell membrane by incubation with 200 molecules per μ m² of ICAM-1 (fluorescently conjugated to AF405 in some experiments), 100 molecules per μ m² of VCAM-1, 100 molecules per μ m² of MAdCAM-1 and 30 molecules per μ m² of anti-CD3 (clone UCHT1). Calibration of recombinant protein densities were performed on BSLBs as described above. After 30 minutes RT incubation SLBs were washed three times with HBS/BSA (1%).

T cell clones were washed and resuspended in HBS/BSA (1%) buffer. 0.5 M T cells were added per well for 15 minutes at 37°C. Cells were immediately fixed with prewarmed 4% paraformaldehyde in PBS containing 2 mM MgCl₂ for 10 minutes at RT. Cells were then washed three times with HBS/BSA (1%). Cells were stained with PE anti-integrin β 7 (1:50 dilution), AF647 anti-CD49d (1:50 dilution) and AF488 anti-CD29 (1:50) overnight at 4°C.

The following morning the chambers were washed three times with HBS/BSA (1%) and allowed to equilibrate to RT prior to performing imaging as described below.

2.9.4 TIRF Microscopy and Fiji image analysis

Total internal reflection fluorescence microscopy (TIRFM) was performed using an Olympus IX83 inverted microscope equipped with four laser-lines: 405-nm, 488-nm, 561-nm and 640-nm. The system was fitted with an Olympus UAPON x 150 TIRF numerical aperture 1.45 objective, and a Photometrics Evolve delta EMCCD camera. Quantification of fluorescence intensity in each synapse was performed with Fiji/ImageJ. A batch measure macro was used to segment cell:SLB contacts based on internal reflection microscopy followed by background subtraction and the measure of fluorescence across different channels. Graphs of fluorescence intensity were generated using GraphPad Prism and representative images of synapses were generated with Fiji/ImageJ.

2.10 Bioinformatics

2.10.1 Bulk RNA sequencing analysis

2.10.1.1 RNA-seq data processing and alignment

FASTQ files were trimmed using Cutadapt²²³ to remove adapter sequences. Reads shorter than 20 nucleotides after trimming were removed. Trimmed reads were aligned to the reference genome (GRCh37, Ensembl release 87) using STAR²²⁴ (v2.6.1). Gene-level read counts were quantified using Subread's featureCounts²²⁵ (v2.0.0) function. The resulting count matrix was imported into R for downstream differential expression analysis.

2.10.1.2 Differential gene expression analysis

Genes with 0 counts or <10 counts in half of the samples were filtered out. Differential gene expression was determined using the generalised linear model implemented in

DESeq2²²⁶ (v.1.48.1) with contrasts defined according to the clone or activation timepoint. Log2 fold changes were estimated using DESeq2's shrinkage approach with the ashhr method²²⁷. Adjusted p -values were computed using the Benjamini–Hochberg procedure to control the false discovery rate (FDR). Genes with adjusted $p < 0.05$ and a LFC value >1 were considered significantly differentially expressed.

For visualisation of differentially expressed genes normalisation of counts was performed. For heatmaps these were scaled across each row. For visualisation of individual genes normalised counts were log transformed.

2.10.2 Proteomics analysis

2.10.2.1 Differential protein analysis

Log normalised protein expression values were analysed using the R package DEP2²²⁸ (v0.5.28.2). Differential expression testing was performed using the test_diff() function from DEP2 with the Benjamini–Hochberg procedure to control for multiple testing. Proteins with an adjusted $p < 0.05$ and a LFC value >0.25 were classified as significantly differentially expressed. Volcano plots were generated using the EnhancedVolcano package (v1.26.0).

2.10.2.2 Pathway analysis

Pathway analysis was performed using enrichr²²⁹ (v3.4) and enrichplot (v1.28.2) using the BioPlanet2019 database²³⁰ to identify enriched pathways. Enrichment was calculated against all detected proteins as the background, and p -values were adjusted for multiple testing using the Benjamini–Hochberg method.

2.10.3 Single cell RNA sequencing analysis

2.10.3.1 Generation of filtered gene and barcode matrices and TCR sequences for scRNA-sequencing

Cell Ranger²³¹ multi pipeline (10X Genomics, v8.0.1) was used align FASTQ sequencing reads to the GRCh38 reference human genome for quantification of gene expression, surface protein expression and to generate consensus TCR sequences. Downstream analysis was performed in Rstudio using Seurat²³² (v5.3.0). The package scRepertoire²³³ (v2.4.0) was used to perform initial TCR analysis with TCR gene usage information then added to Seurat objects as metadata.

2.10.3.2 Quality control and filtering

Low quality cells were excluded based on percentage of ribosomal genes, percentage of mitochondrial genes and library complexity. Specifically, cells with fewer than 700 detected counts, greater than 10% mitochondrial reads, or less than 10% ribosomal reads were removed. Cells with total RNA counts more than three median absolute deviations above the dataset median were also excluded to minimise the inclusion of doublets in downstream analysis. scDblFinder was also used to identify and exclude doublets²³⁴. Using these filtering cut offs, when all samples were combined, 33695 cells remained.

2.10.3.3 Dextramer classification

To establish which cells were dextramer positive, expression values in the antibody derived tag (ADT) assay were normalised using centered log-ratio (CLR) normalisation. Normalised values for the dextramers were assessed and a threshold selected based on separation between positive and negative populations. Cells were classified dextramer-positive for a given specificity (flu-dex, cmv-dex or ebv-dex) when the normalised signal for that dextramer exceeded the threshold of 3 and the negative-control dextramer was below 1.5. Cells not meeting these criteria were classified as dextramer-negative. For

one sample dextramer+ staining was lower so a threshold of 1.5 was used to classify cells as dextramer+.

2.10.3.4 Normalisation, integration and clustering

The individually generated Seurat objects (one for each sample) were normalised using Log Normalisation and SCTransform and PCA analysis was performed. The objects were integrated using reciprocal PCA (RPCA). Dimensionality reduction was performed using PCA and visualized with UMAP based on the first 30 principal components. Clustering was performed using a resolution of 0.4. Clusters were given biologically meaningful names based on expression of surface proteins and gene expression.

2.10.3.5 Differential gene expression

Differential gene expression analysis was performed using the log normalised RNA counts. Differential gene expression testing between cells at different timepoints was performed using the MAST²³⁵ (Model-based Analysis of Single-cell Transcriptomics) test implemented in Seurat (v5). Donor identity, percentage of mitochondrial transcripts, and total RNA counts per cell were included as latent variables to control for variation between donors and cell-level quality effects. Genes with an adjusted p value (using Bonferroni correction) of <0.05 were considered significantly different between timepoints. To perform pseudo bulk analysis Seurat's AggregateExpression function was used to collapse cells from an individual donor and timepoint into a single datapoint. DESeq2 was used to do differential gene expression testing of pseudobulk and genes with an adjusted p value < 0.05 were considered significant, with Bonferroni correction used to adjust for multiple testing.

2.10.3.6 TCR analysis

The package scRepertoire was used to perform initial TCR analysis. For much of the TCR analysis, cells were filtered to only include cells that had fully resolved variable and joining genes for both the TCR α and TCR β chains. For CDR3 analysis cells were filtered to only include cells with fully resolved CDR3 α and CDR3 β , in addition to V and J genes.

For CDR3 similarity analysis, pairwise distances (Levenshtein edit distances) between CDR3 sequences were computed using the stringdist package (v0.9.15). Edges were drawn between clonotypes when their sequence distance ≤ 2 and resulting similarity graphs were constructed using igraph (v2.1.4) and visualized using ggraph (v2.2.1). Simpson and Shannon indices were calculated using the package vegan²³⁶ (v2.7.1) according to the following equations:

$$Gini - Simpson = 1 - \sum_{i=1}^s p_i^2$$

$$Shannon = -\sum_{i=1}^s p_i \ln p_i$$

Where s is the total number of unique clonotypes, and p_i is the relative proportion of the i th clonotype.

2.10.3.7 Visualisation

UMAP plots, violin plots and dot plots were created using in built functions in Seurat. Circos plots were generated using circlize²³⁷ (v0.4.16). Alluvial plots were generated using ggalluvial²³⁸ (v0.12.5). Other plots were generated using ggplot2 (v3.5.2).

2.10.4 TCGA Analysis

RNA-sequencing (RNA-Seq by Expectation-Maximization normalised) and clinical data for TCGA were obtained from the Broad Institute Genome Data Analysis Center (GDAC) standard data release (https://gdac.broadinstitute.org/runs/stddata_2016_01_28/). For each of the tested integrin genes, samples from a particular cancer type were split into high- and low- expression groups based on the optimal cutpoint determined using the R package survminer (v0.5.0). Kaplan-Meier curves and Cox proportional hazards model adjusted curves were fitted with the R package survival (v3.8-3), and statistical significance was assessed using log-rank tests. CD8⁺ T cell proportions in samples were estimated using the R package quantiseq²³⁹ (1.18.0).

3. Profiling age-related changes in influenza M1₅₈₋₆₆-specific CD8⁺ T cells

3.1 Introduction

CD8⁺ T cells can provide cross protection against heterosubtypic influenza viruses^{114, 127, 128, 129} through their recognition of epitopes from conserved viral proteins²⁴⁰. Community based cohort studies have demonstrated pre-existing influenza-specific CD8⁺ T cells can protect against viral shedding and disease severity^{127, 128}, highlighting the importance of studying influenza-specific T cells¹¹⁴. There has been substantial effort to identify which epitopes from influenza T cells can recognise. To date, this has led to the identification of 335 CD8⁺ T cell epitopes, restricted by over 20 HLA allotypes, for IAV (Immune Epitope Database, accessed 4 September 2025).

Of these identified epitopes, the response targeting the M1₅₈₋₆₆ epitope of the matrix 1 (M1) is one of the most immunodominant. This epitope was first identified through studies in the late 1980s and early 1990s^{122, 241, 242}. Gotch and colleagues showed that a peptide derived from the influenza M1 protein could be recognized by CD8⁺ T cells in association with the HLA-A*02:01 molecule¹²². Subsequent work identified the optimal peptide sequence as the amino acids in positions 58-66²⁴²: GILGFVFTL, commonly referred to as GIL²⁴³. Unlike the surface proteins neuraminidase and haemagglutinin, the M1 protein is not prone to mutations¹⁰⁸. In particular the M1₅₈₋₆₆ epitope is highly conserved, with 93% conservation reported for almost 70 tested strains of seasonal and pandemic IAVs²⁴⁴. Due to functional constraints this epitope is intolerant of amino acid substitutions in several positions²⁴⁵, which partially accounts for its conservation. However, extra-epitopic mutations have been identified which could affect the antigen presentation of this epitope^{246 247}.

Since its identification, the M1₅₈₋₆₆-specific response has been found in the majority of people who are positive for HLA-A*02:01^{124, 248}. HLA-A*02:01 has a relatively high frequency across almost all human populations²⁴⁹, highlighting the importance of this immunodominant response. Koutsakos and colleagues showed that in individuals actively infected with IAV, M1₅₈₋₆₆-specific T cells were present at a 4-fold increased

frequency compared to healthy controls¹²⁴. In the infected individuals many of the M1₅₈₋₆₆-specific CD8⁺ T cells were Ki67⁺CD38⁺ suggesting they were activated and contribute to the immune response against the virus. M1₅₈₋₆₆-specific T cells were found in the lungs in human donors¹²⁴ and were shown to be protective against influenza in an HLA-A2/K(b) transgenic mouse model¹²⁵. The protective effects of M1₅₈₋₆₆-specific T cells generated interest in whether the inclusion of the M1 protein in influenza vaccine design could be beneficial^{250, 251}. Powell and colleagues observed that vaccination with a modified vaccinia Ankara construct containing M1 led to more activated M1₅₈₋₆₆-specific CD8⁺ T cells, following vaccine challenge, and that these cells contained higher levels of perforin and granzymes compared to those from unvaccinated controls²⁵¹.

Following identification of the M1₅₈₋₆₆ epitope, researchers aimed to identify which TCR genes were used by T cells to recognise it. Work in the 1990s found that the HLA-A*02:01-restricted M1₅₈₋₆₆-specific TCR repertoire exhibits a strong TRBV19 bias (formerly known as Vβ17)^{252, 253, 254} and relatively restricted TRAV gene usage, with TRAV27 and TRAV12 being overrepresented^{253, 255}. However, a 2017 study looked into the M1₅₈₋₆₆-specific TCR repertoire in great detail and identified over 2400 unique TCRα and 2430 unique TCRβ clonotypes, indicating that the TCR repertoire for this epitope is much more diverse than previously appreciated²⁴³. Many of the identified TCR clonotypes are public, meaning the exact sequence is found in more than one individual. Chen and colleagues found that of total unique TCR sequences, public clonotypes accounted for 17% and 20% of unique M1₅₈₋₆₆-specific TCRα and TCRβ but in terms of weighted total TCRs, these public clonotypes made up 53% and 74% of M1₅₈₋₆₆-specific TCRαs and TCRβs, respectively, indicating that the public clonotypes are more highly expanded than private clonotypes. Through structural and functional analysis, Valkenburg and colleagues demonstrated that the TRAV27-TRBV19 public TCR clonotype can recognise naturally occurring M1₅₈₋₆₆ mutated epitopes²⁵⁶, which provides a potential explanation for why this clonotype is more prone to expansion than others. Analysis of “non-canonical” TCRs (those not utilising TRBV19 and TRAV27) revealed diverse structural solutions for how TCRs can recognise the M1₅₈₋₆₆ peptide^{257, 258}.

Most people are infected by influenza several times throughout their life with the first infection happening in almost 100% of people by the age of seven²⁵⁹. Individuals are

generally infected by influenza repeatedly during childhood and early adulthood, but after the age of 30, infections with IAV become less frequent, occurring on average twice per decade²⁶⁰. Outcomes from influenza infection vary greatly depending on age; in England, over 90% of influenza attributable deaths in hospital occur in people over the age of 65²⁶¹. A recent study looking at the impact of influenza on working age adults in the US found that adults between the ages of 50-64 years had 3-5-fold higher rates of hospitalisation and death than adults aged 18-49 years²⁶². These statistics highlight the need to investigate changes in the influenza-specific immune response as people age.

Ageing is associated with extensive changes to T cells, ranging from shifts in population composition to molecular alterations to individual T cells⁹⁵. Advances in the ease and accessibility of single cell RNA sequencing (scRNAseq) in recent years has allowed for the generation of large datasets profiling immune cell populations across human lifespan^{99, 220, 221}. Several of these studies have identified age-associated changes in T cell populations, such as the accumulation of GZMK⁺CD8⁺ T cells with increased age⁹⁹. Most of these studies focus on profiling T cells from peripheral blood, but a recent study from the Farber group demonstrated differences in ageing between tissue resident and circulating T cell populations²⁶³. While these datasets provide increased granularity and are an excellent resource to furthering our understanding of T cell ageing, they do not address the question of T cell ageing in a pathogen- or antigen-specific manner.

Previous studies have profiled changes in influenza-specific T cells with age. Lee and colleagues observed a reduction in the breadth of the IAV-specific CD8⁺ T cell response in donors over the age of 65²⁶⁴ which was supported by subsequent studies showing a decline in the frequency of both IBV-specific and IAV-specific CD8⁺ T cells with age^{265, 266}. Shifts in epitope immunodominance and restricted diversity in the TCR repertoire of influenza-specific T cells in aged mice has been shown to cause a decline in heterosubtypic immunity^{267, 268}. As well as a numerical decline, functional changes in influenza-specific T cells have been observed; Zhou and McElhaney showed reduced cytolytic activity in effector and effector memory CD8⁺ T cells from older adults responding to influenza A/H3N2 challenge²⁶⁹.

The effects of ageing on the M1₅₈₋₆₆-specific response, have been investigated^{255, 264, 266, 270, 271}. Several studies have demonstrated a reduced frequency of M1₅₈₋₆₆-specific CD8⁺ T cells, determined by *ex vivo* tetramer staining, in older individuals (>60/65 years) compared to younger adults^{266, 271}. Much of the research has focussed on changes in the TCR gene usage with age; the Kedzierska group have demonstrated that in elderly individuals there is a reduction in the frequency of the public TRAV27-TRBV19 public TCRαβ clonotype. Gil and colleagues reported that within M1₅₈₋₆₆-specific cells that do retain the public TRAV27 and TRBV19 gene usage there is a reduction in unique clonotypes (distinct CDR3 sequences), suggesting narrowing of the repertoire with age²⁵⁵. However recent studies have found that older adults have a more diverse TCR repertoire than younger adults, due to the expansion of private clonotypes^{271, 272}. TCRα CDR3 regions from older individuals are longer than those of younger individuals^{255, 273}. At the transcriptomic level, it has been reported that M1₅₈₋₆₆-specific T cells from older adults have lower expression of cytotoxicity genes, including those encoding granulysin and granzymes, compared to younger adults²⁷¹. While profiling M1₅₈₋₆₆-specific from newborns, children, adults and older adults, van de Sandt and colleagues determined that gene expression profiles from older adults were more similar to newborns than adults. They observed that M1₅₈₋₆₆-specific T cells from children and young adults have a greater proliferative capacity than those from older adults. The authors attributed this to the higher frequency of the optimal TRAV27–TRBV19 public TCRαβ clonotype in younger individuals²⁷¹. Longitudinal studies of M1₅₈₋₆₆-specific T cells are rare, largely due to the logistical challenges of repeatedly sampling the same individuals over extended time periods. To the best of our knowledge, only two previous studies have been reported, in which participants were sampled up to three times across a 10-12-year span^{274, 275}, however, this work focused exclusively on TCR gene usage.

Due to high costs, and experimental challenges, most of the single cell transcriptomic analysis of these M1₅₈₋₆₆-specific T cells, has been done in isolation, without useful comparison to other CD8⁺ T cells. Therefore, in this study, we utilise barcoded dextramers to simultaneously capture single-cell transcriptomes and TCR gene usage profiles from M1₅₈₋₆₆-specific CD8⁺ T cells, alongside data from the broader CD8⁺ T cell population. Following initial *in vitro* work with M1₅₈₋₆₆-specific CD8⁺ T cell clones, our

study follows three individuals over a 19-year period and applies single-cell RNA sequencing with the aim of investigating age-associated changes in both transcriptomic profiles and TCR gene usage in M1₅₈₋₆₆-specific T cells. Through comparison to the broader T cell population and EBV/CMV-specific T cells, we utilise these M1₅₈₋₆₆-specific T cells to address the question of how antigen-specificity affect the ageing trajectory of CD8⁺ T cells.

3.2 Results

3.2.1 Functional differences in M1₅₈₋₆₆-specific T cell clones

To investigate the M1₅₈₋₆₆ -specific CD8⁺ T cell response, T cell clones were initially isolated and established by Dr Yanchun Peng (Fig 3.1), after which I performed assays to determine their functionality. This clone system was used as it removes confounding variables such as heterogeneity of CD8⁺ T cell response and allows assessment of functionality using clean *in vitro* assays in an antigen-specific manner.

Clones were isolated from PBMCs by tetramer staining using a PE-conjugated HLA-A*02:01 GILGFVFTL tetramer that binds to TCRs to that recognise this epitope (Fig 3.1). Tetramer⁺ CD8⁺ cells were identified through fluorescence activated cell sorting (FACS) and single cell sorted into the wells of a 96 well plate. Isolated cells were expanded using feeder cell stimulation and clones that grew to sufficient numbers were used to perform functional assays and had their TCR sequenced (Table 3.1). PBMCs collected from the same donor (D1) in 2006 (T1) and 2016 (T2) were used to generate these clones, allowing us to examine antigen-specific T cell ageing over a 10-year period. All the M1₅₈₋₆₆-specific CD8⁺ T cell clones used in these experiments bear the same TCR: TRAV25-TRAJ42-TRBV19-TRBJ2-3 (Table 3.1) which we later confirm to be the dominant TCR for this donor (Fig 3.16).

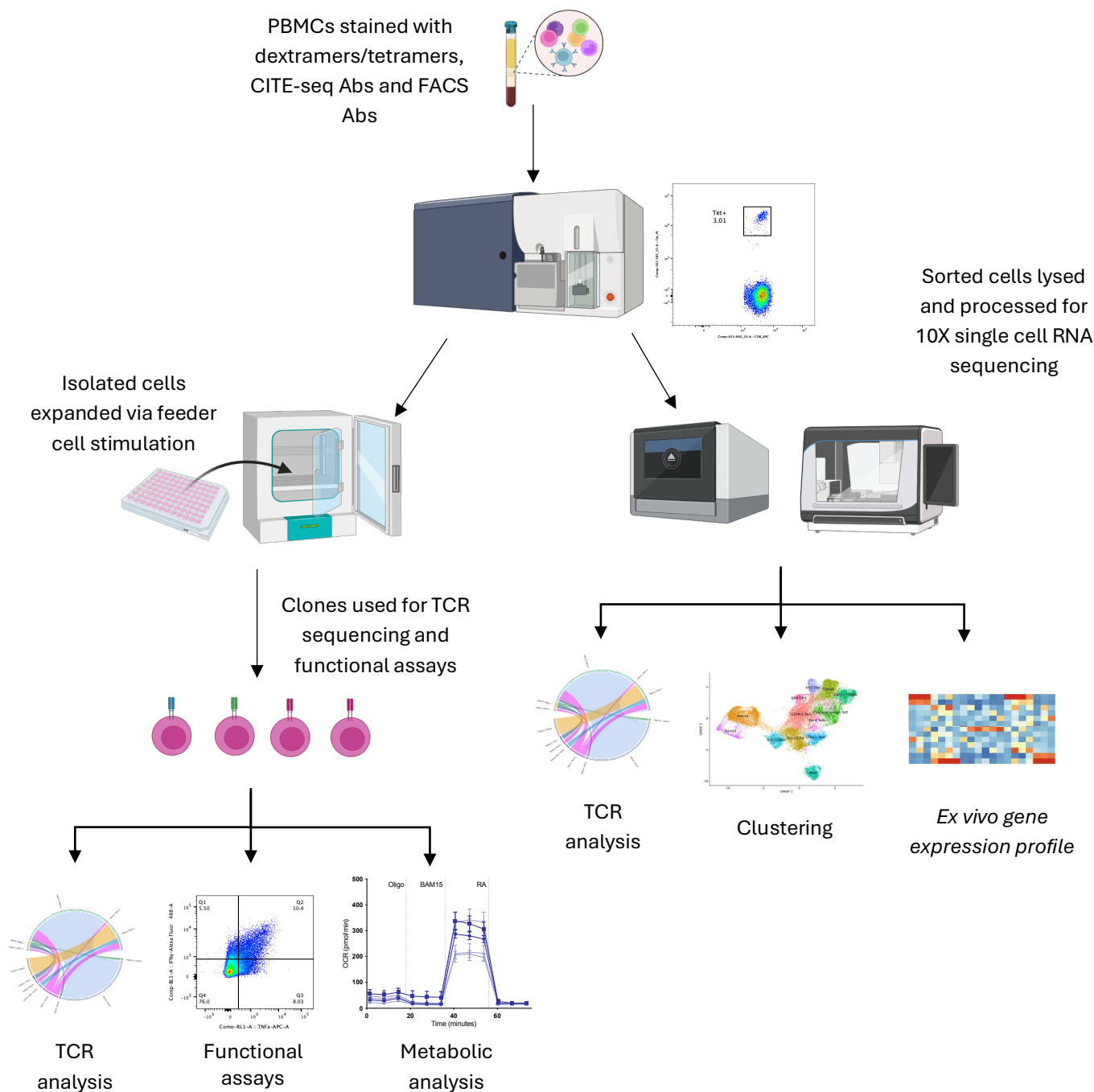


Figure 3.1: Experimental overview for investigating effects of ageing on M1₅₈₋₆₆-specific CD8⁺ T cells.

Left side: Isolated PBMCs from D1 from 2006 and 2016 were stained with FACS antibodies and a PE-conjugated HLA-A*02:01 GILGFVFTL tetramer. Tetramer+ CD8⁺ cells were single cell sorted into the wells of a 96 well plate and expanded using feeder cell stimulation. Clones that grew well were used to perform functional assays and TCR sequencing. Right side: Isolated PBMCs from three HLA-A*02:01 donors at 2-3 timepoints were stained with FACS antibodies, CITE-Seq antibodies and PE-conjugated dextramers. CD8⁺ T cells and Dextramer+ CD8⁺ T cells were sorted and lysed for 10X single cell RNA sequencing. Feature barcode, TCR and gene expression libraries were prepared and sequenced. Abbreviations: TCR, T cell receptor; CITE-seq, cellular indexing of transcriptomes and epitopes by sequencing; FACS, fluorescent-activated cell sorting, Abs, antibodies.

Clone	Timepoint	Year	TRAV	TRAJ	TRA_CDR3	TRBV	TRBJ	TRB_CDR3
A5.1	T1	2006	TRAV25	TRAJ42	CALNYGG SQGNLIF	TRBV19	TRBJ2- 3	CASSSR STDTQYF
B6	T1	2006	TRAV25	TRAJ42	CALNYGG SQGNLIF	TRBV19	TRBJ2- 3	CASSSR STDTQYF
1-3	T2	2016	TRAV25	TRAJ42	CALNYGG SQGNLIF	TRBV19	TRBJ2- 3	CASSSR STDTQYF
1-5	T2	2016	TRAV25	TRAJ42	CALNYGG SQGNLIF	TRBV19	TRBJ2- 3	CASSSR STDTQYF
1-7	T2	2016	TRAV25	TRAJ42	CALNYGG SQGNLIF	TRBV19	TRBJ2- 3	CASSSR STDTQYF

Table 3.1 TCR sequencing of M1₅₈₋₆₆-specific CD8⁺ T cell clones. Variable and joining TCR gene usage and CDR3 sequences of M1₅₈₋₆₆ specific CD8⁺ T cell clones isolated from D1 in 2006 and 2016. TCR sequencing of clones was performed by Dr. Yanchun Peng.

To assess functionality of the M1₅₈₋₆₆-specific T cell clones we investigated their cytokine production and degranulation via intracellular cytokine staining (ICS). The T cell clones were cocultured for four hours with an HLA-A*02:01⁺ B cell line (BCL) that had been loaded with GILGFVFTL peptide. Following the coculture we assessed production of IFN γ and TNF which have direct antiviral and immunostimulatory effects⁶³, as well as the degranulation marker CD107a (Fig 3.2C, Fig 3.2D & Fig 3.2F). Despite bearing the same TCR we observe substantial differences in levels of cytokines produced from the M1₅₈₋₆₆-specific T cell clones (Fig 3.2A, Fig 3.2C & Fig 3.2E). Clone B6 (isolated at T1) shows the highest levels of TNF, IFN γ and CD107a when cocultured with 5 μ g/ml peptide loaded BCLs, while Clone 1-3 (isolated at T2) has the lowest levels of all three markers.

We then assessed the proliferative capacity and killing capacity of the M1₅₈₋₆₆-specific T cell clones. To determine their proliferative capacity, we stained the T cell clones with CFSE, cocultured them with peptide loaded BCL lines for 72 hours and assessed proliferation by CFSE dilution (Fig 3.3A & Fig 3.3B) Again, there were differences in proliferative capacity of the clones (Fig 3.3A) with Clone B6 having the highest

proliferative capacity and Clone 1-3 having the lowest proliferative capacity of only 6.5% when cocultured with 5 µg/ml peptide loaded BCLs.

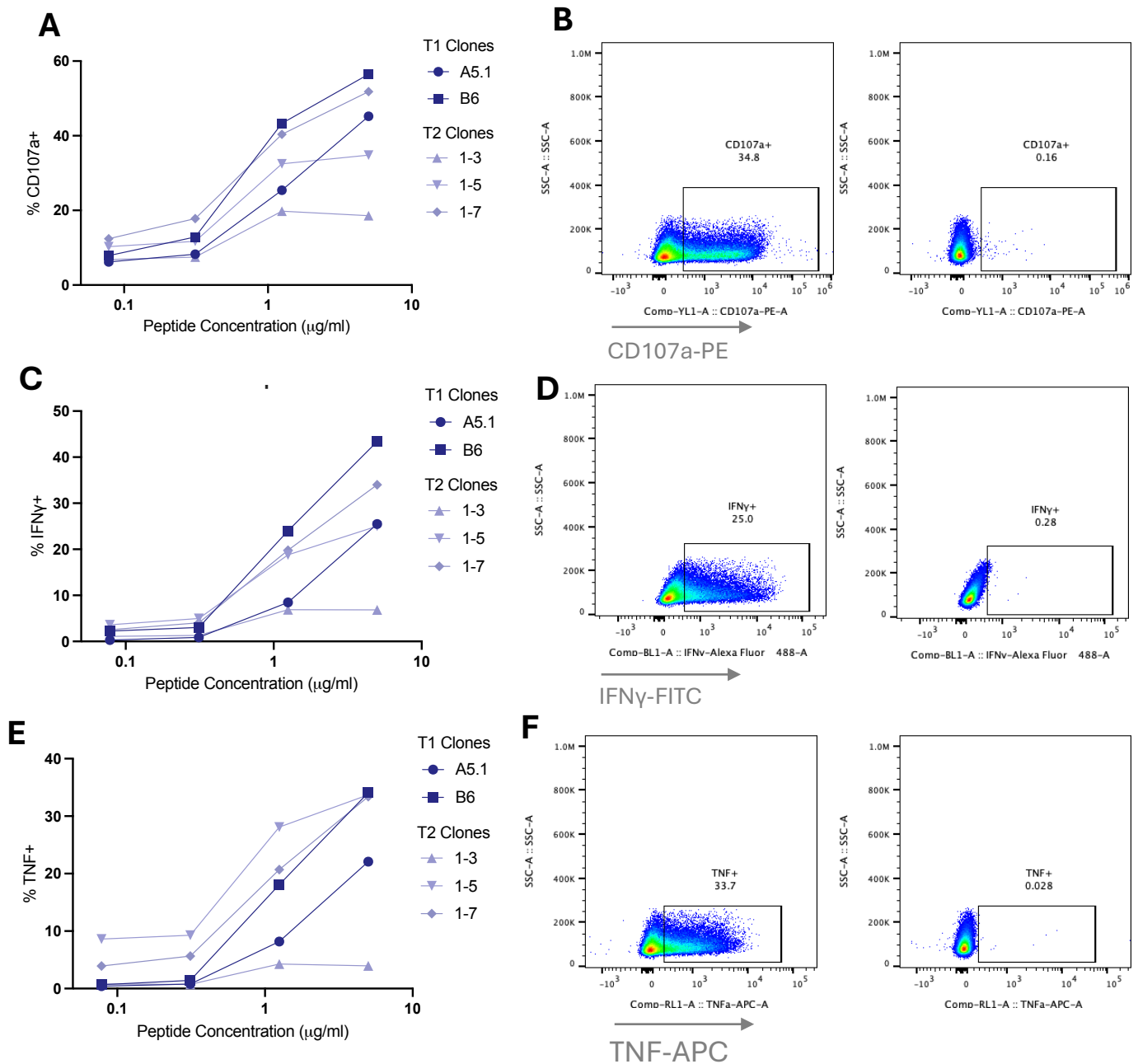


Figure 3.2: Differences in TNF, IFN γ and CD107a production in M158-66-specific CD8⁺ T cell clones. T cell clones were cocultured with BCLs with that had been preloaded with peptide (5 µg/ml, 1.25 µg/ml, 0.3125 µg/ml, 0.078 µg/ml and 0 µg/ml) at an Effector:Target ratio of 10:1. IFN γ , TNF and CD107a expression were assessed by intracellular cytokine staining and flow cytometry. **(A, C, E)** Percentages of cells positive for CD107a **(A)**, IFN γ **(C)** and TNF **(E)**. **(B, D, F)** Representative staining of CD107a **(B)**, IFN γ **(D)** and TNF **(F)** in M158-66-specific T cell clone stimulated with BCL preloaded with 5 µg/ml of peptide and no peptide stimulation control. Abbreviations: BCL, B cell line; IFN γ , interferon gamma; TNF, tumour necrosis factor; T1, timepoint 1; T2, timepoint 2.

To examine killing capacity, we cocultured 2 $\mu\text{g/ml}$ peptide loaded BCLs with the T cell clones at three effector:target ratios for 4 hours (Fig 3.3C). 7-AAD staining was used to determine the proportion live BCLs following coculture with the T cell clones (Fig 3.3D). The T clones isolated at timepoint 1 (A5.1 and B6) exhibited the higher killing capacity at

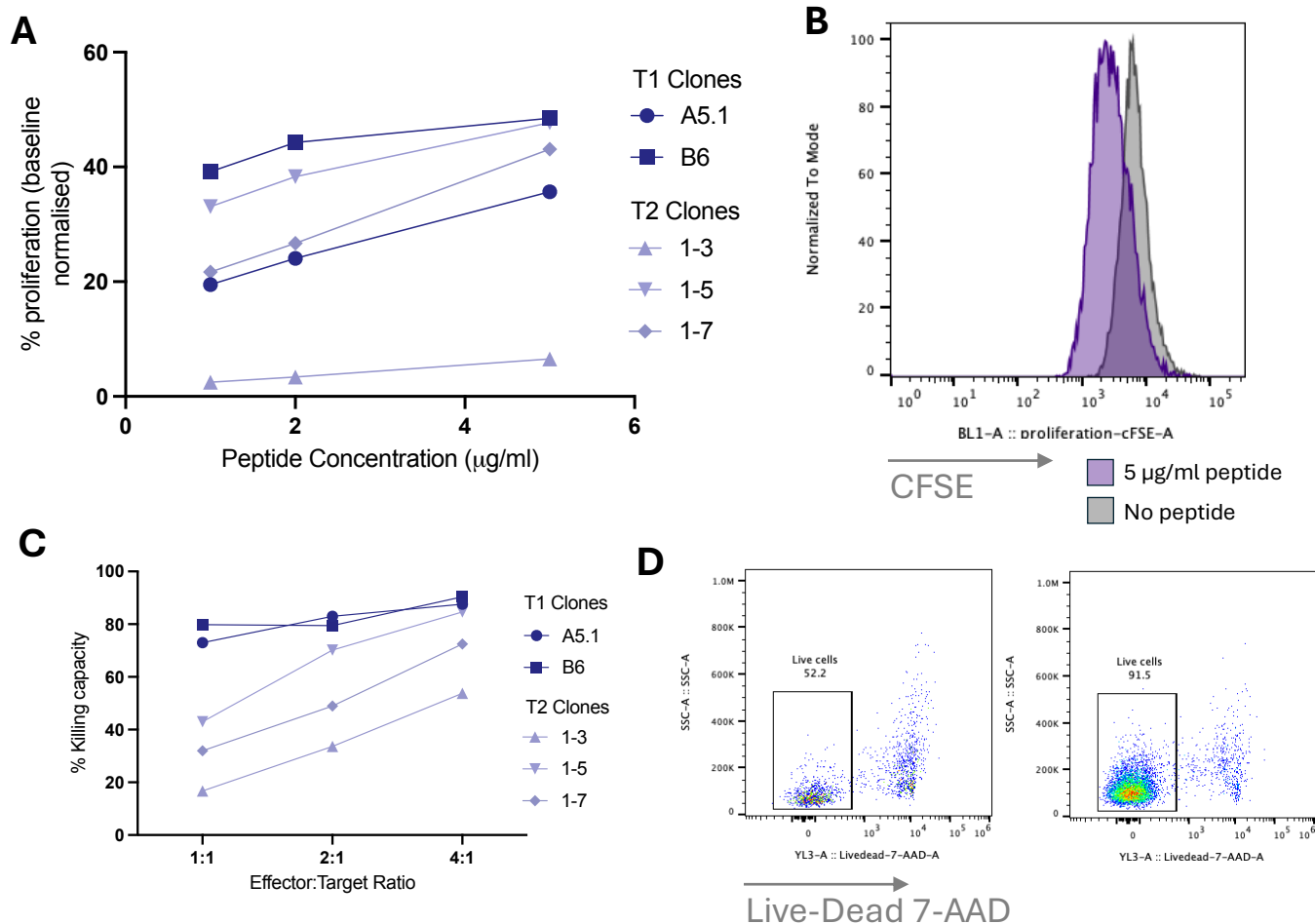


Figure 3.3: Differences in killing and proliferative capacity of M158-66-specific CD8⁺ T cell clones. (A) CFSE-stained T cell clones were cocultured with peptide-pulsed BCLs for 72 hours at an E:T ratio of 10:1. Proliferation was measured by CFSE dilution and proliferation of each clone was normalised to the baseline levels (no peptide BCLs). (B) Representative example of CFSE dilution. (C) CFSE stained peptide (2 $\mu\text{g/ml}$) loaded BCLs (or non-peptide loaded BCLs) were cocultured with T cell clones for 4 hours at three Effector:Target ratios. Live/Dead staining (7-AAD) was used to determine percentage of live BCLs via flow cytometry. Killing capacity was determined by normalizing the % of live peptide-pulsed BCLs at each E:T ratio to the % of live non peptide-pulsed BCLs at the corresponding E:T ratio. (D) Representative example of Live/Dead staining (7-AAD) for peptide loaded BCLs and non-peptide loaded BCLs. Abbreviations: BCL, B cell line.

all E:T ratios tested compared to the clones isolated at timepoint 2. Clone 1-3 had the lowest killing capacity, reaching only 50% at an E:T ratio of 4:1 (Fig 3.3C).

Together these functional assays demonstrate substantial variation in functionality of M1₅₈₋₆₆-specific clones, despite them bearing the same TCR, suggesting other factors must be affecting the functionality of the T cell clones. Thus, it is possible that ageing may influence the function of M1₅₈₋₆₆-specific T cells as the clones isolated from T1 exhibit higher killing capacity than those isolated at T2. However, due to the low number of clones tested no firm conclusions on the impact of ageing can be drawn from this *in vitro* data.

As metabolism is known to underpin T cell function and is altered with ageing⁹⁵ we next examined the metabolic profile of these T cell clones. To do this we used a Seahorse metabolic analyser which measures oxygen consumption rate (OCR) as a proxy for mitochondrial respiration and extracellular acidification rate (ECAR) as a proxy for glycolysis. While these measurements are ongoing a series of compounds are sequentially added to the cells: first oligomycin which inhibits ATP synthase and thus limits mitochondrial respiration, then the mitochondrial uncoupler BAM15 and finally rotenone/antimycin A which block Complex I and III of the electron transport chain. At baseline the clones from T1 have higher ECAR than the clones from T2 (Fig 3.4A), suggesting differences in metabolic profile between the clones from the different ages. Clone 1-3 has the lowest ECAR and OCR of the clones, demonstrating it has a low metabolic capacity (Fig 3.4A and Fig 3.4B) which may underpin its lower functional capabilities compared to the other clones. We also assessed the effect that peptide stimulation had on the metabolic profile of the clones. Upon peptide stimulation ECAR and OCR increased for all clones (Fig 3.4C and Fig 3.4D). Clone 1-3 exhibited the smallest increase, while Clone B6, one of the most functional clones, exhibited the greatest increase. These data suggest that ability to increase metabolic pathway usage upon activation may contribute to differences in functionality of M1₅₈₋₆₆-specific T cells.

Although useful to confirm functional differences between M1₅₈₋₆₆-specific T cells bearing the same TCR, drawing conclusions on the impact of aging from this *in vitro* data is challenging due to several limitations. These include low numbers of clones tested, low

cell counts of the clones (meaning most experiments could not be repeated) and the fact that long term culture outside the human body can alter the phenotype and function of T cells²⁷⁶. Thus, moving forward, we shifted our approach to investigate M1₅₈₋₆₆-specific T cell ageing using scRNAseq, which allows *ex vivo* profiling of a substantially larger number of cells.

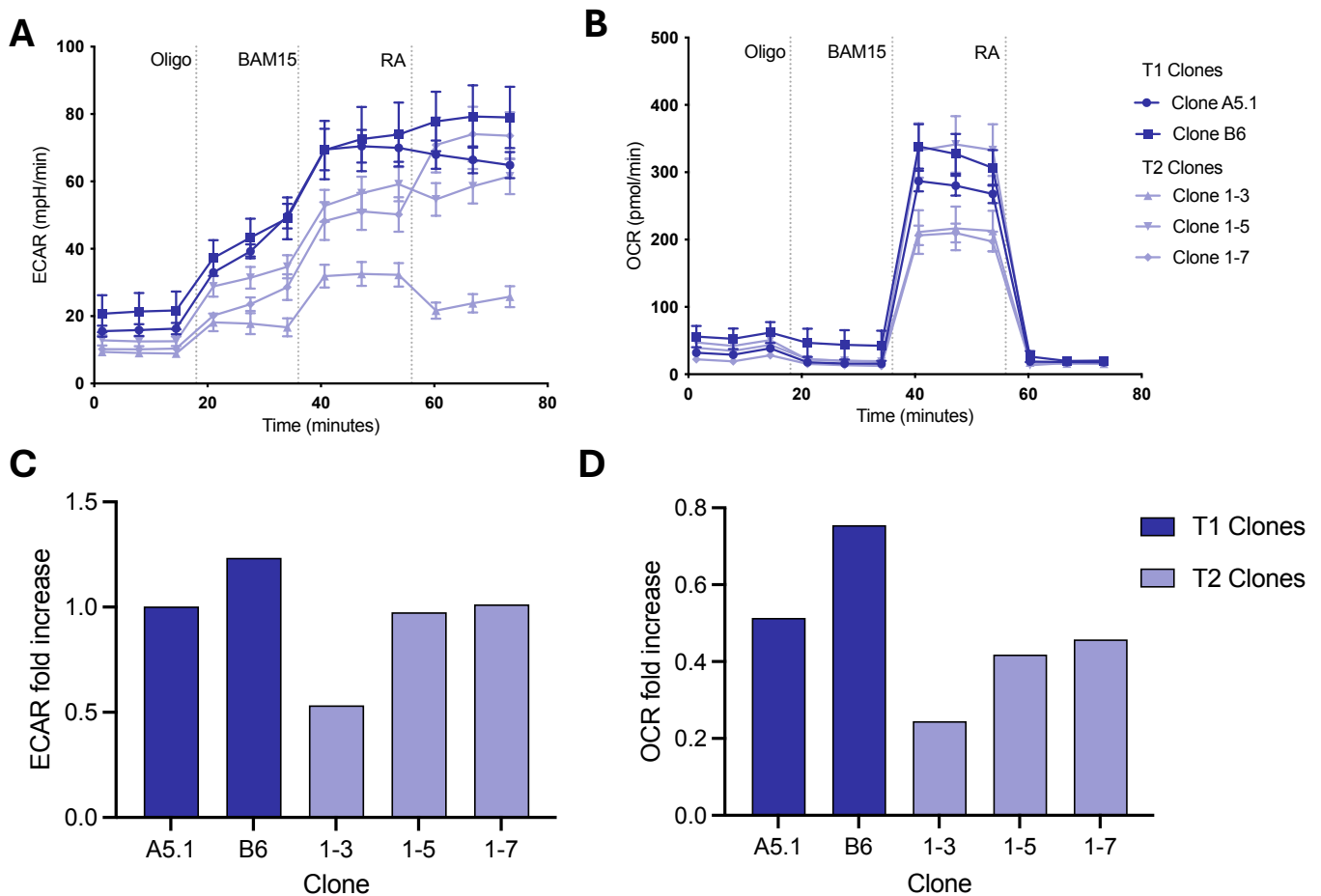


Figure 3.4: Metabolic pathway usage of M1₅₈₋₆₆-specific T cell clones. (A) ECAR and (B) OCR of M1₅₈₋₆₆-specific T cells were measured every 3 minutes. Oligomycin, BAM15 and RA were added at specified intervals. Fold increase in (C) ECAR and (D) OCR of M1₅₈₋₆₆-specific T cells upon addition of GIL peptide. Abbreviations: ECAR, extracellular acidification rate; OCR, oxidative consumption rate; RA, Rotenone and antimycin A.

3.2.2 Single cell dataset generation and phenotype of M1₅₈₋₆₆-specific T cells

3.2.2.1 Single cell RNA sequencing dataset generation

In addition to increasing the number of cells profiled, we aimed to examine M1₅₈₋₆₆-specific T cells from multiple individuals. It proved logistically challenging to increase the size of our donor pool as several inclusion criteria had to be met:

- Donors must be HLA-A*02:01 positive
- Historic PBMC samples (from approx. 2006) available
- Donor available to donate blood in 2023/2024

We found three donors who met these conditions and who were therefore included in the study. At the first sampling timepoint in 2006 the donors were all in their 30s and at the final sampling timepoint in 2023/2024 the donors were aged 51-57 (Fig 3.5A, Fig 3.5B). For two of the three donors we have an additional sample from 2016. Although these ages are younger than donors in many immune ageing studies^{271,277}, a recent study highlighted that individuals aged 50–64 years had increased risk from influenza compared to those aged 18–49 years²⁶². Thus, it was considered it important to study age-related changes in M1₅₈₋₆₆-specific T cells in middle aged individuals.

Before proceeding with scRNAseq, we confirmed that M1₅₈₋₆₆-specific T cells could be identified in each of the donors at each of the timepoints through tetramer staining of the cryopreserved PBMCs (Fig 3.5C). M1₅₈₋₆₆-specific CD8⁺ T cells were identified in PBMCs from all donors, although their frequency varied greatly (Fig 3.5D). The frequency of M1₅₈₋₆₆-specific T cells within CD8⁺ T cells in Donor 1 (D1) and Donor 3 (D3) decreased slightly with age from 2.5% at T1 to 1.67% at T3 for D1 and for D3 from 0.45% to 0.13%. For Donor 2 (D2) we observed the inverse trend, increasing from 2.1% at T1 to 9.3% at T3, indicating expansion of the M1₅₈₋₆₆-specific cells.

Having confirmed the presence of our cells of interest we proceeded with the design scRNAseq experiment (Fig 3.6A). We aimed to profile M1₅₈₋₆₆-specific T cells at the same time as pan CD8⁺ T cells, so that meaningful comparisons between them could be performed. Utilising a PE-conjugated GILGFVFTL-HLA-A*02:01 dextramer with a DNA barcode tag enabled us to do this. We also included two dextramers (dex) for common HLA-A*02:01 restricted epitopes from chronic viruses (pp65 from CMV and BMLF1 from

EBV), allowing us to compare the influenza-specific response to those-specific for chronic viral infections. To assist with phenotyping of the T cells we included several CITE-Seq antibodies targeting surface proteins (CCR7, CD45RA, CD28, CD27, CD57 and CX3CR1). Following staining and sorting (Fig 3.6A-B), feature barcode libraries (for dextramers and CITE-Seq antibodies), VDJ libraries and gene expression libraries were generated and sent for sequencing.

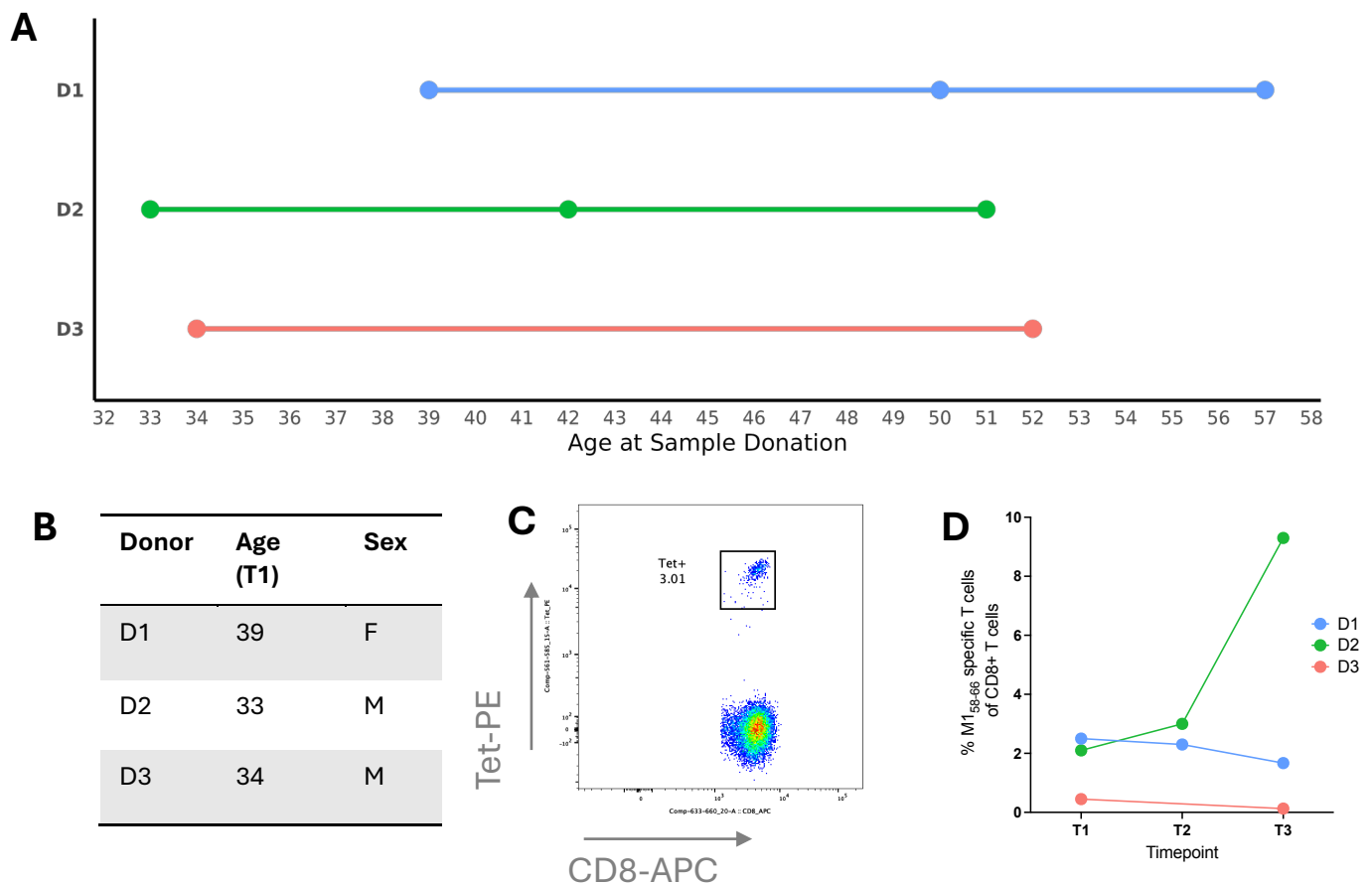


Figure 3.5: Age of donors and frequency of M1₅₈₋₆₆-specific T cells at each timepoint. **(A)** Ages of donors (D1-D3) at each blood sample donation. Dots represent donation. **(B)** Donor characteristics. **(C, D)** PBMCs were isolated from blood at each timepoint and stained with FACS antibodies and PE-conjugated GIL tetramer. **(C)** Representative FACS staining of tetramer and CD8. Gated on Live CD3⁺CD8⁺ cells. **(D)** Frequency of M1₅₈₋₆₆-specific within CD8⁺ T cells at each timepoint. Abbreviations: D1-D3, donor 1-3; FACS, fluorescent activated cell sorting, Tet, tetramer.

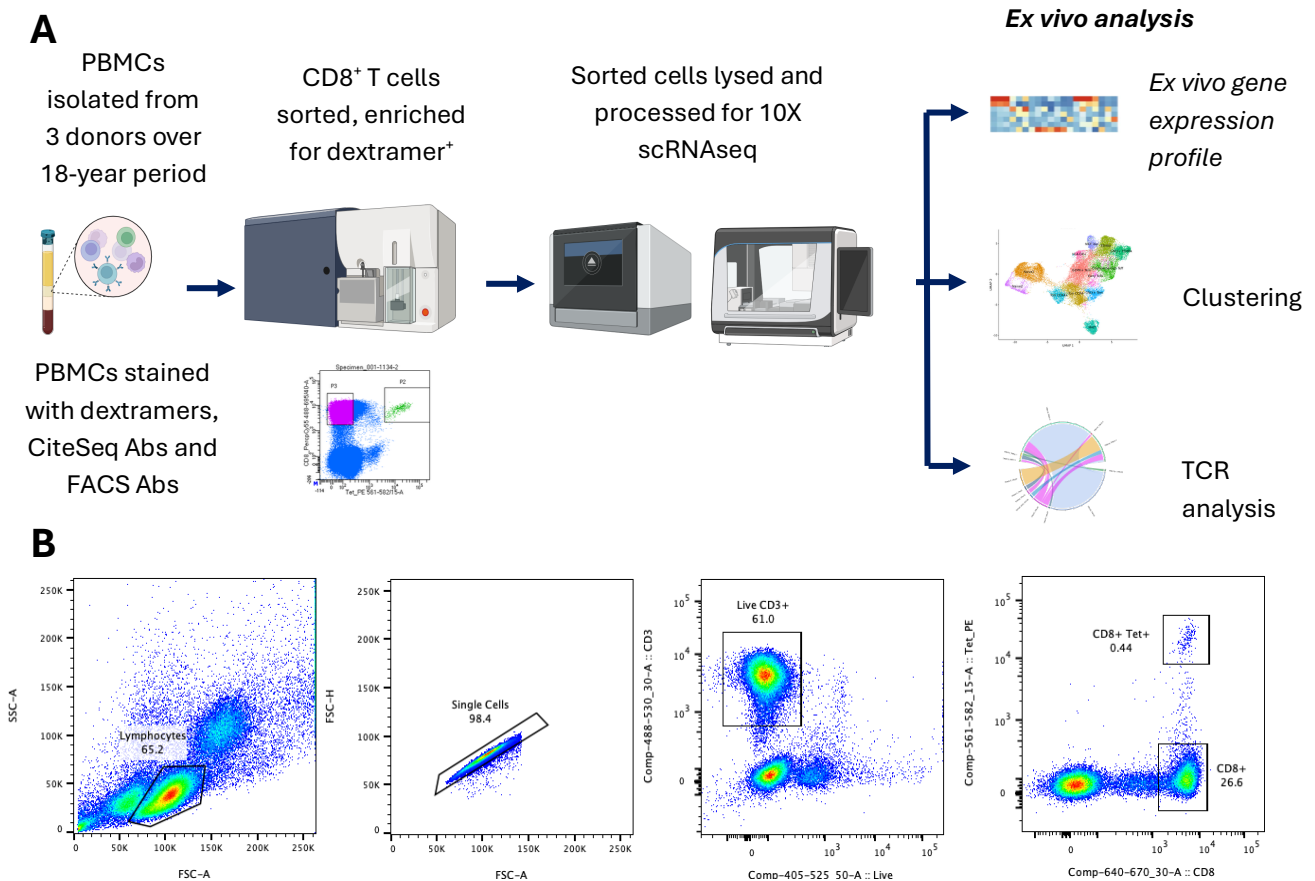


Figure 3.6: Experimental design for isolation of CD8⁺ T cells and Dextramer⁺ CD8⁺ T cells for 10X scRNAseq. (A) Isolated PBMCs were stained with FACS antibodies, CITE-Seq antibodies and PE-conjugated dextramers. CD8⁺ T cells and Dextramer⁺CD8⁺ T cells were sorted and lysed for 10X scRNAseq. Feature barcode, TCR and gene expression libraries were prepared and sequenced. 10X library preparation was done with help and technical assistance from Dr Elie Antoun. **(B)** FACS gating strategy for isolation of CD8⁺ T cells and Dextramer⁺CD8⁺ T cells. Abbreviations: scRNAseq, single cell RNA sequencing; CITE-seq, cellular indexing of transcriptomes and epitopes by sequencing; FACS, fluorescent-activated cell sorting, Abs, antibodies.

After sequencing, quality control, count normalisation, principal component analysis (PCA), integration, and unsupervised clustering were performed, resulting in the identification of twelve clusters. (Fig 3.7A). Surface expression of proteins targeted by CITE-seq antibodies (Fig 3.7B) along with gene expression (Fig 3.8) was used to give the clusters biologically meaningful names. Initially all cells (both Dex⁺ and Dex⁻) were analysed together.

Clusters of naïve CD8⁺ T cells were identified as CCR7⁺CD45RA⁺ (Fig 3.7B) with high expression of *SELL*, *LEF1* and *TCF7* (Fig 3.8). Similarly to a recently published paper⁹⁹ we identified two distinct Tcm clusters, one of which was CCR4⁺ and the other CCR4⁻ (Fig 3.8). Several Tem clusters were identified which we named based on their differential

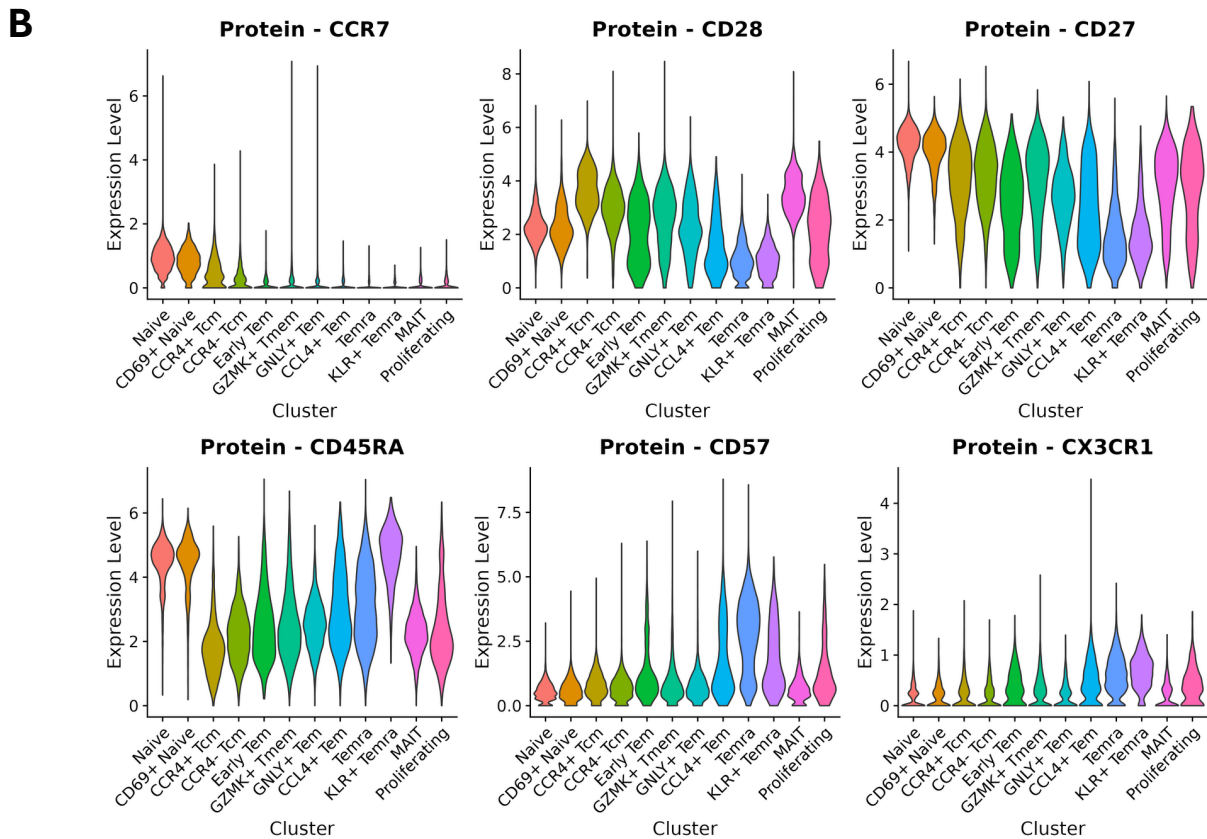
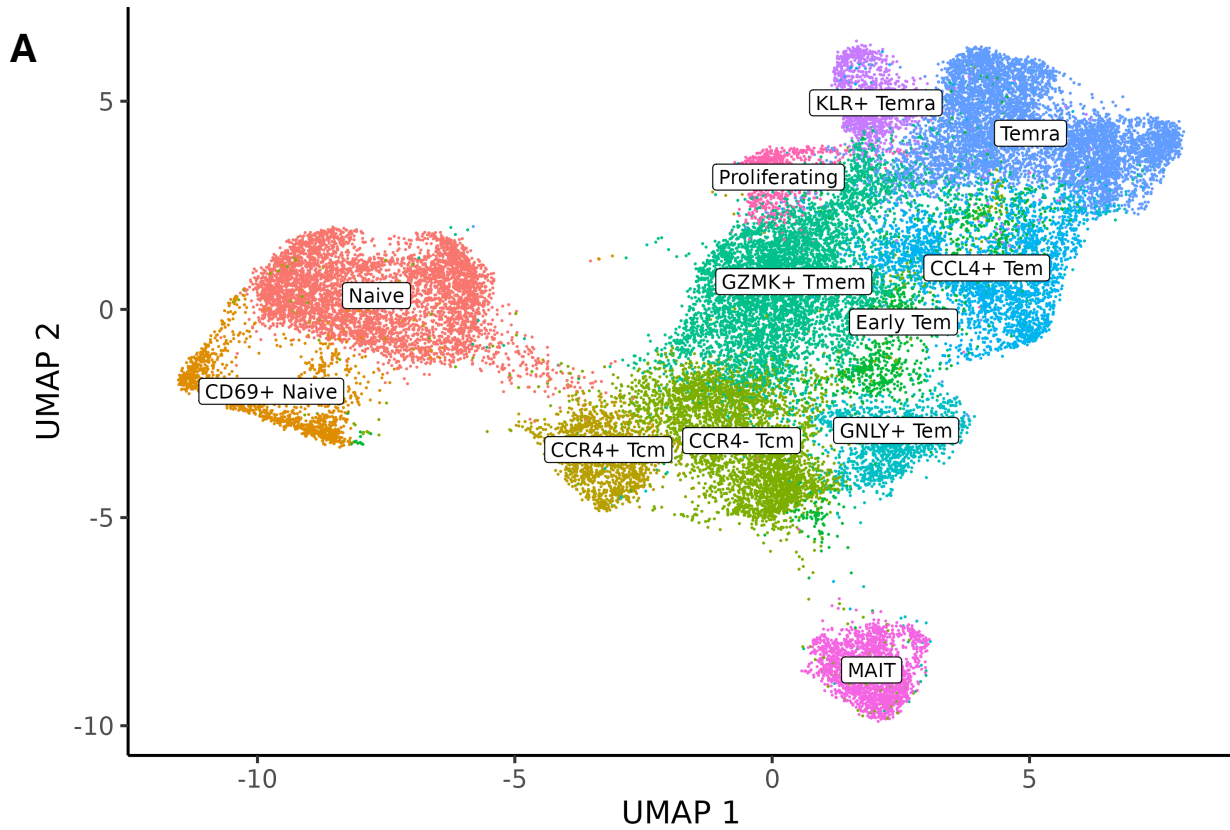


Figure 3.7: CD8⁺ T cell clusters and surface protein expression. (A) UMAP plot showing clusters of CD8⁺ T cells. (B) Expression level of surface proteins CCR7, CD28, CD27, CD45RA, CD57 and CX3CR1 determined by CITE-Seq antibodies across the different clusters. Abbreviations: UMAP, uniform manifold approximation and projection; Tem, T effector memory; Tcm, T central memory; Temra, terminally differentiated effector memory re-expressing CD45RA; MAIT, mucosal associated invariant T cells, CITE-seq, cellular indexing of transcriptomes and epitopes by sequencing.

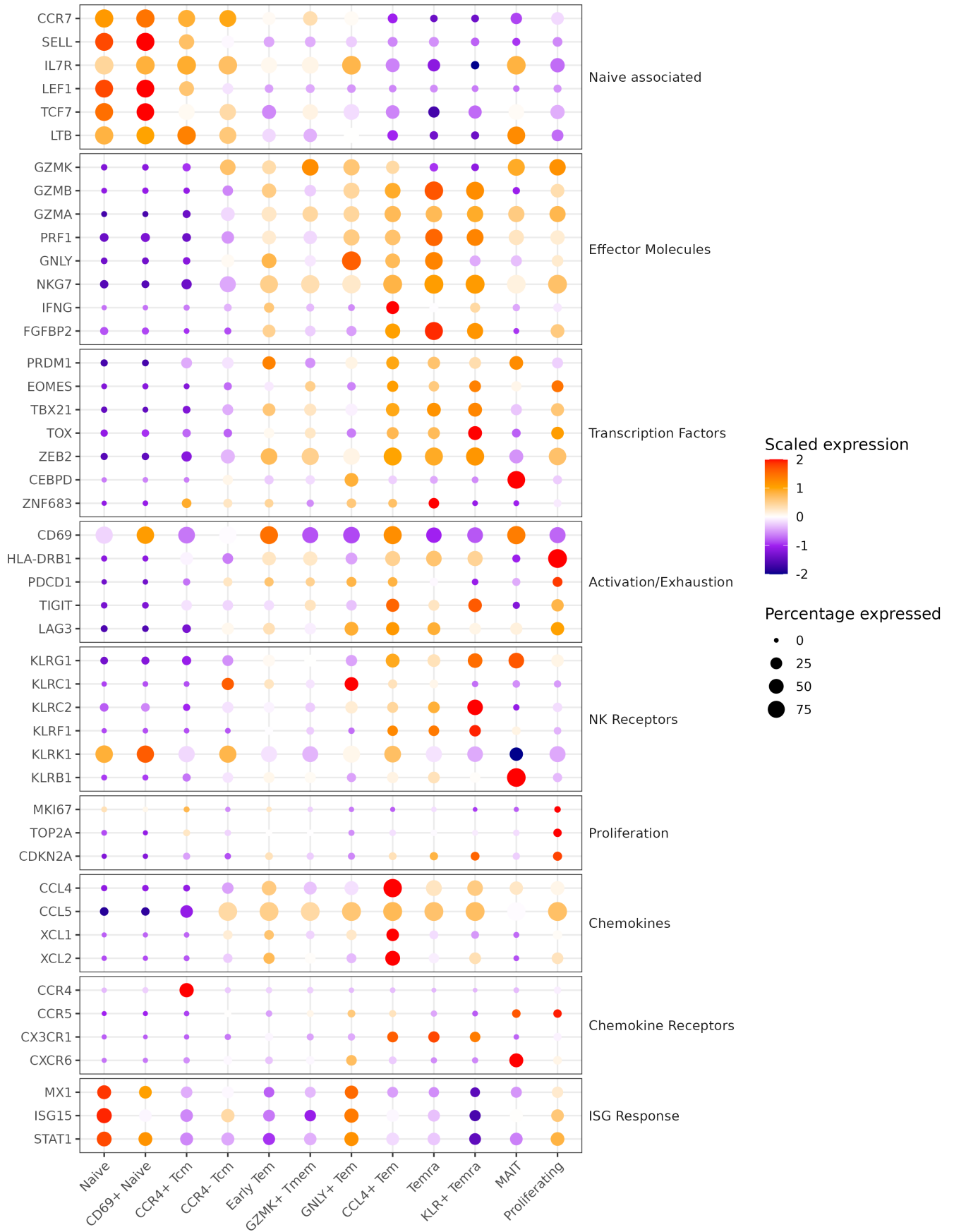


Figure 3.8: Expression of T cell related genes across CD8⁺ T cell clusters. Expression of each gene was scaled independently for plotting. Genes were selected for visualisation to aid in identification of T cell clusters. Abbreviations: ISG, interferon stimulated gene; NK, natural killer.

expression of effector molecules/chemokines (Fig 3.7A, Fig 3.8). Two Temra clusters were identified which were largely positive for CD45RA and CD57, a commonly used marker of senescence (Fig 3.7B).

To ensure that the integration method had performed sufficient mixing of our samples we analysed in which clusters the cells from the different timepoints (Fig 3.9A) and donors (Fig 3.9B) were located. Although we observed higher proportions of cells from some donors compared to others in certain clusters, we believe this reflects true biological variation between the donors. For instance, the MAIT cluster predominantly comprises cells from D2 and D3, with minimal contribution from D1 (Fig. 3.10B). Donor-specific scRNAseq analysis (data not shown) revealed that D1 had very low numbers of MAIT

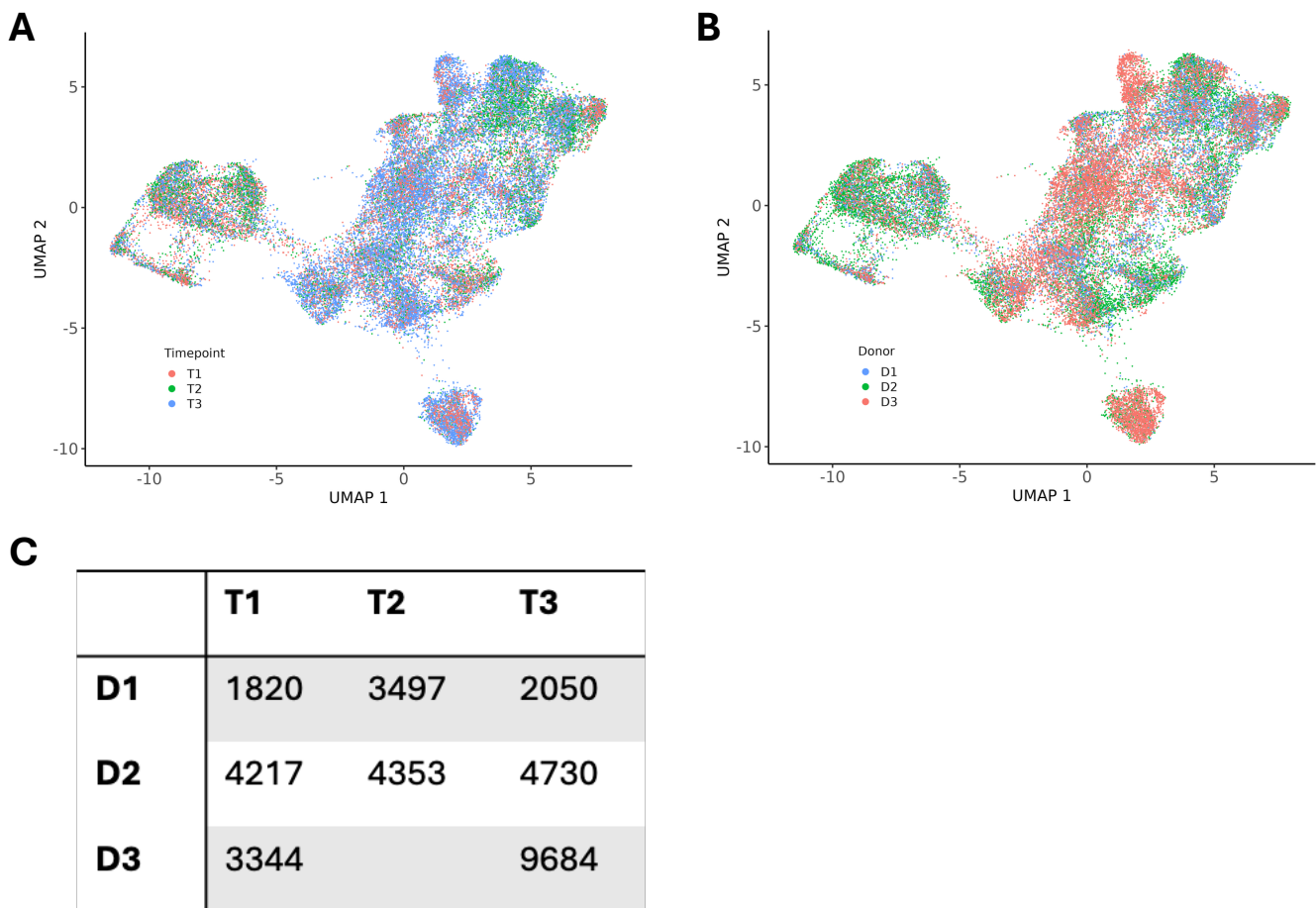


Figure 3.9: Integration of CD8⁺ T cells from each sample. (A) UMAP plot coloured by timepoint. **(B)** UMAP plot coloured by donor. **(C)** Number of cells obtained from each donor at each timepoint, after filtering and quality control. Abbreviations: UMAP, uniform manifold approximation and projection; T1-T3, timepoint 1-3; D1-3, donor 1-3.

cells, while D2 and D3 exhibited higher frequencies. Donor integration was satisfactory for clusters including the naïve clusters, which are typically present at comparable frequencies across individuals of similar ages, indicating that the integration method was adequate.

3.2.2.2 M1₅₈₋₆₆-specific T cells have a memory phenotype

After integrating the data and defining clusters, we examined which clusters contained M1₅₈₋₆₆-specific T cells and assessed their phenotype. M1₅₈₋₆₆-specific T cells (labelled as flu-dex+ in figures) were largely found in the CCR4⁺ Tcm cluster and across several Tem clusters (Fig 3.10A and Fig 3.10B). This is in line with previous studies that have phenotyped M1₅₈₋₆₆-specific T cells^{271, 272}. When examining age-related changes in the distribution of M1₅₈₋₆₆-specific cells across clusters, we observed an increase in the percentage located in the CCR4⁺ Tcm cluster and a decrease in the percentage located in the GNLY⁺ Tem cluster, but neither of these trends reached statistical significance (Fig 3.10B), likely due to only having three donors per timepoint. Using CITE-seq antibodies we determined that the M1₅₈₋₆₆-specific T cells were largely CCR7⁺CD45RA^{low}CD28⁺CD27⁺CD57⁻ (Fig 3.10C). This differed from the CMV-specific cells and some dextramer⁻ cells, which were CD45RA⁺CD57⁺CD28⁻CD27⁻, suggesting a more senescent or Temra like phenotype (Fig 3.10C). This is consistent with the recent findings of van de Sandt and colleagues, in which M1₅₈₋₆₆-specific T cells with a Temra phenotype were also not detected²⁷¹.

Having put the M1₅₈₋₆₆-specific T cells into context of the wider CD8⁺ population, we then aimed to investigate age-related transcriptomic changes in the M1₅₈₋₆₆-specific T cells. Prior to this analysis we randomly downsampled the number of M1₅₈₋₆₆-specific cells from each sample to 350 so that the higher number of cells from D2 did not skew the analysis in favour of this donor.

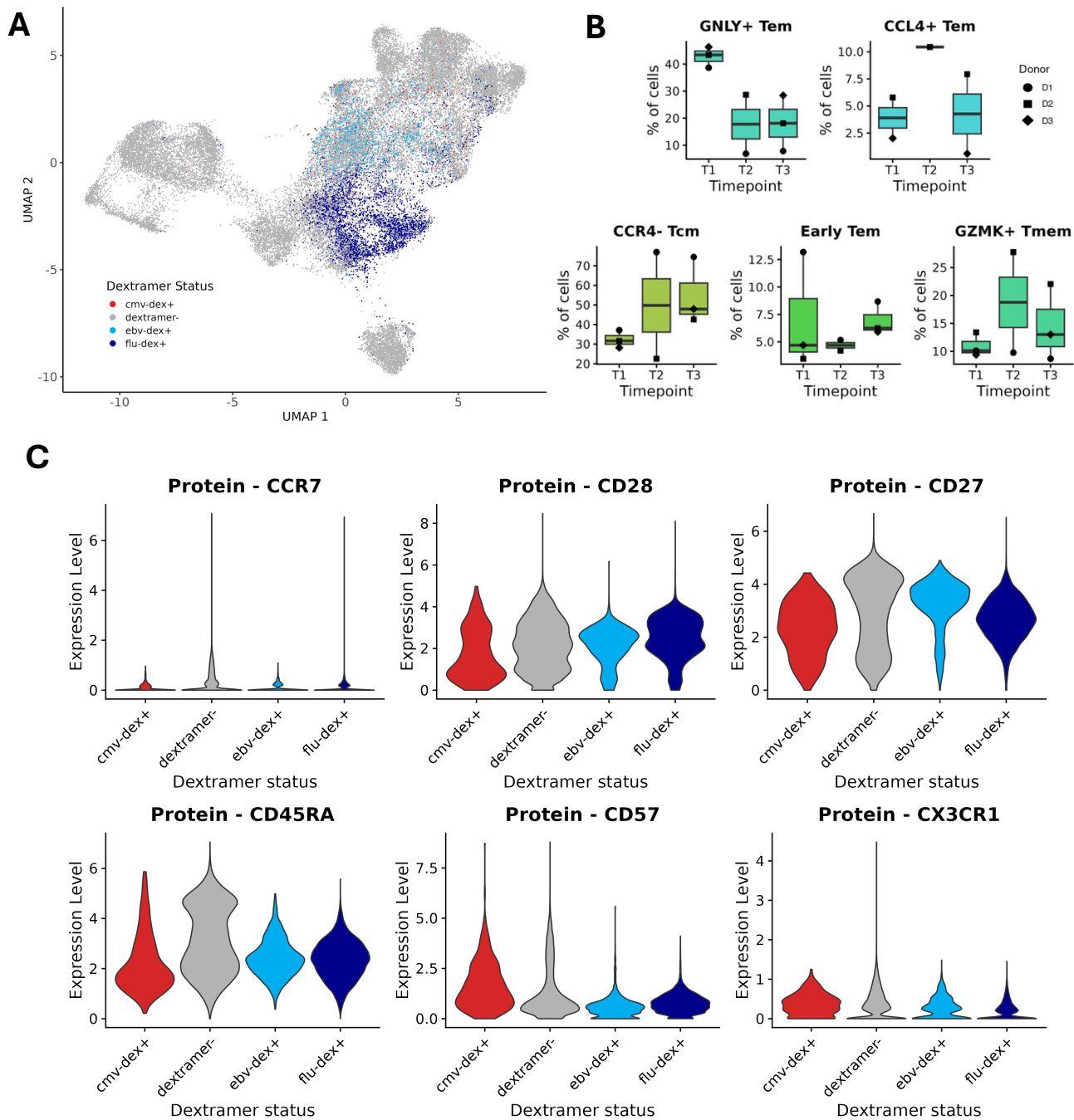


Figure 3.10: M1₅₈₋₆₆-specific CD8⁺ T cells have a memory phenotype. (A) UMAP plot coloured by dextramer status. (B) Percentage of M1₅₈₋₆₆-specific T cells in each cluster across the timepoints. Only clusters with >3% of M1₅₈₋₆₆-specific T cells in them were included. (C) Expression level of surface proteins CCR7, CD28, CD27, CD45RA, CD57 and CX3CR1 determined by CITE-seq antibodies. Abbreviations: UMAP, uniform manifold approximation and projection; CITE-seq, cellular indexing of transcriptomes and epitopes by sequencing; Tem, T effector memory; Tcm, T central memory; dex, dextramer.

3.2.3 Age-related changes in transcriptomic profile of M1₅₈₋₆₆-specific T cells

As we had identified differences in metabolic profile in our *in vitro* data (Fig 3.4) and altered metabolism is key feature of ageing T cells^{95, 278} we investigated metabolic pathway usage at the transcriptomic level. To do this we added module scores for oxidative phosphorylation (OXPHOS), glycolysis and fatty acid oxidation (FAO) using gene lists from the MSigDB hallmark collection²⁷⁹. From T1 to T3, as the donors age from ~30 to ~50, there was a decrease in both OXPHOS and glycolysis scores (Fig 3.11A and Fig 3.11B) but an increase in FAO (Fig 3.11C). FAO is a metabolic pathway frequently utilised by memory T cell subsets²⁸⁰, however recent studies have demonstrated that metabolic flexibility underpins efficient CD8⁺ T cell function²⁸¹ so the decrease in glycolysis and OXPHOS related genes could leave these ageing M1₅₈₋₆₆-specific less able to utilise all fuel sources, thus affecting their functionality. In particular, we observed an age-related reduction in several genes that encode proteins that form Complex I and Complex IV (Fig 3.11D) of the electron transport chain, suggesting potential age-related mitochondrial dysfunction. Age-associated changes in the other electron transport chain complexes were also observed but were less marked. Although more variable we also observed age-related alterations in genes encoding glycolytic enzymes (Fig 3.11E). Collectively, these results demonstrate that M1₅₈₋₆₆-specific T cells undergo age-related shifts in their metabolic profile.

Given that cytotoxicity is a key function of CD8⁺ T cells, and that we observed age-related differences in the killing capacity of M1₅₈₋₆₆-specific T cell clones (Fig. 3.3C), we next examined genes associated with this cytotoxic function. Using a previously published gene list of cytotoxic effectors¹⁴² to add a module score to the M1₅₈₋₆₆-specific cells, we observed no significant differences between T1 and T3. However, T2 was significantly different when compared to both T1 and T3 (Fig 3.12A). When looking at the cytotoxic effectors individually, we observe some significant differences between T1 and T3 (Fig 3.12B). We observed increased expression of Granzyme K with age, which agrees with previous studies looking at T cell ageing^{99, 282, 283}. Granzyme A and NKG7 also exhibited increased expression with age. Conversely, we observed a decrease in granulysin with age (Fig 3.12B).

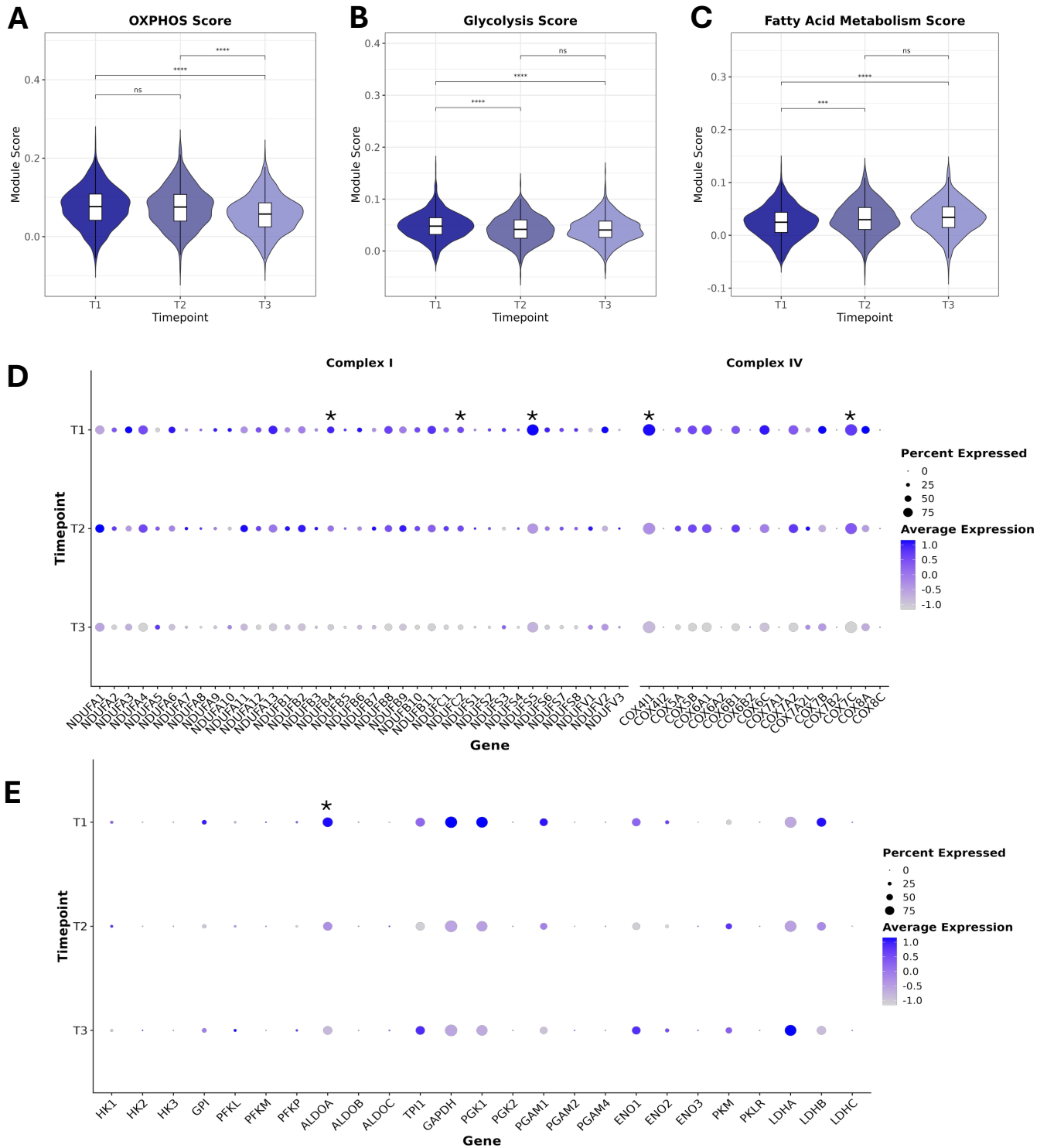


Figure 3.11: M1₅₈₋₆₆-specific CD8⁺ T cells exhibit age-associated changes in metabolism. OXPHOS (A), Glycolysis (B) and Fatty acid metabolism (C) scores were calculated based on expression of the 200 genes in the hallmark OXPHOS, hallmark glycolysis and hallmark FAO gene sets from MSigDB, respectively. Scores were determined for M1₅₈₋₆₆-specific cells at each timepoint. Boxes indicate the interquartile range with the line within each box representing the median. Adjusted *P* values were calculated using the Wilcoxon rank-sum test with Benjamini–Hochberg correction for multiple testing. (D) Dot plot of average expression of Oxphos genes in complex I and complex IV in M1₅₈₋₆₆-specific T cells at each timepoint. (E) Dot plot of average expression of enzymes involved in glycolysis in M1₅₈₋₆₆-specific T cells at each timepoint. Genes marked with a * had a significant adjusted *P* value when comparing with T1 and T3, determined using MAST test with Bonferroni correction for multiple testing. ****, *padj* ≤ 0.0001; ***, *padj* ≤ 0.001; *, *padj* ≤ 0.05; ns, not significant. Abbreviations: OXPHOS, oxidative phosphorylation; FAO, fatty acid oxidation; MSigDB, Molecular Signatures Database.

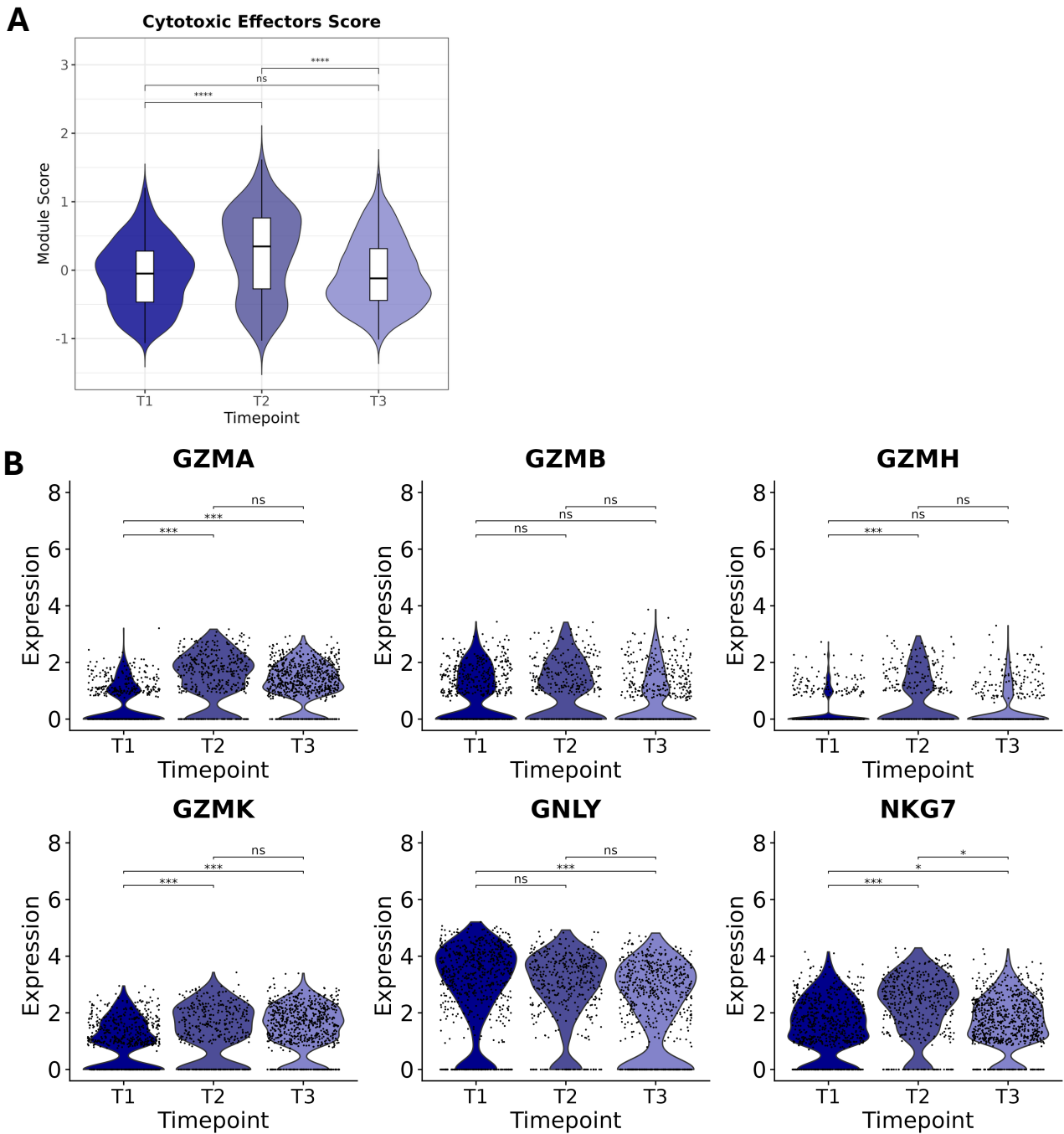


Figure 3.12: M1₅₈₋₆₆-specific CD8⁺ T cells exhibit age-associated changes in cytotoxic effectors. (A) Cytotoxic effector module score calculated based on expression of GZMA, GZMB, GZMK, GZMH, GZMM, GNLY and NKG7. Scores were determined for M1₅₈₋₆₆-specific cells at each timepoint. Boxes indicate the interquartile range, with the line within each box representing the median. *P* values were calculated using the Wilcoxon rank-sum test with Benjamini–Hochberg correction for multiple testing. **(B)** Violin plot of selected cytotoxic effectors at each timepoint. Differential expression was assessed using MAST implemented in Seurat, with the Bonferroni correction for multiple testing. ****, *padj* ≤ 0.0001; ***, *padj* ≤ 0.001; **, *padj* ≤ 0.01; *, *padj* ≤ 0.05; ns, not significant.

Recent papers have highlighted the issue of pseudo replication^{284, 285} within scRNAseq analysis, meaning that significance is obtained due to the sheer the number of very similar cells tested. To mitigate against this, we performed pseudobulk analysis, collapsing all M1₅₈₋₆₆-specific T cells from a donor at each timepoint into a single datapoint. We then performed differential gene expression (Fig 3.12) analysis comparing T1 to T3 (this analysis could not be performed with T2 as we only have samples from 2 donors at this timepoint). Using the pseudobulk approach, we observed several genes which were more highly expressed at T3 compared to T1 (Fig 3.13) including the integrins *ITGB2* and *ITGA6*, the proinflammatory cytokine *IL32*, *GMZA* and genes associated with TCR signalling and activation (*FOSB*, *JUNB* and *AHNAK1*). We observed decreased expression of the death receptor *TNFSFR25* and *TNFSFR10* (which encodes TRAIL) with age.

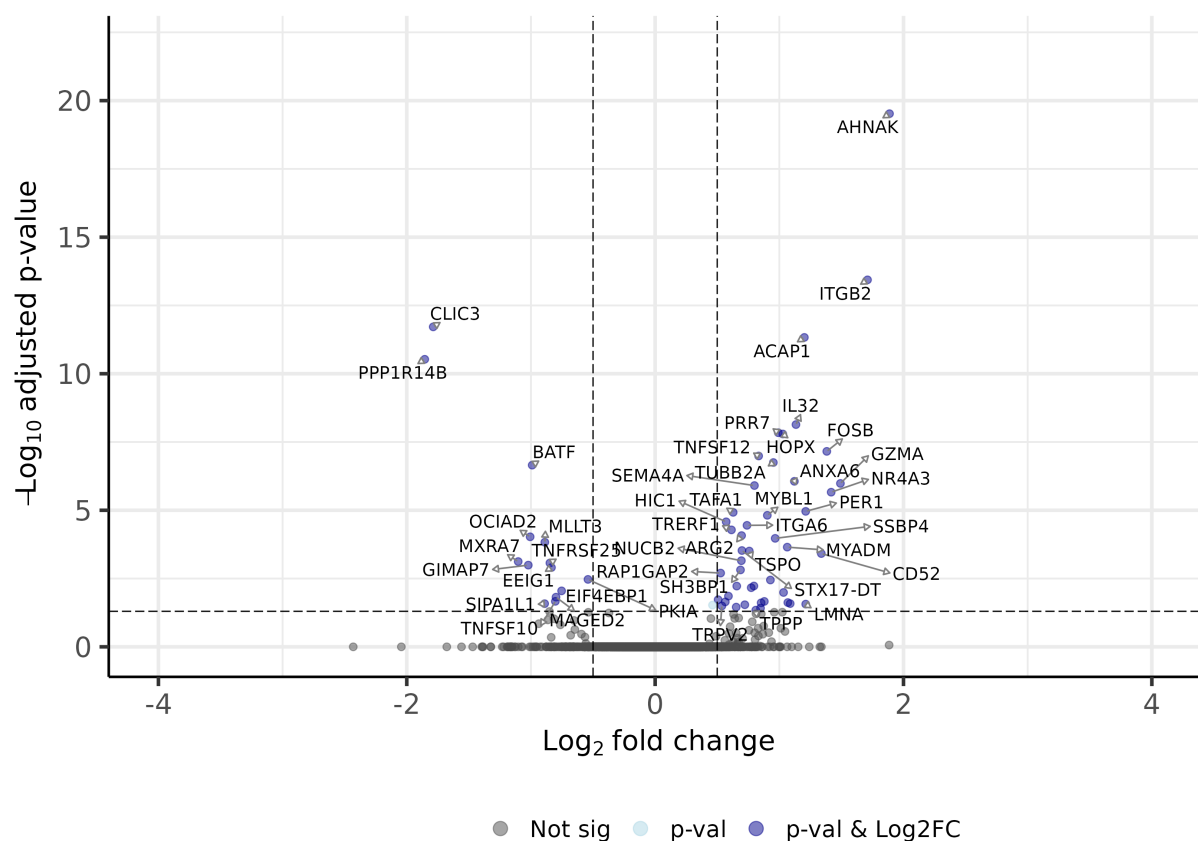


Figure 3.13: Differentially expressed genes in M1₅₈₋₆₆-specific T cells when comparing T1 to T3. Pseudobulk was performed to collapse all M1₅₈₋₆₆-specific cells each sample into a single datapoint. DESeq2 was used to perform differential gene expression analysis to compare T1 to T3. Dotted lines represent the Log₂FC cut off (0.5) and adjusted *P* value (0.05) cut off.

To investigate how antigen-specificity influences ageing trajectory, we then investigated whether these age-related changes were found only in M1₅₈₋₆₆-specific T cells or were reflective of changes to the CD8⁺ T cell population as a whole. To perform this comparison, we first restricted the analysis to the five clusters that M1₅₈₋₆₆-specific T cells were most enriched (Fig 3.10B). M1₅₈₋₆₆-specific cells were then excluded from these clusters, leaving CD8⁺ T cells with other specificities but similar phenotypes. The remaining cells were aggregated into pseudobulk profiles for each donor at each timepoint and used for downstream analysis. We then performed DGE analysis comparing T3 to T1 and then compared the changes seen in these cells to the changes in the M1₅₈₋₆₆-specific cells in the same time frame (Fig 3.14A). Several genes were upregulated to a similar extent in M1₅₈₋₆₆-specific cells compared to related clusters and were significantly different in both comparisons, including *GZMA*, *IL32*, *ITGB2* and *AHNAK* (Fig 3.14A), suggesting that these age-related changes are universal to CD8⁺ T cells. However, several genes were only significantly different in M1₅₈₋₆₆-specific cell, indicating that there are changes unique to, or exacerbated in, ageing M1₅₈₋₆₆-specific cells.

We repeated this analysis, this time, comparing the changes in M1₅₈₋₆₆-specific cells to changes in the CMV and EBV-specific cells (Fig 3.14B). Again, some genes were only significantly different in the M1₅₈₋₆₆-specific T cells, several of which are associated with TCR signalling (*JUND*, *ADGRE5*, *PRR7* and *NR4A3*). This suggests that the intermittent but frequent exposure to antigen may drive the increased expression of these genes in the M1₅₈₋₆₆-specific cells (Fig 3.14A and Fig 3.14B). Visualisation of these genes in the M1₅₈₋₆₆-specific cells across each of the timepoints revealed a step wise increase from T1 to T2 and from T2 to T3 (Fig 3.14C). Additionally, genes linked to metabolism (*ODC1* and *ARG2*) were only differentially expressed in the M1₅₈₋₆₆-specific cells (Fig 3.14A-C).

Together this comparative analysis suggests that some of the age-related changes in the M1₅₈₋₆₆-specific cells are reflective of changes to the wider CD8⁺ T cell population, but that there are some changes which appear to be unique to, or exacerbated in, M1₅₈₋₆₆-specific cells. This suggests that T cells exposed to regular but intermittent antigen stimulation, may experience an altered ageing trajectory and that antigen-specificity influences ageing trajectory.

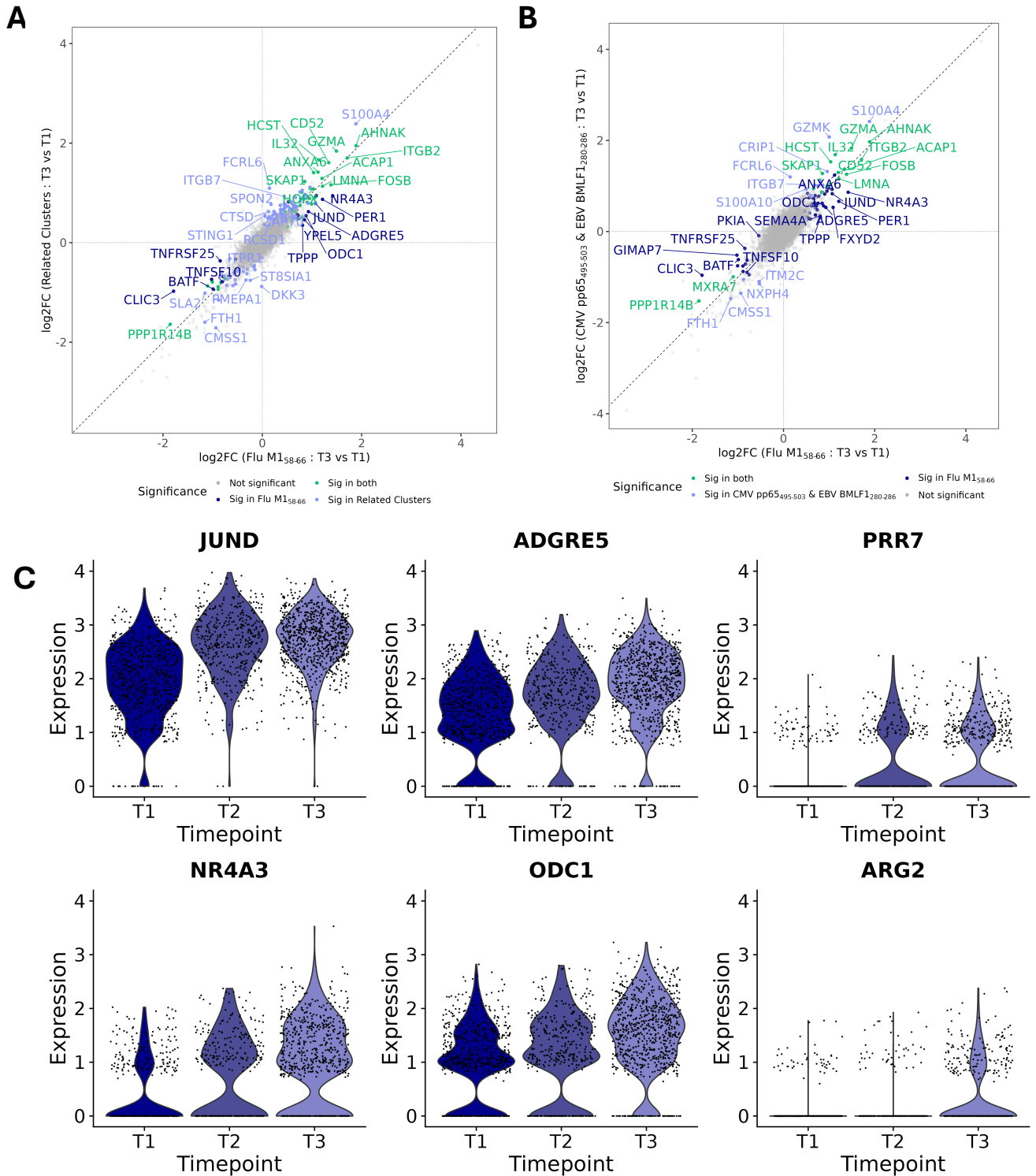


Figure 3.14: Comparison of age-associated differentially expressed genes in M1₅₈₋₆₆-specific T cells and other CD8⁺ T cell populations. (A) Comparison of Log₂FC of Flu-dex+ cells from T1 to T3 to Log₂FC of cells in related clusters from T1 to T3. **(B)** Comparison of Log₂FC of Flu-dex+ cells from T1 to T3 to Log₂FC of CMV-dex+ or EBV-dex+ from T1 to T3. DESeq2 was used to perform differential gene expression analysis to compare T1 to T3 with an adjusted *P* value < 0.05 (calculated using Bonferoni correction) and Log₂FC threshold of 0.5 used to determine significance. **(C)** Violin plots of genes found to be significantly different in M1₅₈₋₆₆-specific T cells when comparing T1 to T3. Abbreviations: Log₂FC, log₂ fold chance; dex, dextramer; T1-T3, timepoint 1-3.

3.2.4 Reduced diversity of the M1₅₈₋₆₆-specific TCR repertoire with age

As recent studies have demonstrated age-related changes in the M1₅₈₋₆₆-specific TCR repertoire we aimed to address this using our longitudinal dataset. To achieve this, we filtered M1₅₈₋₆₆-specific (flu-dex⁺) cells to include only those with fully resolved variable (V) and joining (J) gene segments for both the TCR α and TCR β chains. Of the 4199 flu-dex⁺ cells, 3095 remained after filtering. Due to variation in both the individual repertoire and age-related trends we performed TCR analysis for the three donors individually. We first addressed TRAV and TRBV pairings and whether these changed with age (Fig 3.15A-C).

For all donors, at all timepoints analysed, *TRBV19* was the dominant TCR β V gene used (Fig 3.15A-C), which is in line with previous literature showing expansion of clonotypes bearing *TRBV19*^{243, 252, 254}. In addition to *TRBV19*, the donors utilised between two and five other TRBV genes in their M1₅₈₋₆₆-specific repertoire but none of these other genes were shared between the donors. The TRAV gene usage was much more variable, with *TRBV19* alone pairing with between 7 and 11 different TRAV genes in each of the donors (Fig 3.15A-C). However, there is a noticeably dominant TRAV-TRBV pairing for each of the donors; for D2 and D3 this is *TRAV27-TRBV19* (very public, sometimes considered to be the “canonical” pairing for M1₅₈₋₆₆-specific TCRs²⁵⁸) while for D1 the dominant pairing was *TRAV25-TRBV19*. For D1 and D2 the dominant pairing becomes increasingly dominant with age (Fig 3.15A-B), although this is less obvious for D2 as the *TRAV27-TRBV19* pairing is already very dominant at T1.

As J genes contribute substantially to TCR diversity and the composition of the CDR3 region, we next examined J genes in combination with V genes. For D1, the dominant clonotype is TRAV25_TRAJ42_TRBV19_TRBJ2-3, which is expressed by 52% of the T cells at T1, increasing to 75% at T2 and 92% at T3 (Fig 3.16A). There is a corresponding reduction in other subdominant TCRs such as TRAV8-3_TRAJ37_TRBV19_TRBJ2-7 decreasing from 16% to less than 1%. Additionally, some clones are lost completely, with 16 unique clonotypes present at T1 compared to only 7 at T3 (Fig 3.16A).

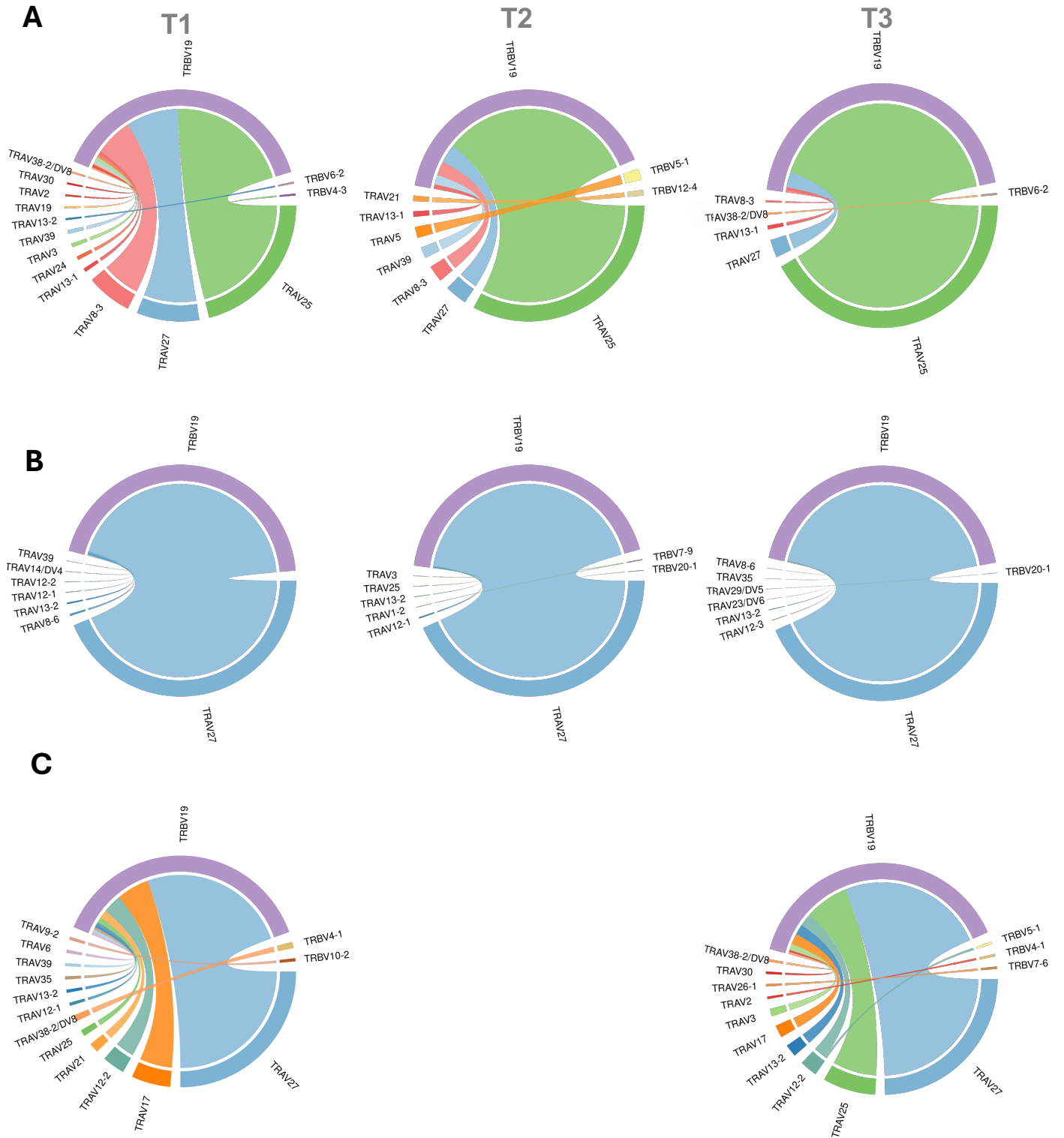


Figure 3.15: Age-associated changes in TRAV-TRBV pairing in M1₅₈₋₆₆-specific TCRs. M1₅₈₋₆₆-specific T cells were filtered to only include cells that had fully resolved variable and joining genes for both TCR α and TCR β . Circos plots were generated to show pairing of TRBV genes with TRAV genes. Connecting lines indicated TRAV-TRBV gene pairing coloured based on their TRAV usage. The thickness of each connecting segment is proportional to the number of T cells with the particular TRAV-TRBV. **(A)** TRAV-TRBV pairings for D1 from T1-T3. **(B)** TRAV-TRBV pairings for D2 from T1-T3. **(C)** TRAV-TRBV pairings for D3 from T1-T3. Abbreviations: TCR, T cell receptor; D1-D3, donor 1-3; T1-3, timepoint 1-3.

As many previously identified M1₅₈₋₆₆-specific TCR are public, meaning the exact sequence is found in more than one individual, we investigated whether any of the TCRs in our dataset were public. Initially we did this analysis within our own dataset but due to the very small donor pool, no paired public TCR $\alpha\beta$ sequences were identified. We therefore used a recently published dataset (from van de Sandt and colleagues) of TCR sequences of M1₅₈₋₆₆-specific T cells from a cohort of over 50 donors²⁷¹. Clonotypes in our dataset were classified as public if they were also found in at least one donor in the van de Sandt dataset. Public clonotypes were identified in every donor in our dataset (Fig 3.17) but the frequency of these varied greatly between the donors; the repertoire of D2 was dominated by a public clonotype while D1 and D3 had largely private repertoires.

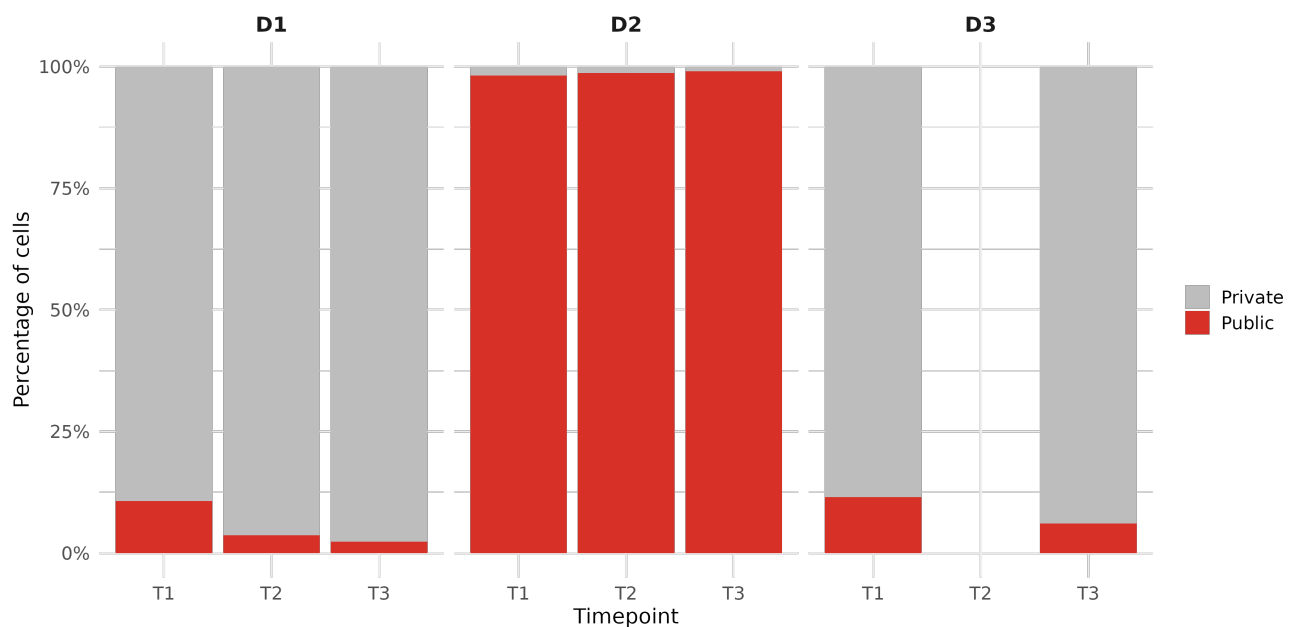


Figure 3.17: Percentage of cells bearing public and private M1₅₈₋₆₆-specific TCR clonotypes across timepoints. Cells were filtered to only include cells with fully resolved TCR α and TCR β chains (CDR3s, variable and joining genes). Clonotypes were classified as public if found in at least one donor in our dataset and at least one donor in a published dataset of M1₅₈₋₆₆-specific TCRs from 50 individuals. Abbreviations: TCR, T cell receptor.

We then analysed CDR3 sequences from the three donors together in more detail. We filtered cells to include those that had fully resolved CDR3 α and CDR3 β sequences (3148 and 3830 cells, respectively). We quantified CDR3 similarity by computing pairwise Levenshtein distances between the amino-acid sequences for the CDR3 sequences for

TCR α and TCR β separately (Fig 3.18A and Fig 3.18B). Edges were drawn between clonotypes when sequences had Levenshtein distances ≤ 2 , meaning connected CDR3 sequences differ from each other by a maximum of two amino acids. We identified several public CDR3 sequences within our dataset, as well as several clonotypes that contain motifs such as “GGG” in TCR α and “RS” in TCR β that have previously been identified to be public motifs²⁷⁴ (Fig 3.18A and Fig 3.18B). We determined the percentage of cells at each timepoint that have a TCR containing a public motif (Fig 3.18C and Fig 3.18E). The majority of cells at all timepoints have CDR3 sequences public motifs but there is an increase in the percentage from T1 to T3, suggesting that the M1₅₈₋₆₆-specific repertoire may be becoming more public as the donors age from their 30s to their 50s.

Finally, we examined the diversity of the M1₅₈₋₆₆-specific TCR repertoire using the Gini-Simpson and Shannon indices²⁸⁶ and compared these to those for the dextramer⁻ cells, as well as the CMV-specific and EBV-specific repertoires. The Gini-Simpson index, also known as Simpsons diversity index, accounts for the number of unique TCR sequences, and the relative abundance of each TCR within the repertoire, emphasising the contribution of abundant clonotypes to overall diversity. A value closer to 1 indicates greater repertoire diversity. The Shannon index accounts for the number of unique clonotypes and their evenness, giving greater weight to rare clonotypes than the Gini-Simpson index. The Shannon index increases when several clonotypes are present at similar frequencies and decreases when the repertoire is dominated by a few expanded clonotypes. Although there is variation in the number of cells (Fig 3.19C, Fig 3.19F, Fig 3.19I) with fully resolved TCRs at each timepoint these do not appear to have a profound effect on the calculated diversity indices. Across all 3 donors there is a reduction in both the Gini-Simpson and Shannon indices for M1₅₈₋₆₆-specific cells from T1 to T3, demonstrating reduced TCR diversity with age (Fig 3.19A-B, Fig 3.19D-E, Fig 3.19G-H). This reduction is very minor for D2 as the TCR repertoire is already very homogenous at T1 (Fig 3.19D-E). As expected, the dextramer⁻ cells show a substantially more diverse TCR repertoire than any of the antigen-specific responses. For D1 and D2 the M1₅₈₋₆₆-specific repertoire is less diverse than the CMV-specific responses for these donors, while for D3 the M1₅₈₋₆₆-specific repertoire is more diverse than the EBV-specific response. Again, this

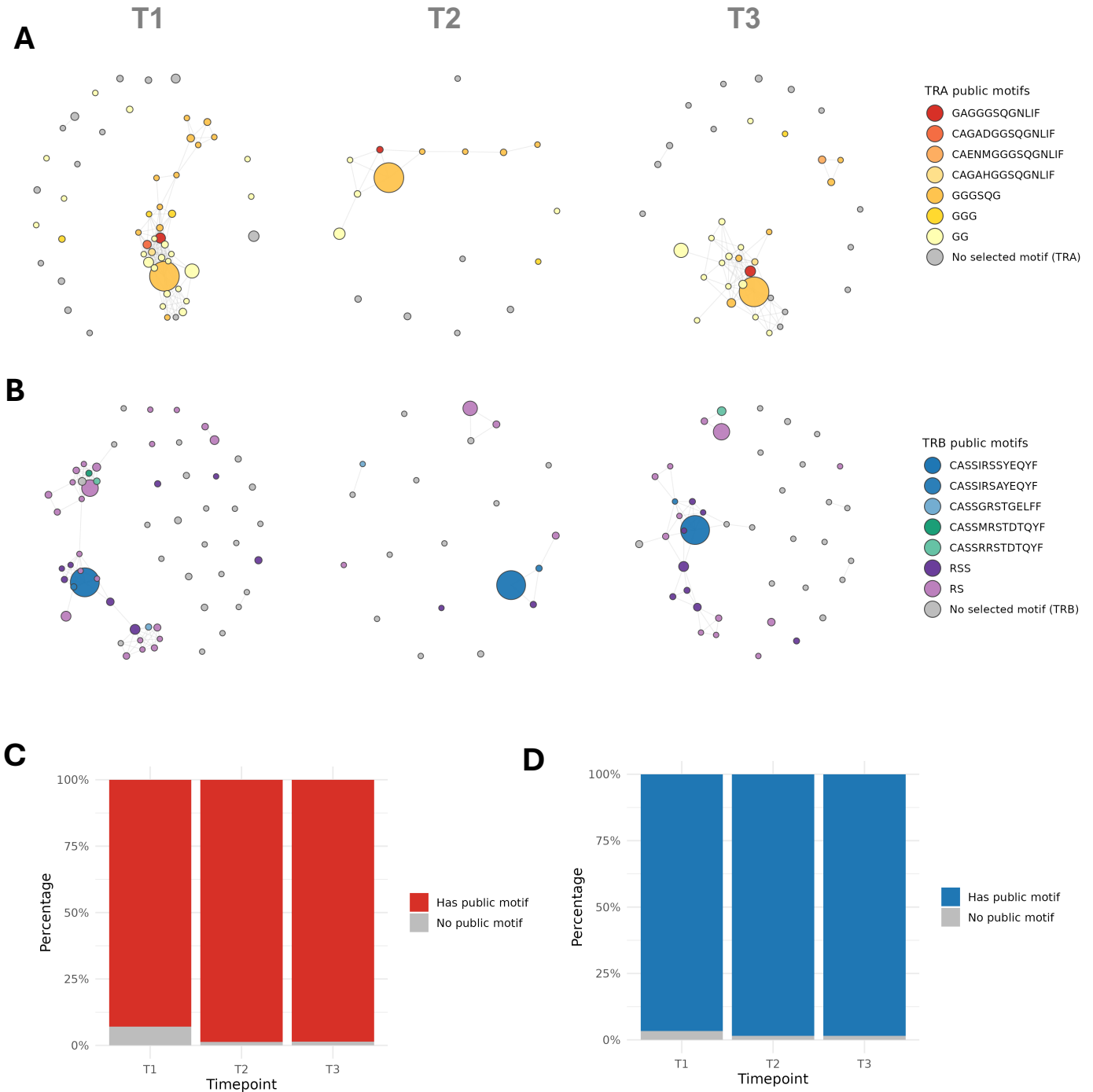


Figure 3.18: Public motifs in CDR3 sequences in TCR α and TCR β chains from M1₅₈₋₆₆-specific TCRs. Cells were filtered to only include cells with fully resolved TCR α and TCR β chains (CDR3s, variable and joining genes). Levenshtein distances between the amino-acid sequences for the CDR3 sequences were calculated and edges were drawn between clonotypes when CDR3 sequences had Levenshtein distances ≤ 2 . Size of the dot refers to size of the clonotype. Public CDR3 sequences, identified in our dataset, and CDR3 sequences with previously identified public motifs were coloured. Motif labels are mutually exclusive, with longer motifs taking precedence over shorter motifs. **(A)** TCR α CDR3 sequence similarity graphs for each timepoint. **(B)** TCR β CDR3 sequence similarity graphs for each timepoint. **(C)** Percentage of cells with TCR α CDR3 sequences containing public motifs. **(D)** Percentage of cells with TCR β CDR3 sequences containing public motifs. Abbreviations: TCR, T cell receptor, CDR, complementarity determining region.

may be due to the lower frequency of M1₅₈₋₆₆-specific cells in D3, compared to D1 and (Fig 3.5D) which suggests that these clones have not undergone hyper expansion.

Altogether our data suggest that the M1₅₈₋₆₆-specific repertoire becomes less diverse through the ages 30 to 57, largely due to expansion of dominant clonotypes such as TRAV27_TRAJ42_TRBV19_TRBJ2-7. However, our data indicate that there is significant inter-donor variability in the age-related changes in the M1₅₈₋₆₆-specific repertoire, highlighting the importance of longitudinal studies.

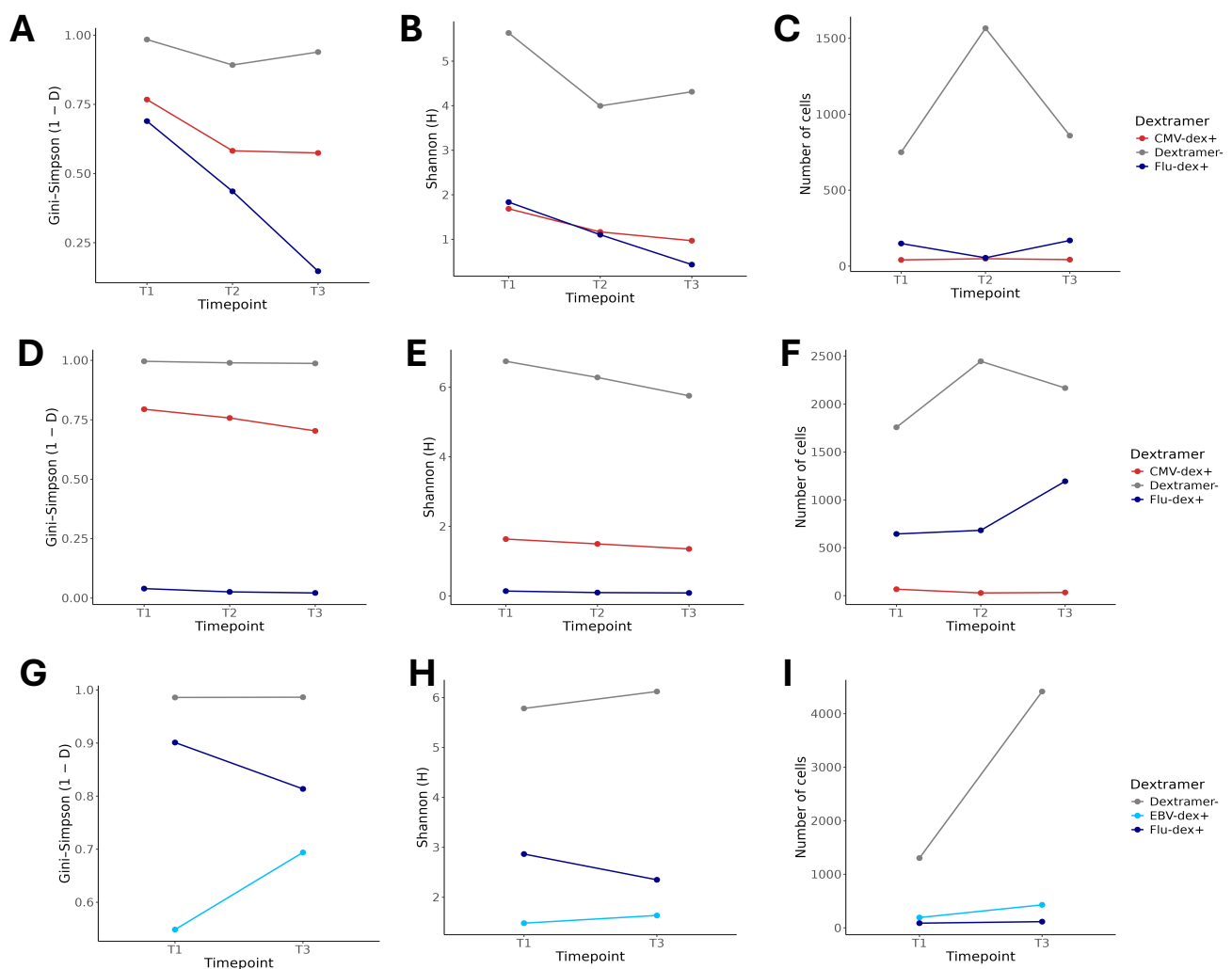


Figure 3.19: Changes in diversity of M1₅₈₋₆₆-specific TCR repertoire with age. Cells were filtered to only include cells with fully resolved TCR α and TCR β chains (CDR3s, variable and joining genes). Shannon and Gini-simpson diversity indices were calculated using the vegan package in R. **(A, D, G)** Gini-Simpson Index for D1 **(A)**, D2 **(D)** and D3 **(G)**. **(B, E, H)** Shannon Index for D1 **(B)**, D2 **(E)** and D3 **(H)**. **(C, F, I)** Number of cells with fully resolved TCRs for **(C)** D1, **(F)** D2 and **(I)** D3. Abbreviations: TCR, T cell receptor, CDR, complementarity determining region.

3.2.5 Influenza infection temporarily boosts aging memory T cells

We had a unique opportunity to analyse changes in M1₅₈₋₆₆-specific following recent influenza infection as one of the donors (D1) had a suspected acute flu infection and donated a blood sample the following week. We also collected an additional sample seven months after infection, giving us a total of five samples for this donor (Fig 3.20A). We first determined the frequency of M1₅₈₋₆₆-specific CD8⁺ T cells in the two additional samples (Fig 3.20B). At T4 (suspected infection) the frequency of M1₅₈₋₆₆-specific increased to 4.65% of CD8⁺ T cells and then at T5 decreased to 1.1%.

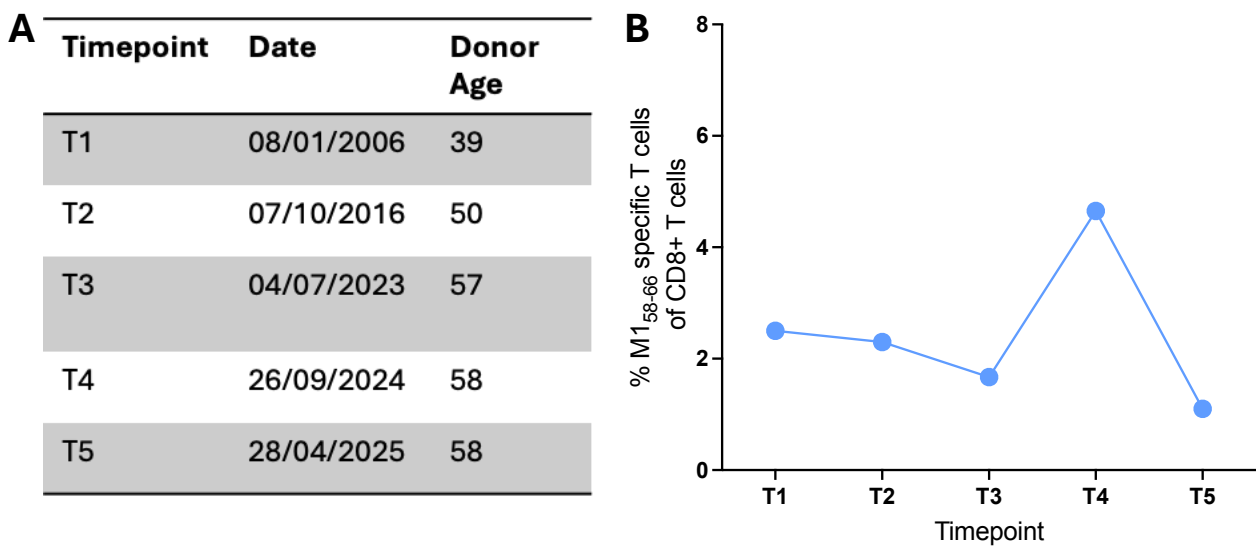


Figure 3.20: Frequency of M1₅₈₋₆₆-specific of T cells across donor 1 timepoints. (A) Dates and age of D1 at each blood sample donation. **(B)** Frequency of M1₅₈₋₆₆-specific within CD8⁺ T cells at each timepoint. PBMCs were isolated from the blood at each of the timepoints and stained with FACS antibodies and PE conjugated GIL tetramer. Abbreviations: PBMC, peripheral blood mononuclear cell; FACS, fluorescence activated cell sorting.

We then performed sorting and 10X scRNAseq of these two additional samples, as previously described (Fig 3.6A-B). Following quality control and filtering we integrated these two samples together with the first three samples from D1 and performed clustering (Fig 3.21A). Most of the clusters we observed were the same, or very similar to the clusters identified in our previous analysis (Fig 3.7A), however we observed a distinct cluster of Activated Tem. This cluster had high expression of several markers associated

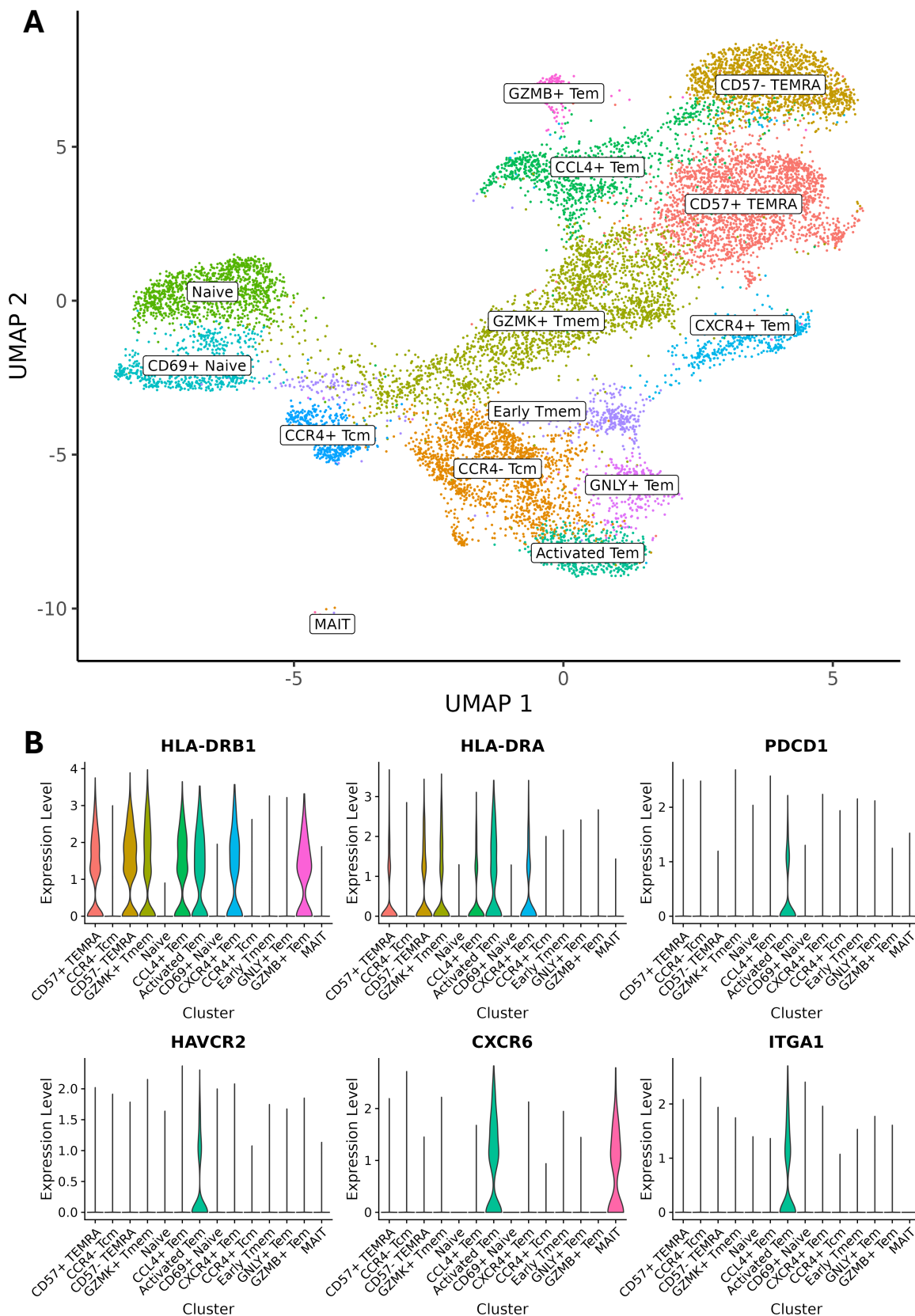


Figure 3.21: Clustering of donor 1 CD8⁺ T cells. (A) UMAP plot showing clusters of CD8⁺ T cells. (B) Expression of selected genes across clusters. Abbreviations: UMAP, Uniform manifold approximation and projection; Tem, T effector memory; Tcm, T central memory; Temra, terminally differentiated effector memory re-expressing CD45RA; MAIT, mucosal associated invariant T cells.

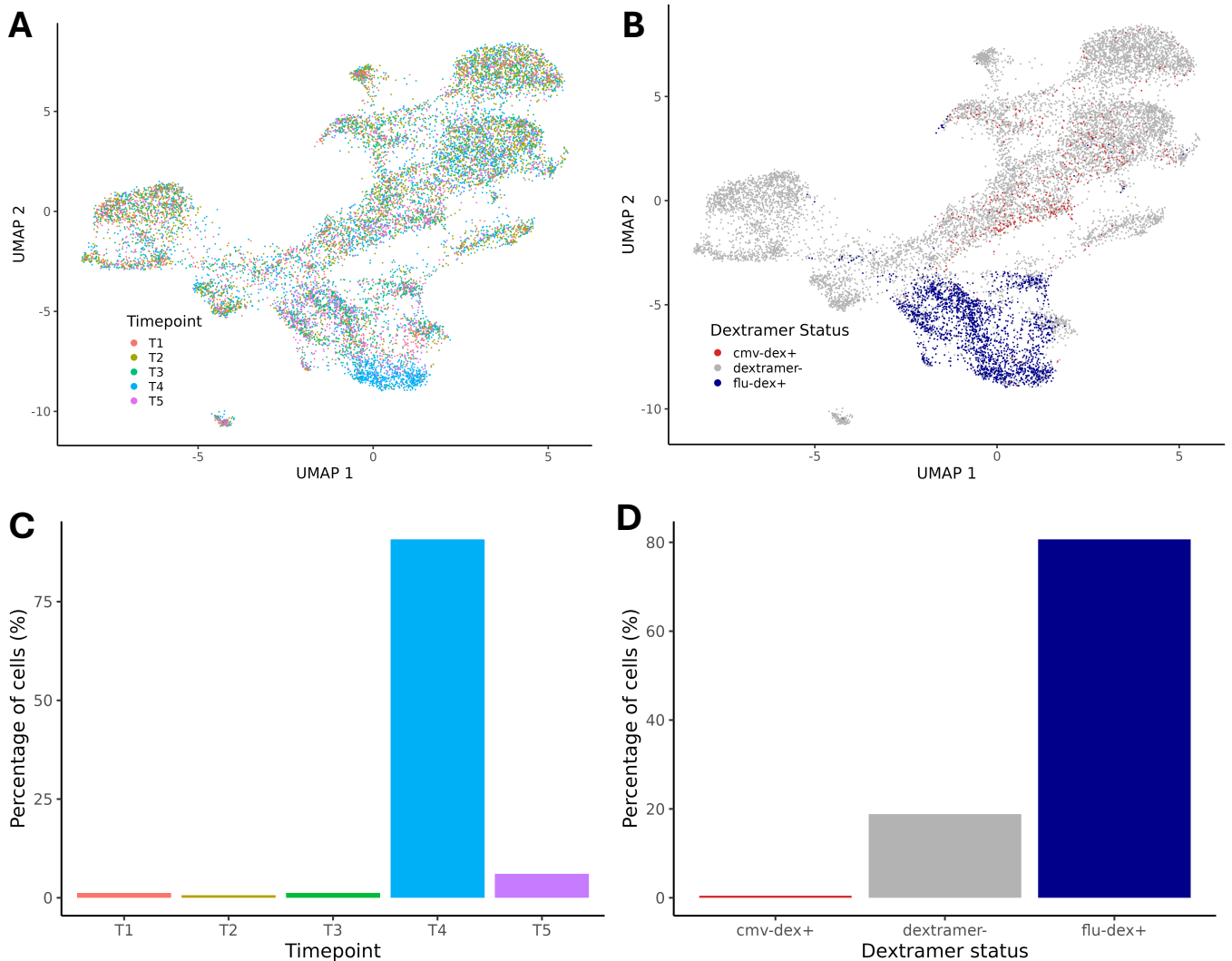


Figure 3.22: Activated Tem cluster contains M1₅₈₋₆₆-specific cells from timepoint 4. (A) UMAP coloured by timepoint. **(B)** UMAP coloured by dextramer status. **(C)** Percentage of cells from each timepoint in activated Tem cluster. **(D)** Percentage of cells of each dextramer status in activated Tem cluster. Abbreviations: UMAP, Uniform manifold approximation and projection, Tem, T effector memory; dex, dextramer.

with activation including *HLA-DRA*, *PDCD1* and *HAVCR2* (Fig 3.21B). The activated cluster had high expression of genes encoding certain homing receptors, including *CXCR6* and *ITGA1* (Fig 3.21B).

Over 90% of the cells in the activated cluster were from T4 (Fig 3.22A and Fig 3.22C) and 80% of them were flu-dex⁺, meaning they were M1₅₈₋₆₆-specific, (Fig 3.22B and Fig 3.22D), providing further evidence of the suspected influenza infection at this timepoint. We analysed the activation markers for M1₅₈₋₆₆-specific T cells across all five timepoints and

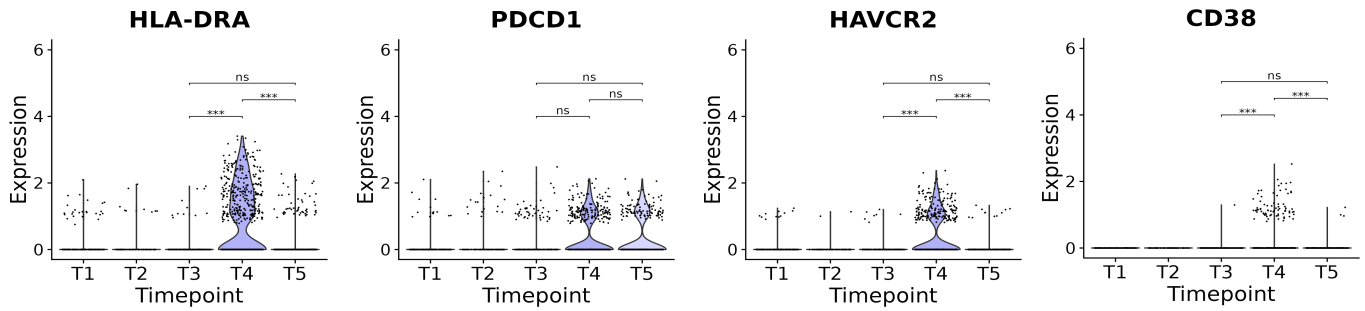


Figure 3.23: Expression of activation markers in M1₅₈₋₆₆-specific CD8⁺ T cells across timepoints. Differential expression was assessed using MAST implemented in Seurat, while controlling for donor and technical covariates. *P* values were adjusted for multiple testing using the Bonferroni method. ****, *padj* ≤ 0.0001; ***, *padj* ≤ 0.001; **, *padj* ≤ 0.01; *, *padj* ≤ 0.05; ns, not significant.

found that the activation markers *HLA-DRA*, *HAVCR2* (which encodes TIM-3) and *CD38* were all significantly upregulated at T4 compared to T3 (Fig 3.23). These activation markers then reduced to levels in line with T3 by T5, suggesting that the upregulation due to influenza infection is relatively short-lived (less than 7 months). Although not reaching significance, we observed a higher proportion of cells expressing *PDCD1* T cells at T5 compared to T3, suggesting that infection may have had relatively long lasting effects on the activation/exhaustion status of these cells.

There was significant upregulation of all cytotoxic effectors (other than *GZMK*) at T4, compared to T3 (Fig 3.24A and Fig 3.24B), suggesting that these activated cells would be effective at killing cells infected with influenza virus. However there is some evidence that high expression of Granzyme B can cause immunopathology during influenza infection²⁸⁷. At T5 the levels of all cytotoxic effectors returned to levels comparable to T3 (Fig 3.24A and Fig 3.24B), implying that this heightened cytotoxic capacity was not sustained.

We then addressed the question of whether infection altered the M1₅₈₋₆₆-specific TCR repertoire. Analysis of V and J gene usage over the five timepoints revealed variation in temporal trends (Fig 3.25). The dominant TCR (TRAV25_TRAJ42_TRBV19_TRBJ2-3) increased in frequency from T1 to T3 (52% and 92%, respectively) but then decreased during infection to 84% at T4. This decrease was associated with a corresponding increase in the frequency of subdominant TCRs, as well as an increase in the number of

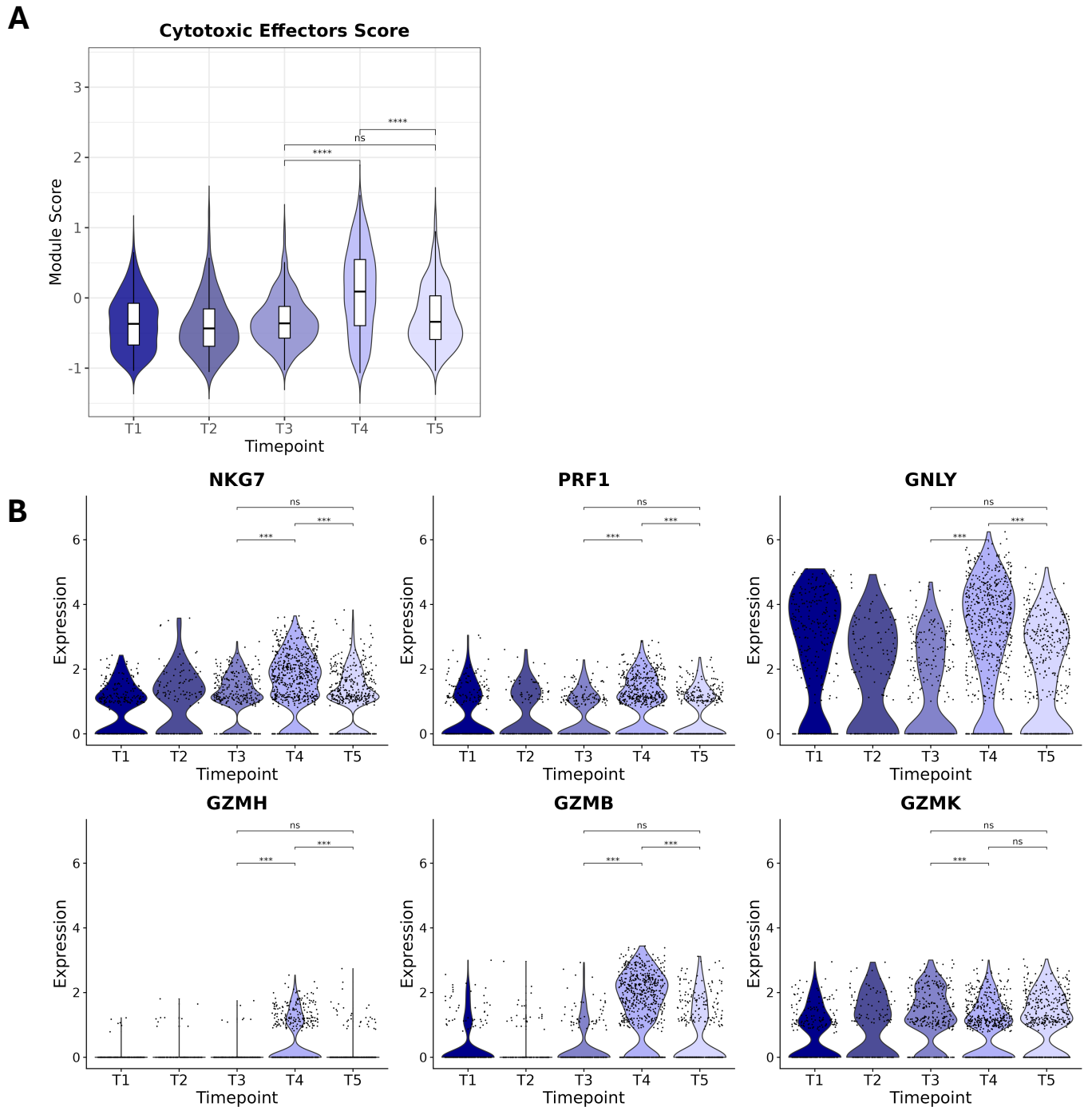


Figure 3.24: M1₅₈₋₆₆-specific CD8⁺ T cells exhibit increased expression of cytotoxic effectors during infection. (A) Cytotoxic effector module score calculated based on expression of GZMA, GZMB, GZMK, GZMH, GZMM, GNLY and NKG7. Scores were determined for M1₅₈₋₆₆-specific cells at each timepoint. Boxes indicate the interquartile range with the line inside each box representing the median. P values were calculated using the Wilcoxon rank-sum test with Benjamini–Hochberg correction for multiple testing. (B) Violin plot of selected cytotoxic effectors at each timepoint. Differential expression was assessed using MAST implemented in Seurat, while controlling for donor and technical covariates. P values were adjusted for multiple testing using the Bonferroni method. ****, $padj \leq 0.0001$; ***, $padj \leq 0.001$; **, $padj \leq 0.01$; *, $padj \leq 0.05$; ns, not significant.

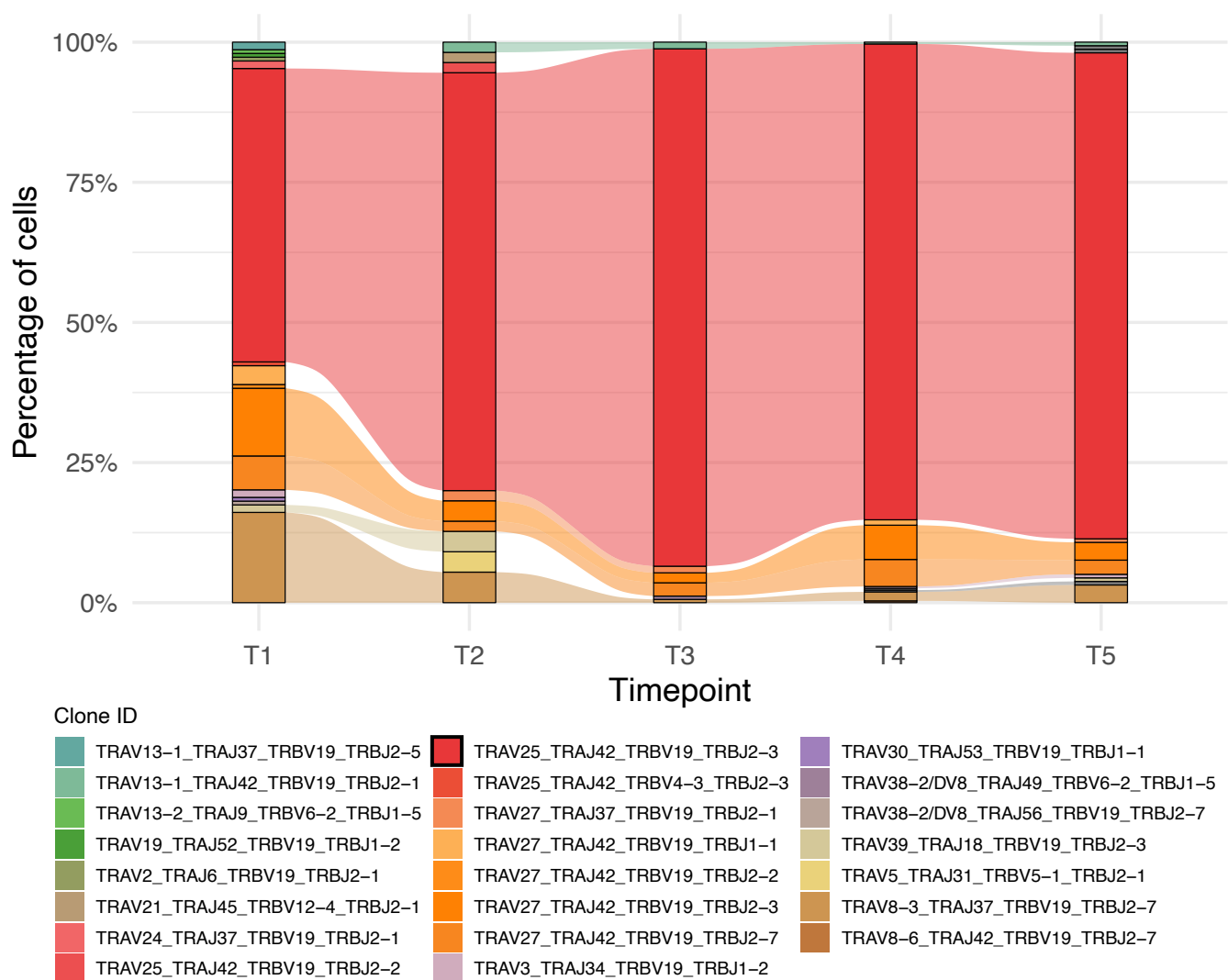


Figure 3.25: Changes in proportions of M1₅₈₋₆₆-specific TCR clonotypes during infection. M1₅₈₋₆₆-specific T cells were filtered to only include cells that with fully resolved variable and joining genes for both the TCR α and TCR β chains. Each cell was assigned a clone ID based on TRAV_TRAJ_TRBV_TRBJ the percentage of cells with each clonotype at each timepoint were plotted. Abbreviations: TCR, T cell receptor.

unique clonotypes. At T5 the dominant clonotype increased slightly to 86% but did not return to the levels seen prior to infection. Additionally, we observed an increase in the Shannon and Gini-Simpson diversity index from T3 to T4 which was largely maintained at T5 (Fig 3.26A and Fig 3.26B). Together these data suggest that acute flu infection boosts the diversity of the M1₅₈₋₆₆-specific TCR repertoire, and that this increase in diversity is maintained for at least 7 months post infection.

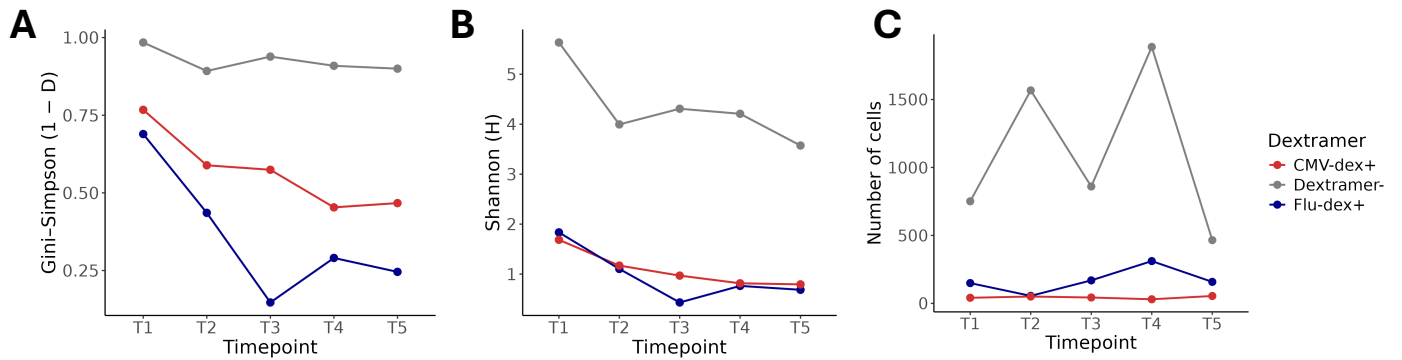


Figure 3.26: Increase in diversity of M1₅₈₋₆₆-specific TCR repertoire during infection. Donor 1 cells were filtered to only include cells with fully resolved TCR α and TCR β chains (CDR3s, V and J genes). Shannon and Gini-simpson diversity indices were calculated using the vegan package in R. **(A)** Gini-Simpson Index for D1. **(B)** Shannon Index for D1. **(C)** Number of cells with fully resolved TCRs. Abbreviations: TCR, T cell receptor; V, variable; J, joining; CDR, complementarity determining region.

3.3 Discussion

In this chapter we use longitudinal samples over a 19-year period to investigate age-related changes in influenza M1₅₈₋₆₆-specific CD8⁺ T cells and how antigen-specificity shapes ageing trajectory of CD8⁺ T cells. Initial *in vitro* data demonstrated differences in the functionality of M1₅₈₋₆₆-specific T cells bearing the same TCR, encouraging us to do an in depth scRNAseq study to profile these cells and their age-related changes. The scRNAseq analysis revealed changes in both the transcriptome and TCR repertoire in ageing M1₅₈₋₆₆-specific CD8⁺ T cells.

In particular, we observed alterations in the metabolic profile with a reduction in both oxidative phosphorylation and glycolysis module scores, suggesting that M1₅₈₋₆₆-specific T cells become less metabolically flexible with age which could underpin alterations in functionality. We also observed changes in expression of cytotoxic effectors and identified age-associated changes that were unique to M1₅₈₋₆₆-specific CD8⁺ T cells. Although age-related changes in the TCR repertoire were unique to each donor, overall we observed a reduction in the diversity of the M1₅₈₋₆₆-specific TCR repertoire with age. We found that infection temporarily increased the frequency, expression of cytotoxic effectors and TCR diversity of M1₅₈₋₆₆-specific T cells. TCR diversity remained increased 7 months after infection, implying that infection has longer lasting effects on the TCR

repertoire than on the transcriptomic profile of the cells. This suggests that infection may recruit new naïve clonotypes that then become memory T cells following resolution of infection.

Profiling M1₅₈₋₆₆-specific cells in parallel with the broader CD8⁺ T cell population, allowed us to accurately determine the phenotype and differentiation status of this population. The majority of the M1₅₈₋₆₆-specific cells in our dataset had a Tcm or Tem phenotype, which is in agreement with previous studies^{272, 274}. Interestingly, despite a significant percentage of CD8⁺ T cells in the broader population having a Temra phenotype, less than 3% of M1₅₈₋₆₆-specific cells were in the Temra cluster. Additionally, the M1₅₈₋₆₆-specific cells do not show signs of immunosenescence or terminal differentiation – they do not express high levels of CD57 and are largely CD27⁺CD28⁺. This finding is in agreement with van de Sandt and colleagues' recent study which profiled M1₅₈₋₆₆-specific in individuals up to the age of 88 and M1₅₈₋₆₆-specific T cells with a Temra phenotype were not identified, even in these elderly individuals²⁷¹. Collectively, these findings indicate that M1₅₈₋₆₆-specific CD8⁺ T cells may undergo a distinct ageing trajectory relative to other CD8⁺ T cell populations. It has been suggested that the initial activation conditions of CD8⁺ T cells can affect their ageing trajectory, many years later²⁸⁸. Thus, the lack of Temra cells within M1₅₈₋₆₆-specific population suggests that influenza infection, or at least stimulation with the GIL peptide, does not drive cells to terminal differentiation.

In the past decade significant progress has been made in profiling changes to CD8⁺ T cells, and other immune cell populations, during the ageing process. Many of these studies have sampled hundreds of donors, generating massive datasets that can be probed to address many questions^{99, 220, 221}. However, a major limitation of these generated datasets is that they do not report antigen-specificity of the T cells, thus cannot control for the confounding variable of how often the T cells are likely to have experienced antigen and the situation in which they experienced antigen. Recently Sturmlechner and colleagues highlighted the importance of accounting for antigen-specificity when addressing questions of ageing; they profiled age-related changes in EBV-specific T cells with different antigen-specificities. Not only did the authors observe different age-related changes in EBV-specific T cells compared to other T cells but also

observed different ageing signatures within T cells-specific for different EBV antigens²⁸⁸. Our current study reinforces this finding by demonstrating that some age-related transcriptomic changes identified are unique to the M1₅₈₋₆₆-specific T cells and are not observed to the same extent in cells of related clusters or EBV/CMV-specific cells. Together these findings suggest that it may be overly simplistic to treat ageing CD8⁺ T cells as a monolith as T cells with different antigen-specificities appear to undergo distinct ageing trajectories.

Some of the changes we observed in ageing M1₅₈₋₆₆-specific T cells are consistent with previously identified age-related changes. For example, we found increased expression of granzyme K in M1₅₈₋₆₆-specific T cells in the later timepoints compared to T1. Several recent studies have shown an increased frequency of GZMK⁺ CD8⁺ T cell subsets with age^{99, 282}. GZMK cleaves many components of the complement cascade, including C2, C3, C4 and C5, which collectively contribute to the activation of this proinflammatory pathway²⁸⁹, suggesting that the increased frequency of GZMK⁺ T cells with age may contribute to inflammaging, a previously identified hallmark of T cell ageing⁹⁵. Several of the genes that we found to be uniquely significantly upregulated in M1₅₈₋₆₆-specific T cells with age were associated with TCR activation, suggesting that the regular but intermittent activation of these T cells due to reoccurring influenza infections could contribute to increased expression of these genes.

Most studies addressing age-related TCR repertoire changes do so with a focus on older individuals (>65 years), however, given the recent finding that adults aged 50-64 have 3-5 fold higher rates of hospitalisation and death from influenza infection than adults aged 18-49²⁶² we decided to profile influenza-specific CD8⁺ T cells throughout middle age. We observed a decrease in the diversity of the M1₅₈₋₆₆-specific TCR repertoire as the donors aged from their 30s to their 50s, although the extent of these changes varied greatly between the donors. In their recent study, longitudinally analysing the M1₅₈₋₆₆-specific TCR repertoire, van de Sandt and colleagues included younger adults (aged between 25 and 52) as a comparison to older individuals. They sampled the younger adults over a 9-12 year period and also observed a reduction in the Gini-Simpson index²⁷⁴ in this stage of life. However, these findings in younger adults contrast with recent findings in elderly

individuals, which show increased diversity in the M1₅₈₋₆₆-specific TCR repertoire^{271, 272, 274}. Along with increased diversity these studies demonstrate a reduction in the cells using public TCR clonotypes and the public motif “RS” in the CDR3 β which has been described as the optimal way to recognise the GIL peptide. The authors conclude that this reduction in optimal TCRs and increased proportion in suboptimal private TCRs may be contribute to increased susceptibility to severe influenza in elderly individuals.

Taking the findings from our study and these recent studies in elderly individuals together, I hypothesise that there could be two phases of M1₅₈₋₆₆-specific T cell ageing. The first occurring between the ages of 30 and 60 in which there is a reduction in repertoire diversity and an expansion of dominant clonotypes. The reduction in TCR diversity in this phase may account for increased susceptibility to severe influenza in individuals aged 50-60. The second phase of ageing occurs after the age of 65 in which there is a loss of optimal clonotypes containing the “RS” motif and increasing diversity of the TCR repertoire, largely due to expansion of private suboptimal TCRs. The increased susceptibility to influenza infection in individuals over the age 65 may be due to this loss of TCRs with the “RS” motif which van de Sandt and colleagues found to have favourable gene expression profiles and functional characteristics. It is plausible that repeated influenza infections throughout life result in the expansion and subsequent contraction of the clonotypes with the highest functional properties during phase one, which eventually reduces their frequency in phase two. Thus, in an ideal situation we would maintain a delicate balance through both phases of ageing in which we have some degree of expansion of the optimal clonotypes but also maintain TCR diversity.

This raises the question of whether the inclusion of the M1 protein in influenza vaccines would be beneficial. Most current influenza vaccines are inactivated and do not focus on inducing CD8⁺ T cell immunity, with their primary aim being boosting antibody levels against the HA and NA or currently circulating influenza strains. In fact, several studies have demonstrated that inactivated influenza vaccines are not able to boost influenza-specific CD8⁺ T cells²⁹⁰. However clinical trials have been conducted assessing vaccination with modified vaccinia Ankara containing the influenza M1 protein. Changes

in frequency of M1₅₈₋₆₆-specific cells following challenge after vaccination were not observed but there were some phenotypic differences in M1₅₈₋₆₆-specific cells from vaccination individuals²⁵¹. Thus, it is likely possible to vaccinate individuals in a way to boost M1₅₈₋₆₆-specific T cells. Care should be taken when doing so and further studies are needed to assess the effects of this as it is possible that increased frequency of activation of these cells could drive them through their phases of ageing at a faster rate, thereby leaving younger individuals with more “aged” M1₅₈₋₆₆-specific T cells. However, it is plausible that there is a period of life (potentially between the ages of 30 and 40) in which boosting M1₅₈₋₆₆-specific cells could maintain TCR diversity and prevalence of optimal clonotypes into later life.

Given the logistical challenges of repeatedly sampling the same donor over long time periods, most TCR repertoire ageing studies are done using cross-sectional cohorts. While there have been longitudinal studies profiling the M1₅₈₋₆₆-specific TCR repertoire over 10-12 year periods^{274, 275}, our current study is, to the best of our knowledge, the first to extend the sampling period to 19 years and to combine TCR sequencing with transcriptomic analysis. The significant variation in the M1₅₈₋₆₆-specific TCR repertoire and its age-related changes between the donors in our cohort highlights the importance of longitudinal studies.

We recognise that our current study has several limitations. Our initial *in vitro* study is limited by the low number of clones isolated at each timepoint and the fact that functional assays could not be repeated due to limited cell numbers. The major limitation of our scRNAseq study is the small size of our cohort; ideally our findings would be confirmed in a larger cohort. However longitudinally stored samples from HLA-A*02:01 individuals are difficult to acquire and therefore previous studies longitudinally profiling this response have also been limited to 3-7 donors²⁷⁴. Additionally our study solely focusses on CD8⁺ T cells from circulating blood, which may not be entirely reflective of CD8⁺ T cells in tissues²⁶³ where active immune responses occur. However previous studies have shown a high overlap in the M1₅₈₋₆₆-specific TCRs in the blood and tissues²⁷⁰ suggesting that the blood can act as a relatively good proxy for harder-to-obtain tissue samples. Additionally, due to differences in the frequency of M1₅₈₋₆₆-specific T

cells between the donors and variation in the percentage of fully resolved TCRs in each sequencing run, the numbers of M1₅₈₋₆₆-specific TCR vary between samples. However, we were still able to calculate diversity indices and determine expanded clonotypes with the number of TCRs obtained. Our study focuses on T cells specific for only one epitope from influenza virus and we could have included dextramers-specific for other HLA-A*02:01 restricted epitopes from the influenza virus, but the lower frequency of these would have made it technically challenging to obtain a meaningful number of them to proceed with sequencing.

In conclusion, through longitudinal profiling of middle-aged individuals, our study identifies age-related changes in M1₅₈₋₆₆-specific CD8⁺ T cells including reduction in TCR diversity and transcriptomic alterations. Additionally, we identify changes unique to M1₅₈₋₆₆-specific CD8⁺ T cells, highlighting that T cells of different antigen-specificity undergo different ageing trajectories. These changes in influenza specific CD8⁺ T cells, along with other age-associated immune changes may partially explain the increased risk of severe disease from influenza infection as people age.

4. Functional and molecular profiling of SARS-CoV-2 ORF3a₂₀₇₋₂₁₅ - specific CD8⁺ T cells associated with severe disease

4.1 Introduction

As T cells play a central role in antiviral immunity, the onset of the COVID-19 pandemic prompted a rapid effort by the immunology community to identify SARS-CoV-2 epitopes recognised by CD4⁺ and CD8⁺ T cells^{135, 136, 137, 139}. Epitopes within the structural proteins spike, NP and M, as well as within the ORF proteins were identified^{136, 137, 138}. In attempt to identify immune correlates of protection, researchers have also investigated the association of SARS-CoV-2-specific T cells and disease outcome. Studies found that patients with mild disease and rapid viral clearance tended to have early induction of functional SARS-CoV-2-specific T cells^{136, 140, 141}. The depletion of CD8⁺ T cells in non-human primates has highlighted the importance of T cells in limiting the disease caused by SARS-CoV-2²⁹¹.

Although important contributors to anti-viral immune responses, T cells do not only have beneficial effects¹⁴⁶. Studies using animal models of viral infection have shown that T cells can contribute to immunopathology – tissue damage and inflammation caused by the overproduction of cytokines and cytotoxic molecules from immune cells. Using the well-studied model of LCMV infection and T cell deficient mice, Cornberg and colleagues demonstrated that the pathology, including pulmonary oedema and interstitial lung infiltration, is largely caused by T cells, rather than the virus itself¹⁴⁵. Early in the pandemic, evidence emerged suggesting that T cells may contribute to immunopathology in COVID-19, with the observation that the breadth and magnitude of T cell responses were significantly higher in patients with severe compared to mild disease¹³⁶. Transcriptomic analysis of CD4⁺ T cells from hospitalised and non-hospitalised patients with COVID-19 revealed reduced proportions of Tregs in hospitalised patients²⁹². This reduction in Tregs in hospitalised patients may lead to insufficient regulation of effector T cell responses, resulting in tissue damage and contributing to disease severity. Additionally, Pena and colleagues found that progression to severe COVID-19 was accompanied by stronger cellular immune

responses and more extreme CD4⁺ T cell activation²⁹³. Highly activated lung-homing CXCR4⁺ T cells have been associated with fatal COVID-19²⁹⁴.

Studies looking directly into lung tissue have also suggested T cell involvement in immunopathology. Early in the pandemic post mortem studies demonstrated lymphocytic infiltration in the lungs of deceased COVID-19 patients, a preliminary suggestion that T cells could be contributing to tissue damage²⁹⁵. Using spatial transcriptomics to study immune infiltration in the lungs, researchers found enriched TCR signalling and cytotoxicity in areas of pathology¹⁵⁰. It has been hypothesised that the activation of T cells within the lung may be spatially removed from the virus and thus activated T cells which remain and cause damage after viral clearance may be a key contributor to immunopathology. These spatial studies have also revealed physical associations between CD8⁺ T cells and other potentially damaging immune cell subtypes such as immature neutrophils^{149, 150}. In the context of other respiratory viral infections T cell recognition of antigen on alveolar epithelial cells has been shown to cause extreme inflammation and contribute to lung tissue damage²⁹⁶.

Together these studies highlight the fine balance needed when initiating CD8⁺ T cell immunity against a virus: in an ideal situation we want early moderate activation of virus-specific CD8⁺ T cells that do not continue to execute effector functions upon resolution of the virus. As antigen-specificity plays a significant role in determining the functional capabilities of T cells it is important to study virus specific T cells in an antigen specific manner when looking for correlates of protection/association with severe disease. When looking in detail at the immunodominant HLA-B*07:02-restricted SARS-CoV-2 NP₁₀₅₋₁₁₃-specific T cell response, Peng and colleagues found that it significantly associated with mild disease. This response was shown to be the dominant response against the NP protein in HLA-B*07:02-positive patients. Within the HLA-B*07:02 patient cohort, individuals with mild symptoms had a higher frequency and greater magnitude of NP₁₀₅₋₁₁₃-specific T cells compared to those with severe disease¹⁴².

Previous work in Tao Dong's group also identified an immunodominant CD8⁺ T cell response against the ORF3a₂₀₇₋₂₁₅ epitope (FTSDYYQLY, referred to as FTS from herein) from SARS-CoV-2 within a cohort of donors from the first wave of the pandemic in

2020¹³⁶. Subsequently, CD8⁺ T cells specific for this epitope were identified in both acute and convalescent patients in several other studies^{297, 298, 299}. Within HLA-A*01:01⁺ individuals, T cells specific for this epitope were identified in 90% of people, highlighting its immunodominance (Fig 4.1A). Interestingly when this HLA-A*01:01 positive cohort

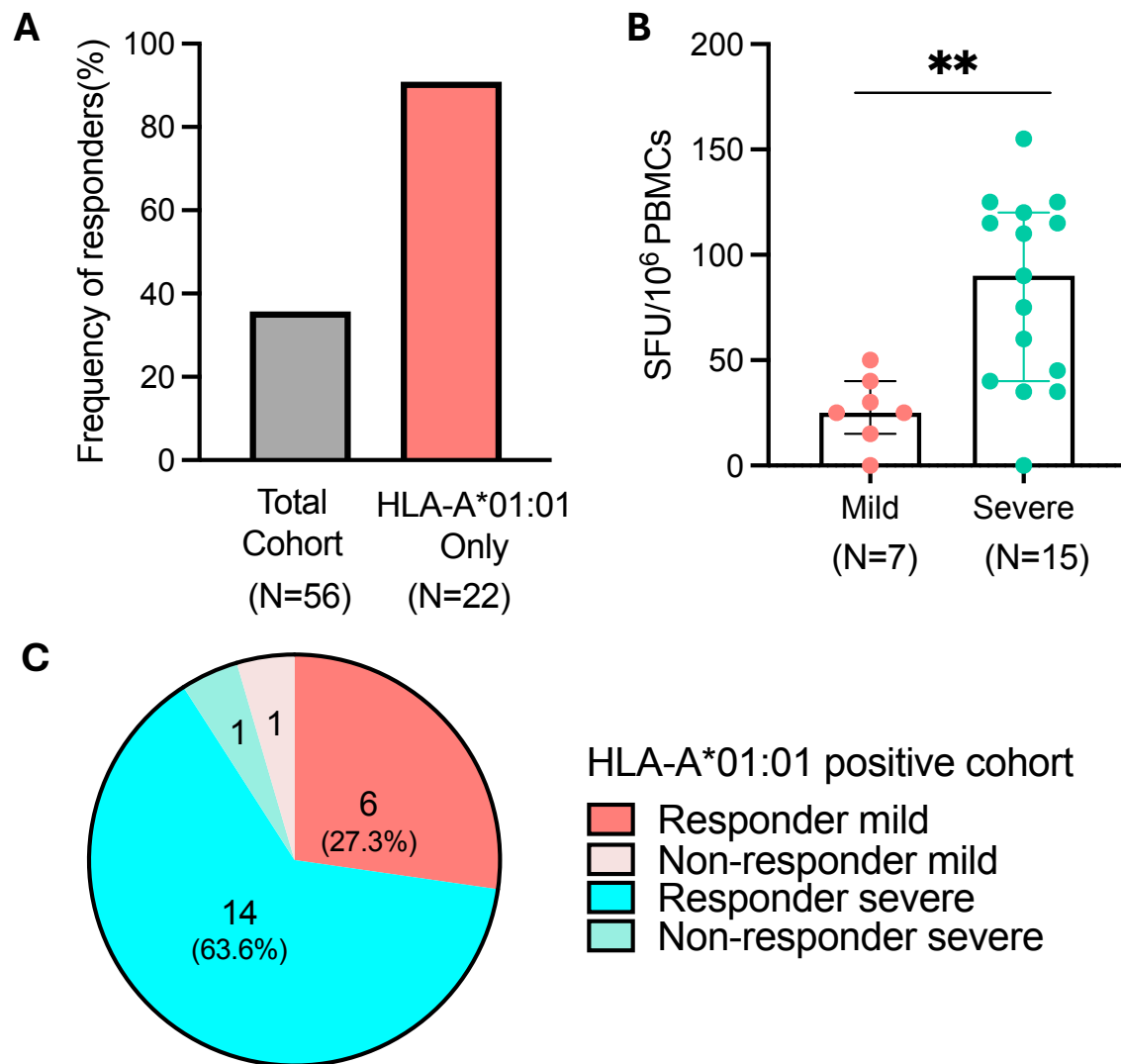


Figure 4.1: Association of ORF3a₂₀₇₋₂₁₅-specific T cell response with disease severity in HLA-A*01:01 cohort. Blood samples were collected from patients during the first wave of the COVID-19 pandemic. **(A)** Frequency of patients that responded to the FTS peptide within the whole cohort and the HLA-A*01:01⁺ cohort. Response was determined via ELISpot with PBMCs. **(B)** Number of spot forming cells per 10⁶ PBMCs when stimulated with FTS peptide. Patients were split based on severity of disease. Patients were classified as having mild symptoms if they did not require oxygen or if their symptoms were managed at home. Severe infection was defined as one of the following conditions in a patient confirmed as having COVID-19: respiratory distress with a respiratory rate of >30 breaths per minute; blood oxygen saturation of <93%; or arterial oxygen partial pressure/FiO₂ < 300 mmHg. Significance was determined by Mann-Whitney test. **(C)** Proportion of donors that responded to ORF3a₂₀₇₋₂₁₅ epitope within HLA-A*01:01 cohort, split by severity of disease. Data were generated by Yanchun Peng and previous members of the Dong group. **, $p < 0.01$. Abbreviation: ELISpot, enzyme-linked immunosorbent spot.

was split into those with mild and severe COVID-19, T cells specific for the ORF3a₂₀₇₋₂₁₅ epitope were identified at a higher frequency in PBMCs from patients with severe disease (Fig 4.1B). Within HLA-A*01:01 individuals who responded to this epitope a higher percentage of them had severe disease compared to mild disease (Fig 4.1C). Together this data demonstrates that this T cell response is associated with disease severity and thus that these T cells may possess features that allow them to cause immunopathology.

In this thesis chapter we investigate features of ORF3a₂₀₇₋₂₁₅-specific T cells that could enable them to contribute to severe COVID-19. To investigate the features of these cells, we utilised CD8⁺ T cell clones, providing a controlled *in vitro* system that removes several confounding variables such as differences in TCR gene usage. Utilising T cell clones that bear the same TCR but differ in their functional avidity can allow for the identification of features affecting T cell function. T cell functional avidity is biological readout that reflects T cell responsiveness upon antigen-specific stimulation and thus differs from the biophysical measurements of TCR affinity or TCR avidity³⁰⁰. Previous studies in Tao Dong's lab have utilised comparison of T cell clones through omics analysis to identify features associated with T cell functionality and link them to disease outcome. For example, RNA sequencing of high and low functional avidity SARS-CoV-2 NP₁₀₅₋₁₁₃-specific CD8⁺ T cell clones identified several differently expressed genes between the high and low functional avidity clones with *IL10RA*, *PARK7* and *LTA4H*¹⁴² expressed at higher levels in the high functional avidity clones. As these genes encode for immune dampening molecules it is possible that the presence of these high functional avidity T cell clones could help in the prevention of exacerbated pathology and illness and thus explain the association of this T cell response with mild disease. Additionally proteomic analysis of tumour associated antigen-specific T cell clones with differences in functionality identified the integrin CD61 as a novel regulator of CD8⁺ T cell function³⁰¹.

Thus, in this thesis chapter, we identify pairs of ORF3a₂₀₇₋₂₁₅-specific T cell clones with identical TCR sequences but differences in their functional avidity. By stratifying ORF3a₂₀₇₋₂₁₅-specific T cell clones based on their functional avidity we utilise the high functional avidity clones as an extreme example of these T cells that potentially contribute to immunopathology. To investigate features of these high functional avidity

clones we perform proteomics and RNA-sequencing of the clones with and without stimulation.

4.2 Results

4.2.1 Functional differences in ORF3a₂₀₇₋₂₁₅-specific CD8⁺ T cell clones that bear the same TCR

To investigate the ORF3a₂₀₇₋₂₁₅-specific T cell response in detail, T cell clones were isolated from PBMCs obtained from COVID-19 patients during acute infection or 1-3 months post infection. T cell clones were generated from PBMCs by tetramer staining using a fluorescently conjugated HLA-A*01:01 FTS tetramer that binds to TCRs specific for ORF3a₂₀₇₋₂₁₅ epitope. Tetramer⁺CD8⁺T cells were identified by FACS sorting and single cell sorted into the wells of a 96 well plate. Isolated clones were expanded using feeder cell stimulation and clones that grew sufficiently were used to perform functional assays and TCR sequencing.

TCR sequencing of ORF3a₂₀₇₋₂₁₅-specific T cell clones revealed several different TCR gene used by T cells specific for this epitope. However, we identified several clones with the same TCR (Table 4.1). In all subsequent analysis the T cell clones are grouped by which TCR they have. To establish the functional avidity of the ORF3a₂₀₇₋₂₁₅-specific T cells IFN γ ELISpots were performed. T cell clones were cocultured with B cell lines (BCLs) that had been preloaded with a range of concentrations of the FTS peptide. Spot forming cells were counted (Fig 4.2A) and calculated as a percentage of total T cells added to the well (Fig 4.2B-D). To assign a numerical value to functional avidity, the EC50 (the peptide concentration at which half the maximal number of T cells respond to peptide epitope) of each clone was calculated. The T cell clones with TCR-3 all had similar EC50s. In contrast, despite having the same TCR, clones with TCR-1 had a ~3-fold difference in EC50 (Fig 4.2E). This phenomenon was also observed in clones with TCR clonotype 3, having up to an ~8-fold difference in EC50 despite bearing the same TCR (Fig 4.2E). This variation in response to peptide stimulation suggests that there are factors, other than the TCR, affecting the functionality of the T cell clones. T cell clones with an EC50 of less than 0.01 were classed as high functional avidity clones and clones with an EC50 of

TCR clonotype	TRBV	TRBJ	CDR3 β	TRAV	TRAJ	CDR3 α	Clones
TCR-1	TRBV13	TRBJ1-2	CASSLIGQGGYTF	TRAV8-6	TRAJ22	CAVSGPQGSARQLTF	CL1.1, CL1.2, CL1.3
TCR-2	TRBV2	TRBJ2-2	CASSDPTSGELFF	TRAV20	TRAJ35	CAVQAEGFGNVLHC	CL2.1, CL2.2, CL2.3, CL2.4, CL2.5, CL2.6, CL2.7, CL2.8, CL2.9
TCR-3	TRBV6-6	TRBJ2-2	CASREDQNGELFF	TRAV1-2	TRAJ42	CAVRRYGGSQGNLIF	CL3.1, CL3.2, CL3.3, CL3.4

Table 4.1: TCR gene usage of ORF3a₂₀₇₋₂₁₅-specific T cell clones. Variable (V), joining (J) TCR gene usage and CDR3 sequences of ORF3a₂₀₇₋₂₁₅-specific CD8⁺ T cell clones. TCR sequencing was performed by Yanchun Peng.

greater than 0.01 were classed as low functional avidity. Clones with the largest differences in EC₅₀ from TCR clonotype 1 and 2 were then selected for further functional analyses (Fig 4.2F). This selection was also dependent on viability and growth of the clones, as only well-growing clones could be used for subsequent analysis. Prior to functional assays, tetramer staining was performed to confirm that levels of TCR expression were comparable between the T cell clones (data not shown) and therefore unlikely to account for differences in functionality.

To further interrogate the cytokine production of the ORF3a₂₀₇₋₂₁₅-specific T cell clones we performed intracellular cytokine staining and Luminex. To perform these assays T cells were cocultured with peptide-loaded BCLs. IFN γ , TNF and CD107a expression were determined via intracellular staining following 4 hour coculture. Levels of a wider panel of cytokines were assessed in the supernatant of the clones following a 48-hour coculture. Across both techniques, higher levels of the key effector cytokine TNF were observed from the high functional avidity clones compared to the low functional avidity clones (Fig 4.3D-E, Fig 4.3A-B). Levels of RANTES, also known as CCL5, a chemokine that plays a role in sustaining CD8⁺ T cell responses to viral infections³⁰² varied between the clones with two of the high functional avidity clones secreting it at the highest levels (Fig 4.4A-B). Levels of GM-CSF and IL-13 also varied between the clones (Fig 4.4A-B), indicating that factors other than TCR must be influencing cytokine production from ORF3a₂₀₇₋₂₁₅-specific T cell clones.

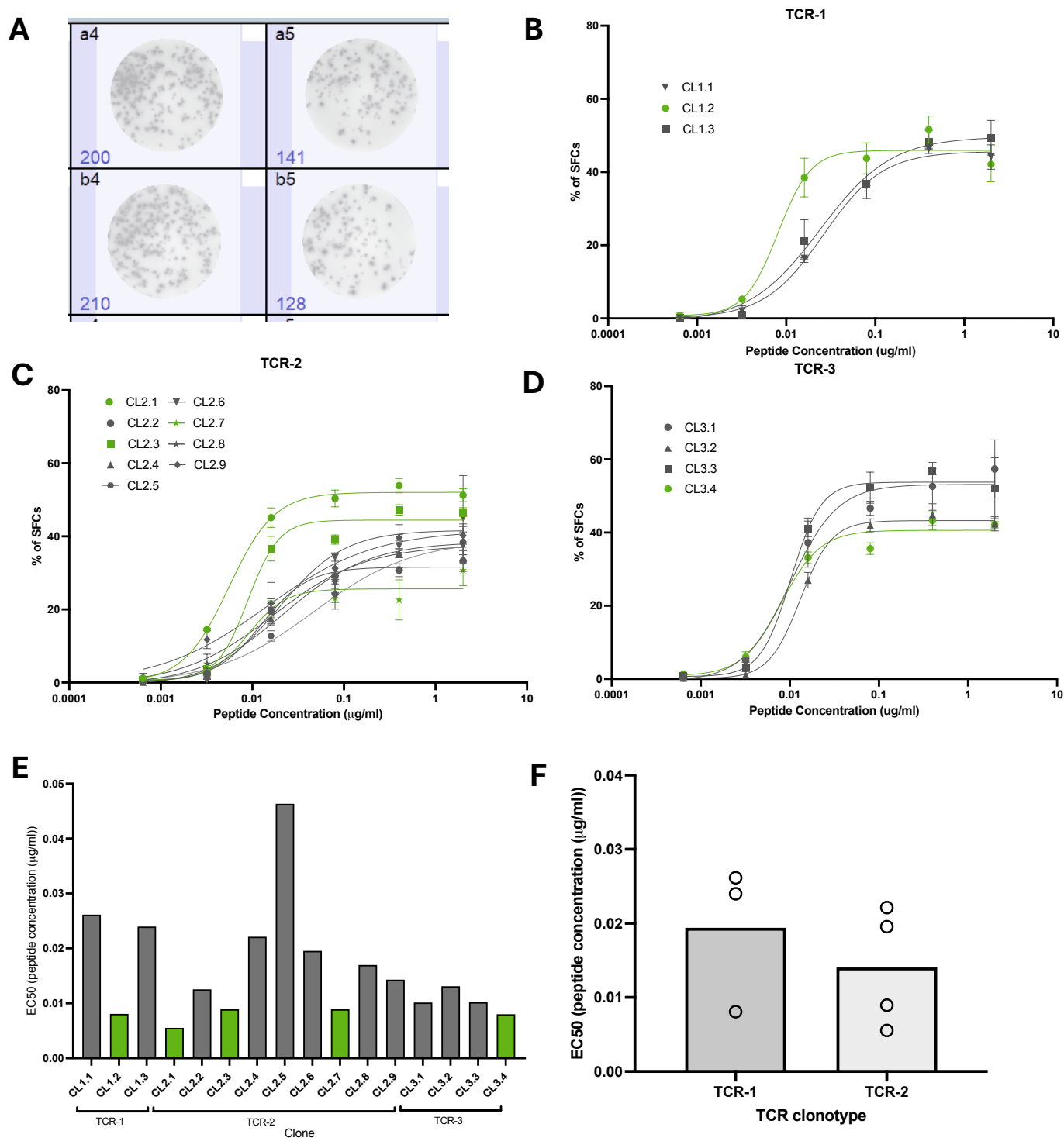


Figure 4.2: ORF3a₂₀₇₋₂₁₅-specific T cell clones with the same TCR differ in functional avidity. ORF3a₂₀₇₋₂₁₅-specific T cell clones were cocultured with BCLs that had been preloaded with a range of concentrations of peptide (2 μg/ml, 0.4 μg/ml, 0.08 μg/ml, 0.016 μg/ml, 0.0032 μg/ml, 0.0016 μg/ml) in pre-coated and blocked plates. After plates were developed an ELISpot plate reader was used to count the number of SFCs which correspond to IFN γ producing cells. SFCs at each peptide concentration are expressed as a percentage of total T cells added to each well (400). **(A)** Representative wells of ELISpot. T cells clones are grouped by TCR clonotype: **(B)** TCR-1 clones, **(C)** TCR-2 clones, **(D)** TCR-3 clones. The EC₅₀ of each T cell clone was determined by a nonlinear regression with variable slope (four parameters) in a dose-response-stimulation model using GraphPad Prism **(E, F)**. **(E)** EC₅₀ of individual clones. **(F)** EC₅₀s of selected clones, grouped by TCR clonotype. Bars show mean EC₅₀ for clonotype. Individual points represent EC₅₀s of individual clones. Clones classed as high functional avidity (EC₅₀<0.01) are coloured in green, clones classed as low functional avidity (EC₅₀>0.01) are coloured in grey. Abbreviations: SFC, spot forming cell; TCR, T cell receptor; IFN γ , interferon gamma.

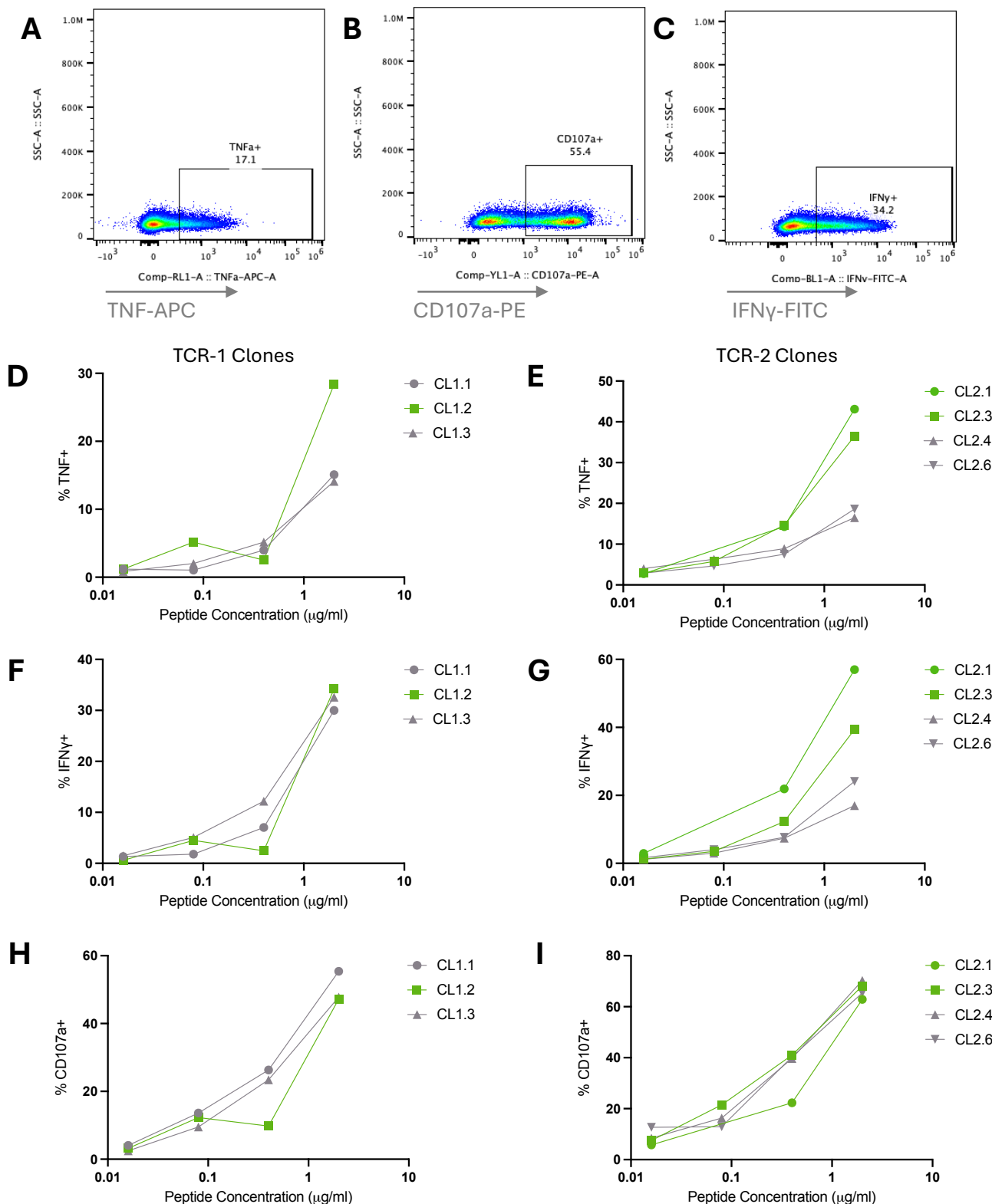


Figure 4.3: TNF, IFN γ and CD107a expression differ in ORF3a₂₀₇₋₂₁₅-specific T cell clones with the same TCR. T cell clones were cocultured with BCLs with that had been preloaded with a range of concentrations of peptide (2 μ g/ml, 0.4 μ g/ml, 0.08 μ g/ml, 0.016 μ g/ml and no peptide). IFN γ , TNF and CD107a expression were assessed by intracellular cytokine staining and flow cytometry. **(A-C)** Representative staining of TNF **(A)**, CD107a **(B)** and IFN γ **(C)** expression in ORF3a₂₀₇₋₂₁₅ T cell clone stimulated with BCL preloaded with 2 μ g/ml of peptide. **(D-E)** Percentages of TNF+ T cells after stimulation with BCLs preloaded with peptide. **(F-G)** Percentages of IFN γ + T cells after stimulation with BCLs preloaded with peptide. **(H-I)** Percentages of CD107a+ T cells after stimulation with BCLs preloaded with peptide. **(D, F, H)** TCR-1 clones. **(E, G, I)** TCR-2 clones. Clones classed as high functional avidity are coloured in green, clones classed as low functional avidity are coloured in grey. Abbreviations: TNF; tumour necrosis factor; IFN γ , interferon gamma; BCL, B cell line; TCR, T cell receptor.

Cytokine	Clone	Functional avidity	Peptide Concentration (µg/ml)			
			0.016	0.08	0.4	2
IFN γ	CL1.2	High	-	-	-	++++
	CL1.1	Low	-	-	-	+
	CL1.3	Low	-	-	-	+
TNF	CL1.2	High	-	-	++	++++
	CL1.1	Low	-	-	+	++
	CL1.3	Low	-	-	+	++
IL-13	CL1.2	High	-	-	++	++++
	CL1.1	Low	-	+	++	+++
	CL1.3	Low	-	-	+	++
GM-CSF	CL1.2	High	-	-	++	+++
	CL1.1	Low	-	-	+	+
	CL1.3	Low	+	+	++	++++
RANTES	CL1.2	High	-	-	-	++++
	CL1.1	Low	-	-	-	++
	CL1.3	Low	-	-	-	++

Figure 4.4A: Cytokine secretion profiles of ORF3a₂₀₇₋₂₁₅-specific T cell clones from TCR-1 clones. ORF3a₂₀₇₋₂₁₅-specific T cell clones were cocultured with BCLs with that had been preloaded with a range of concentrations of peptide (2 µg/ml, 0.4 µg/ml, 0.08 µg/ml, 0.016 µg/ml) for 48 hours. Where possible technical replicates were included and means of technical replicates were calculated. Supernatants from the coculture were collected and Luminex was performed to determine levels of secreted cytokines. IFN γ : +++++>5000 pg/ml; +>500 pg/ml, -<500 pg/ml. TNF: +++++>3000 pg/ml, ++>900 pg/ml, +>400 pg/ml, -<400. IL-13: +++++>3000 pg/ml, +++>2000 pg/ml, ++>1000 pg/ml, +>500 pg/ml, -<500 pg/ml. GM-CSF: +++++>5000 pg/ml, +++>4000 pg/ml, ++>3000 pg/ml, +>2000 pg/ml, -<2000 pg/ml. RANTES: +++++>1000 pg/ml, ++> 200 pg/ml, -<200 pg/ml. Abbreviations: TNF; tumour necrosis factor; IFN γ , interferon gamma; BCL, B cell line; TCR, T cell receptor; RANTES, regulated upon activation, normal T cell expressed and secreted; IL, interleukin; GM-CSF, Granulocyte-Macrophage Colony-Stimulating Factor.

Cytokine	Clone	Functional Avidity	Peptide Concentration (µg/ml)			
			0.016	0.08	0.4	2
IFN γ	CL2.1	High	-	-	+	++++
	CL2.3	High	-	-	-	+
	CL2.4	Low	-	-	+	+++
	CL2.6	Low	-	-	-	+
TNF	CL2.1	High	+	+	++	++++
	CL2.3	High	-	+	++	++++
	CL2.4	Low	+	++	++	+++
	CL2.6	Low	-	-	+	++
IL-13	CL2.1	High	+	++	+++	++++
	CL2.3	High	+	+	+++	++++
	CL2.4	Low	++	+++	+++	++++
	CL2.6	Low	+	+	++	+++
GM-CSF	CL2.1	High	-	-	+	+++
	CL2.3	High	-	-	+	++++
	CL2.4	Low	+	+	+	++++
	CL2.6	Low	-	-	+	++
RANTES	CL2.1	High	+	++	+++	++++
	CL2.3	High	-	-	+	++
	CL2.4	Low	-	+	+	++
	CL2.6	Low	-	-	++	++

Figure 4.4B: Cytokine secretion profiles of ORF3a₂₀₇₋₂₁₅-specific T cell clones from TCR-2 clones. ORF3a₂₀₇₋₂₁₅-specific T cell clones were cocultured with BCLs with that had been preloaded with a range of concentrations of peptide (2µg/ml, 0.4µg/ml, 0.08µg/ml, 0.016µg/ml) for 48 hours. Where possible technical replicates were included. Supernatants from the coculture were collected and Luminex was performed to determine levels of secreted cytokines. Averages of cytokine levels were calculated. IFN γ : ++++> 1000 pg/ml, +++>500 pg/ml, +>100 pg/ml, -<100 pg/ml. TNF: ++++>1500 pg/ml, +++>1000 pg/ml, ++>500 pg/ml, +>200 pg/ml, -<200 pg/ml. IL-13: ++++>4000 pg/ml, +++>3000 pg/ml, ++>2000 pg/ml, +>1000 pg/ml, -<1000 pg/ml. GM-CSF: ++++>700 pg/ml, +++>500 pg/ml, ++>300 pg/ml, +>100 pg/ml, -<100 pg/ml. RANTES: ++++>10000 pg/ml, +++>7500 pg/ml, ++>4000 pg/ml, +>2000 pg/ml, -<2000 pg/ml. Abbreviations: TNF; tumour necrosis factor; IFN γ , interferon gamma; BCL, B cell line; TCR, T cell receptor; RANTES, regulated upon activation, normal T cell expressed and secreted; IL, interleukin; GM-CSF, Granulocyte-Macrophage Colony-Stimulating Factor.

To address other functions of CD8⁺ T cells we assessed the proliferative capacity of ORF3a₂₀₇₋₂₁₅-specific T cell clones via dilution of CFSE. The T cell clones were cocultured with peptide loaded BCLs for 72 hours and CFSE levels were then assessed via flow cytometry (Fig 4.5A). Again, differences in proliferative capacity were observed between the clones. Within the TCR-1 clones, the high functional avidity clone (CL1.2) exhibited greater proliferation than the other clones (Fig 4.5B). This phenomenon was also

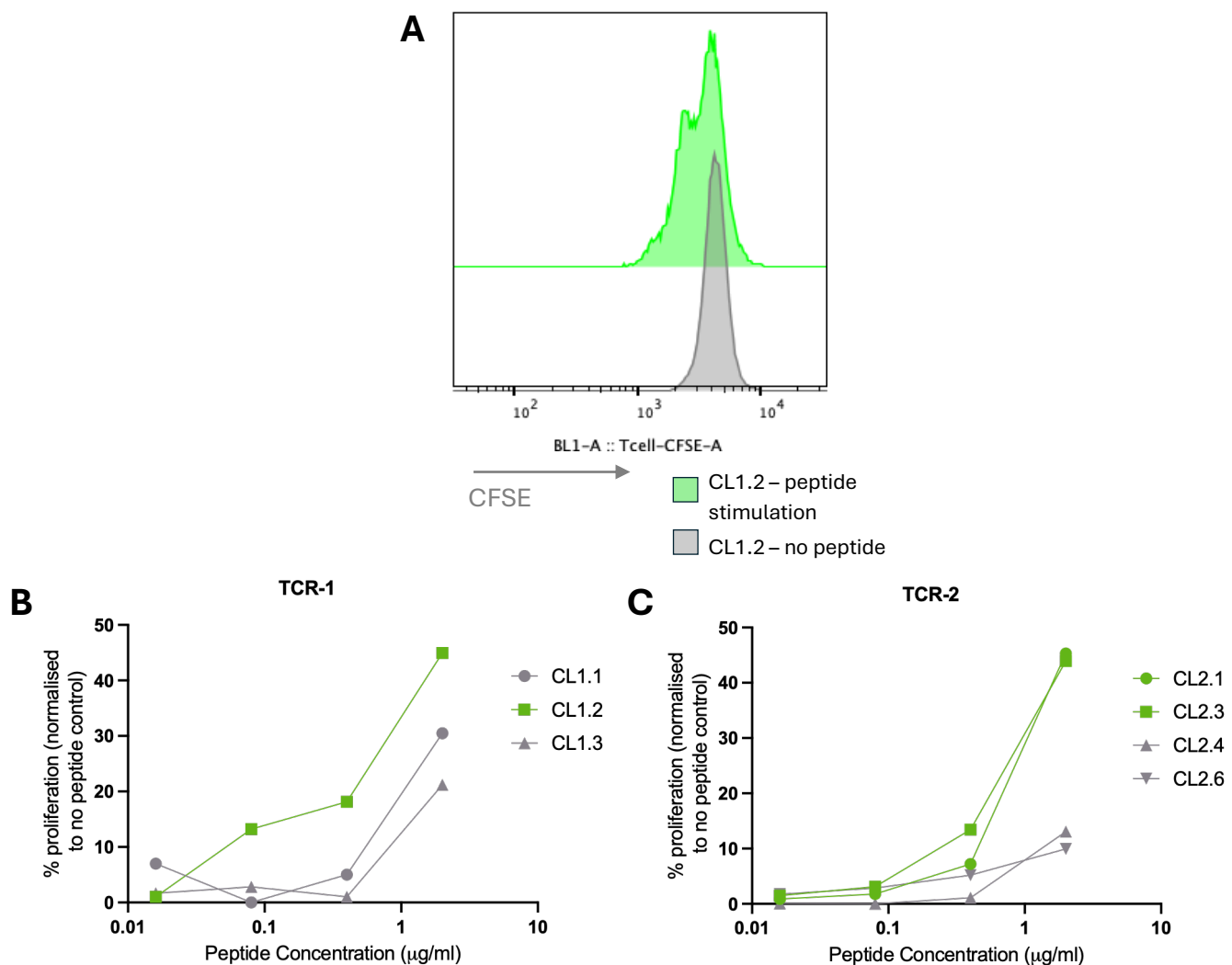


Figure 4.5: ORF3a₂₀₇₋₂₁₅-specific clones with the same TCR differ in their proliferation capacity.

CFSE-stained T cell clones were cocultured with peptide loaded BCLs for 72 hours at an E:T ratio of 10:1. Proliferation was measured by CFSE dilution and proliferation of each clone was normalised to the baseline levels (no peptide BCLs). **(A)** Representative CFSE staining for T cell clone cocultured with no peptide BCLs (grey) and 2 µg/ml peptide-pulsed BCLs (green). **(B, C)** Normalised proliferation levels for ORF3a₂₀₇₋₂₁₅-specific clones with TCR-1 **(B)** and TCR-2 **(C)**. Clones classed as high functional avidity are coloured in green, clones classed as low functional avidity are coloured in grey. Abbreviations: TCR, T cell receptor; BCL, B cell line; E:T, effector: target.

observed for TCR-2 clones (Fig 4.5C), although was particularly apparent at the top peptide concentration.

To assess the ability of ORF3a₂₀₇₋₂₁₅-specific T cell clones to kill, the T cell clones were cocultured with BCLs that had been preloaded with peptide at a range of effector: target ratios. Live dead staining was performed to assess the viability of the BCLs (Fig 4.6A-B).

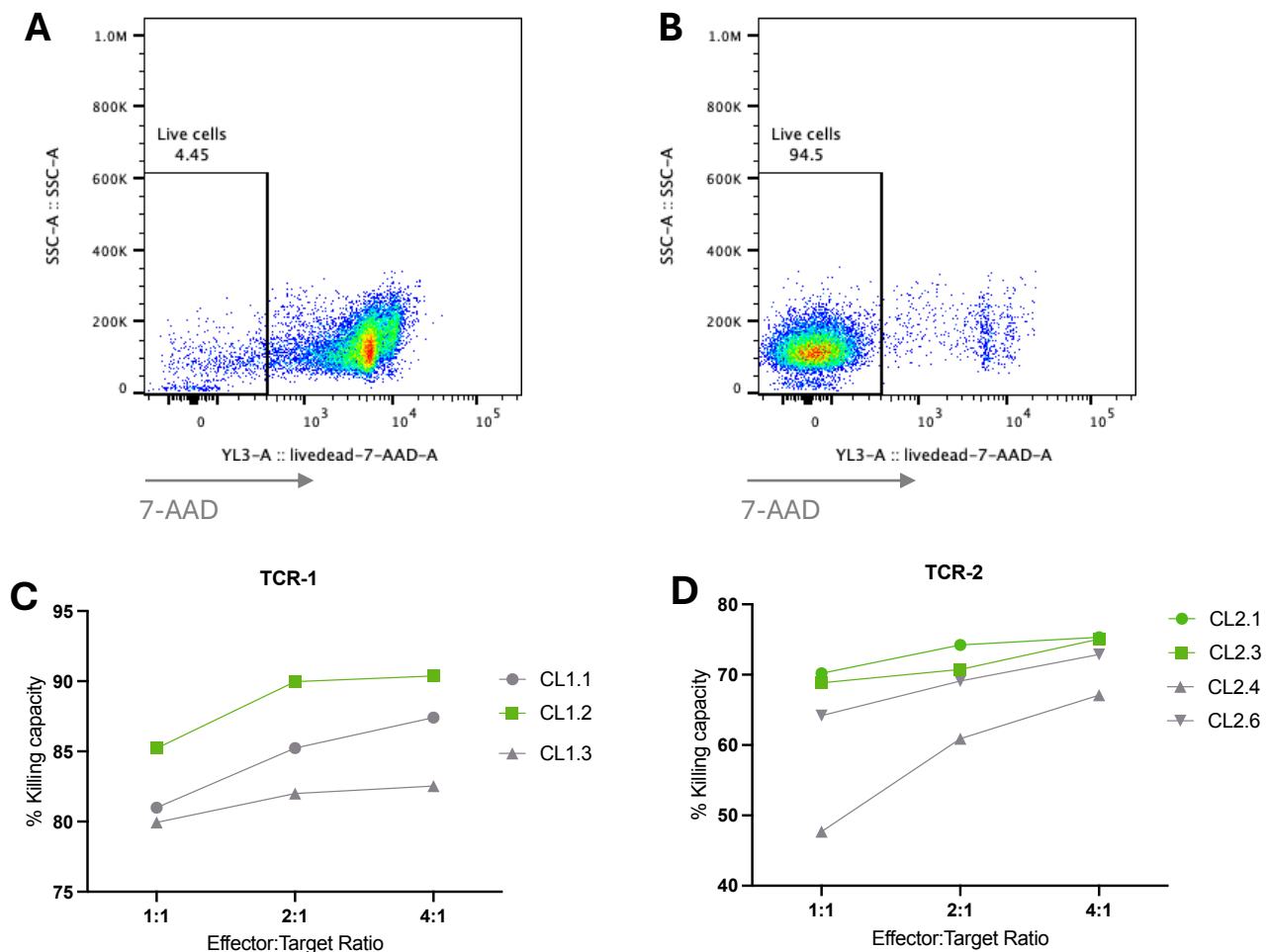


Figure 4.6: ORF3a₂₀₇₋₂₁₅-specific T cell clones with the same TCR differ in their killing capacity. CFSE stained peptide-pulsed BCLs (or non-peptide-pulsed BCLs) were cocultured with T cell clones for 4 hours at the following E:T ratios: 4:1, 2:1, 1:1. Live/Dead staining (7-AAD) was used to determine what proportion of the BCLs had been killed by each of the T cell clones at the E:T ratios. The killing capacity was determined by normalising the % of live peptide-pulsed BCLs at each E:T ratio to the % of live non peptide-pulsed BCLs at the corresponding E:T ratio. **(A, B)** Representative flow plots of proportion of live BCLs at the E:T ratio of 4:1 when the BCLs were preloaded with peptide **(A)** or control BCLs (not loaded with peptide) **(B)**. **(C, D)** Killing capacity of ORF3a₂₀₇₋₂₁₅ clones with TCR-1 **(C)** or TCR-2 **(D)** when BCLs had been preloaded with 0.4 µg/ml peptide. Abbreviations: TCR, T cell receptor; BCL, B cell line; E:T, effector: target.

Across both TCR clonotype groups the high functional avidity clones exhibited the greatest killing capacity at all tested effector: target ratios (Fig 4.6C-D).

Together these data demonstrate substantial functional variability across ORF3a₂₀₇₋₂₁₅-specific T cell clones, despite bearing the same TCR. Therefore, we hypothesised that there are other factors affecting the functionality of these clones that could potentially play a role in the association of this T cell response with severe disease. Thus, moving forward, we use the high functional avidity clones as an extreme example of T cells specific for this response to identify features associated with their enhanced functionality, which could potentially contribute to immunopathology. By comparing ORF3a₂₀₇₋₂₁₅-specific clones with distinct functional profiles but identical TCRs and epitope specificity, we can control for these variables and focus on other factors that may underlie their functional differences.

4.2.2 Optimisation and design of omics experiment

Having identified ORF3a₂₀₇₋₂₁₅-specific T cell clones with differences in their functionality we aimed to perform multi-omics analysis of these clones with and without stimulation. In previous functional assays we utilised FTS peptide-loaded BCLs to activate the T cell clones in an antigen-specific manner. However, this activation method is not possible when preparing samples for omics analysis as it would result in contamination from the BCLs in the samples. Thus, we optimised an alternative method of stimulation which yielded similar levels of activation as stimulation using peptide loaded BCLs. We used ICS to test stimulation via staphylococcus enterotoxin B (data not shown) and anti-CD3/CD28 in comparison to peptide loaded BCLs (Fig 4.7). TNF and IFN γ levels were assessed via flow cytometry. Stimulation with anti-CD3/CD28 at 25 μ l/ml after 4 hours gave comparable percentage of TNF⁺IFN γ ⁺ cells as stimulation with 0.4 μ g/ml peptide-pulsed BCLs (Fig 4.7) and thus this stimulation method was selected for the omics experiments. As substantial differences in the percentage TNF⁺IFN γ ⁺ cells between 2 hour and 4 hours of stimulation were observed we planned to do omics analysis at both timepoints to investigate temporal changes in activation.

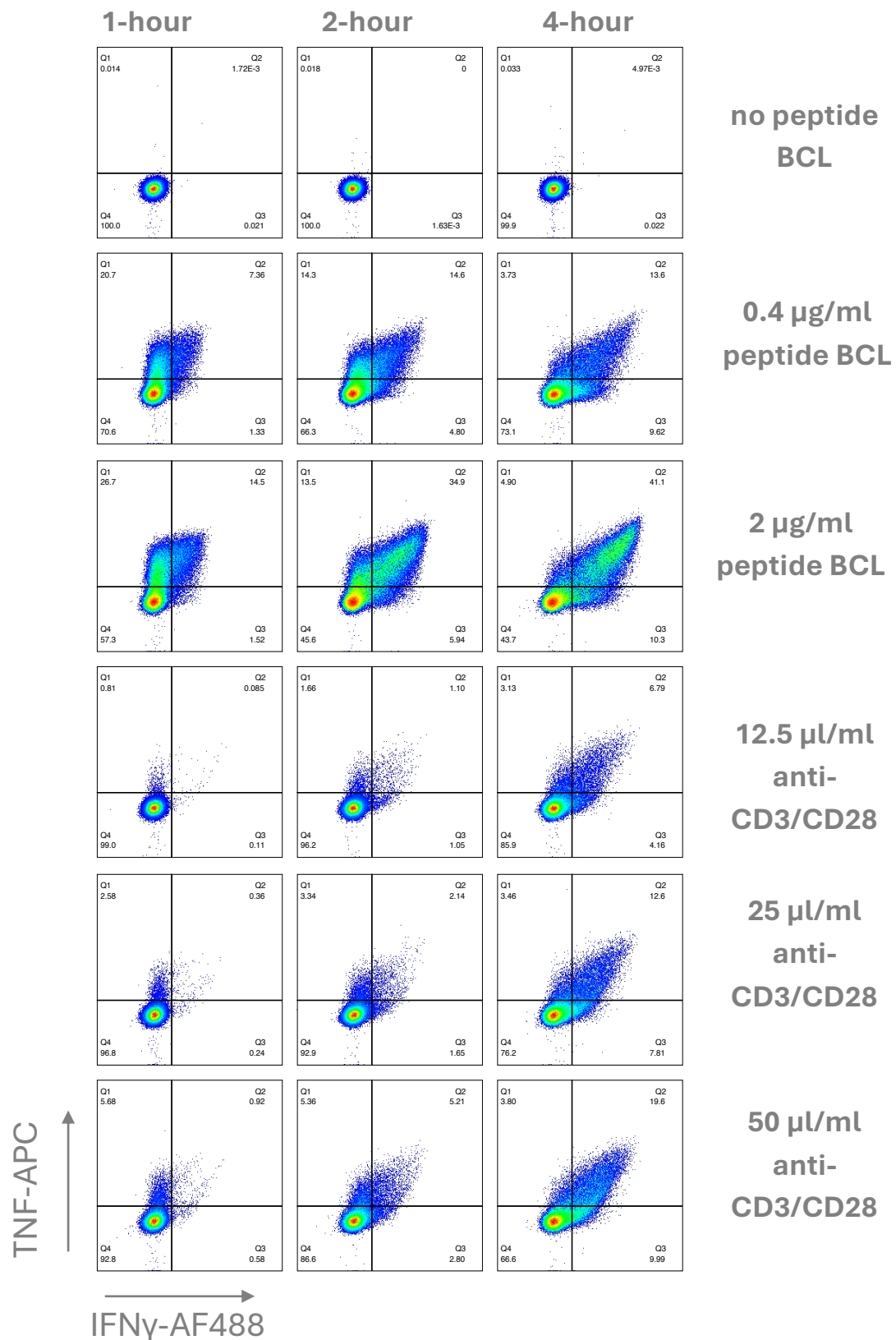


Figure 4.7: Optimisation of stimulation conditions of T cell clones for omics experiment. T cell clones were cocultured with BCLs with that had been preloaded with a peptide (0.4 µg/ml, 2 µg/ml, no peptide control) or stimulated with anti-CD3/CD28 antibody (12.5 µl/ml, 25 µl/ml, 50 µl/ml) for 1, 2 or 4 hours. IFN γ and TNF expression were assessed by intracellular cytokine staining and flow cytometry. Representative FACS plots of TNF and IFN γ staining are shown for CL1.2 with various stimulation conditions. Cells were gates on single cells, live CD8 $^{+}$. Abbreviations: BCL, B cell line; TNF; tumour necrosis factor; IFN γ , interferon gamma.

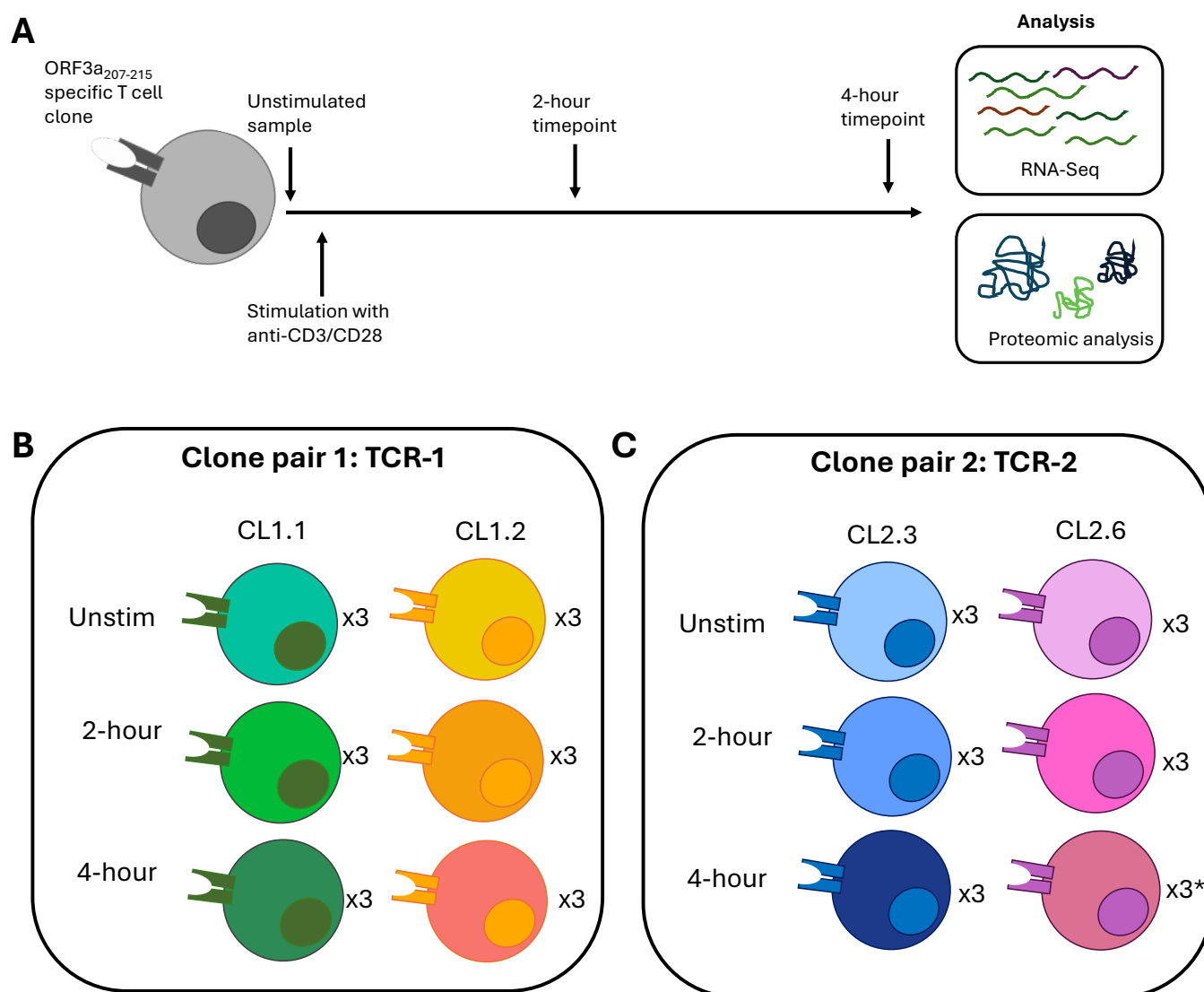


Figure 4.8: Experimental design and samples for omics analysis of ORF3a₂₀₇₋₂₁₅-specific T cell clones. Two clone pairs were selected for omic analysis. T cell clones were stimulated in triplicate with 25 μ l/ml of anti-CD3/CD28 for either 2 or 4 hours. Unstimulated samples were also included. Following stimulation T cell clones were lysed for proteomics and RNA-sequencing. **(A)** Overview of experimental set up for omics experiment. **(B)** Samples generated for TCR-1 clones for RNA sequencing and proteomics. **(C)** Samples generated for TCR-2 clones for RNA sequencing and proteomics. 4-hour stimulated samples of CL2.6 were not included for proteomics analysis due to insufficient cell numbers.

A high and a low functional avidity clone from each of the TCR clonotype groups were selected for omics analysis, giving two pairs of clones. To prepare the samples for omics analysis the clones were stimulated for 2/4 hours with anti-CD3/CD28 in triplicate. Unstimulated samples of the clones were also included. Following stimulation cells

were lysed for RNA extraction and proteomics analysis (Fig 4.8A). For one of the clones (CL2.6) insufficient cells were available so only 2-hour stimulation samples and unstimulated samples were included for proteomics.

4.2.3 Transcriptomic differences in ORF3a₂₀₇₋₂₁₅-specific CD8⁺ T cell clones

To analyse features that could cause functional differences between the ORF3a₂₀₇₋₂₁₅-specific T cell clones we first analysed the transcriptomic data generated from the bulk RNA-sequencing of the clones with and without stimulation. Principal component analysis (PCA) showed that the major source of variance (PC1: 61%) was driven which by which TCR the clone had (Fig 4.9A). The two separate pairs of clones were isolated from the patient at different timepoints (TCR-1 clones were isolated during acute infection, TCR-2 clones were isolated at 1-3 months post infection) which likely influences their transcriptomic profile. As our focus was on differences between clones within each TCR-matched pair, all subsequent analyses were conducted separately for each pair. PCA was then performed separately for each pair of clones (Fig 4.9B and Fig 4.9C). The stimulation status of the clones was the dominant source of variation, contributing 49% of the total variance captured by PC1 for TCR-1 clones and 73% for TCR-2 clones. For TCR-1 clones the second major source of variance appeared to be which clone the sample belonged to, with the 2- and 4-hour stimulation samples for CL1.2 clustering close together (Fig 4.9B). However, for TCR-2 clones the length of stimulation was the second major source of variation with the 2-hour stimulation samples from both clones clustering very closely together (Fig 4.9C).

4.2.3.1 Transcriptomic differences in ORF3a₂₀₇₋₂₁₅-specific CD8⁺ at baseline

We then investigated differences between the clones, at baseline, without any stimulation. We performed differential gene expression testing using DESeq2 and genes were classified as significantly different between the clones in they had a LFC of >1 and an adjusted *P* value of <0.05. For TCR-1 clones, a total of 753 genes were differentially expressed between the clones; 466 of these were more highly expressed by the high functional avidity clone CL1.2 and 287 were expressed at a higher level by CL1.1. This

demonstrates substantial transcriptomic differences between these clones, despite them having been isolated from the same patient, at the same timepoint and bearing the same TCR. Differentially expressed genes relevant to T cell biology were selected for visualisation in a heatmap (Fig 4.10A). CL1.2 had higher expression genes encoding of several costimulatory receptors and chemokine receptors in (Fig 4.10A). Of particular interest, *CX3CR1*, which has been shown to correlate with the degree of effector T cell differentiation³⁰³, was more highly expressed by the high functional avidity clone. The high functional avidity clone also had higher expression of several effector cytokines,

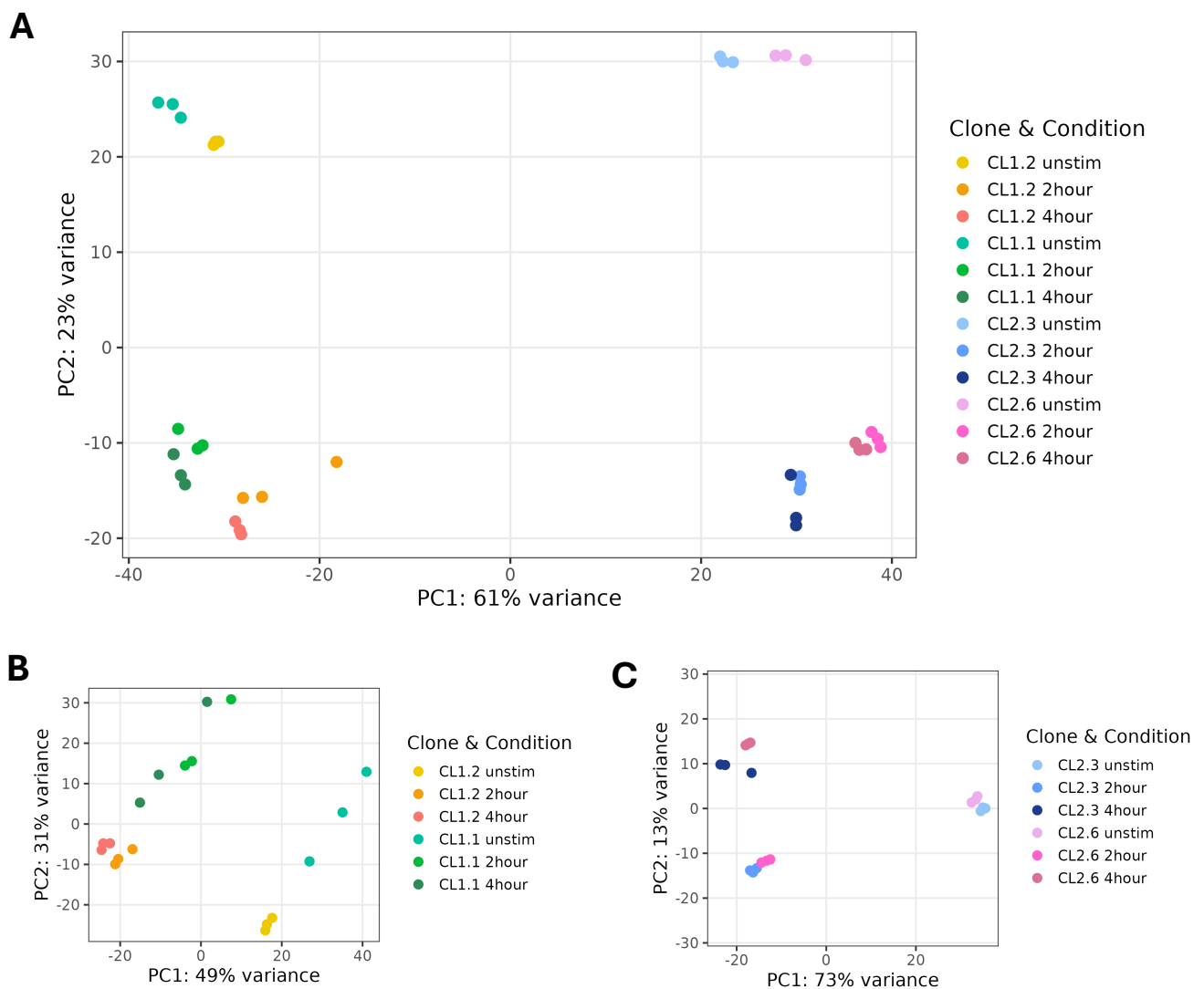


Figure 4.9: Principal component analysis of ORF3a₂₀₇₋₂₁₅-specific T cell clones. PCA was performed on the top 1000 genes using variance stabilising normalised counts in DESeq2. Colours correspond to individual clones and stimulation conditions. **(A)** PCA analysis of all clones. **(B)** PCA analysis of TCR-1 clones. **(C)** PCA analysis of TCR-2 clones. Abbreviations: PCA, principal component analysis; TCR, T cell receptor.

including *IL7* and *IL24*, while the low functional avidity clone exhibited higher expression of the immunomodulatory cytokine *TGFB1*. We observed higher expression of the transcription factor *ZNF683*, which encodes as HOBIT in CL1.2, which is typically associated with expression by tissue resident memory cells and long-lived effector CD8⁺ T cells³⁰⁴. Despite being unstimulated CL1.2 also expresses several genes associated with activation, including *HLA-DRA*, suggesting that it may have a low threshold for activation and be poised to execute effector function.

When comparing the second pair of clones (TCR-2) to each other we identified 162 significantly differentially expressed genes between the clones at baseline. 79 of these were more highly expressed by CL2.3 and 83 of them were more highly expressed by CL2.6. CL2.3 (high functional avidity) had higher expression of the chemokine receptors *CCR4* and *CCR9* while the low functional avidity clone (CL2.6) had higher expression of some interferon stimulated genes, including *IFI44L* (Fig 4.10B). Showing the same trend as TCR-1 clones, the high functional avidity clone of this pair also exhibited higher expression of the transcription factor *ZNF683*.

Together these data indicate that the high functional avidity clones in both TCR-matched pairs have a transcriptomic profile that phenocopies that of highly differentiated effector CD8⁺ T cells, which fits with their enhanced performance in the functional assays. Additionally, their transcriptomic profile suggests that the cells are primed for activation, suggesting they would require low levels of antigen to induce their effector functions.

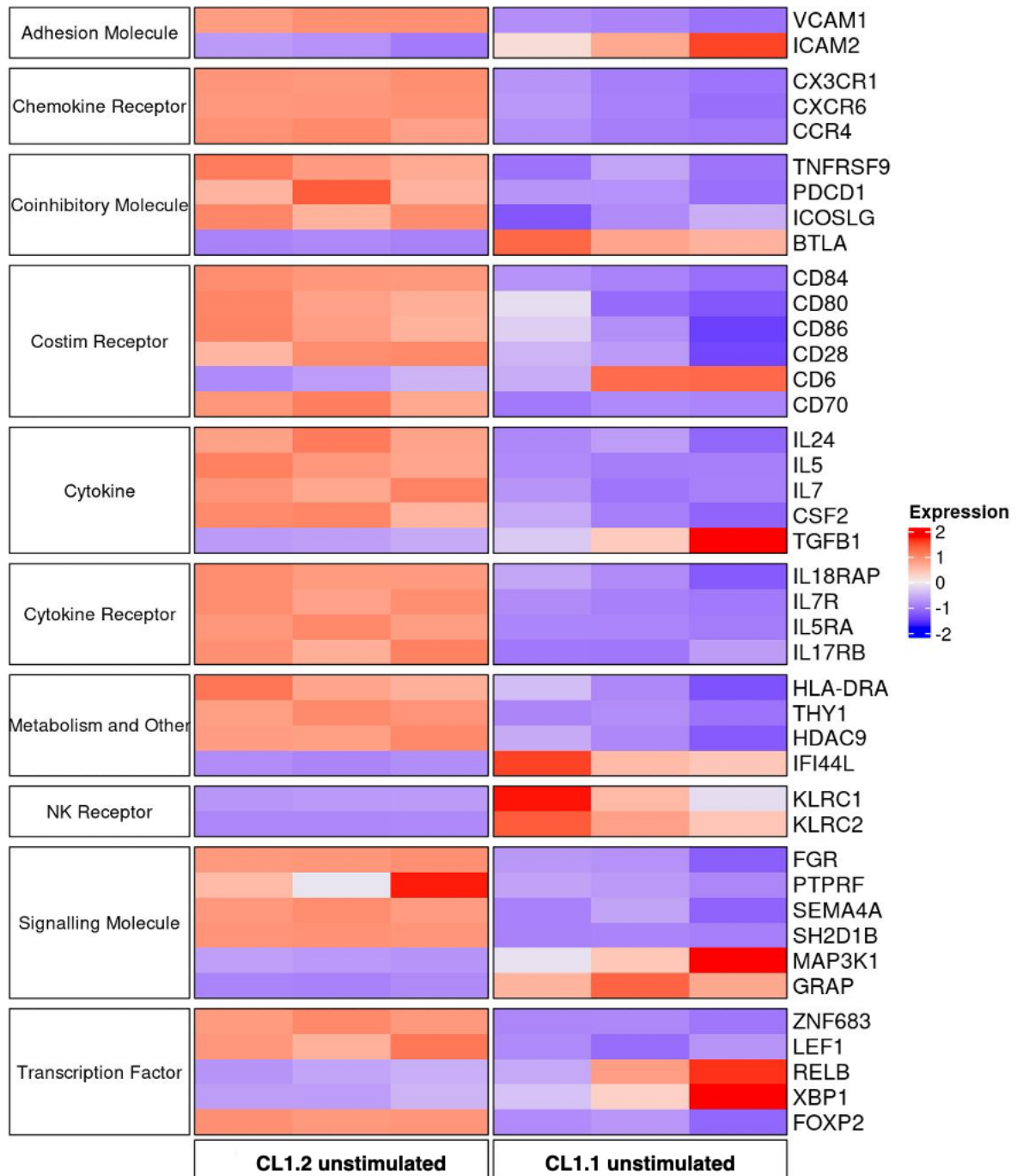


Figure 4.10A: Differentially expressed genes of interest between TCR-1 ORF3a₂₀₇₋₂₁₅-specific T cell clones at baseline. Differential gene expression between unstimulated ORF3a₂₀₇₋₂₁₅-specific T cell clones was performed using DESeq2 with Benjamini–Hochberg method to adjust for multiple testing. LFC shrinkage was performed using the ash method. A subset of significantly different genes (adjusted *P* value <0.05 and LFC>1) were included in the heatmap. The heatmap shows normalised and scaled counts. Abbreviations: TCR, T cell receptor; LFC, log2 fold change; ash, adaptive shrinkage.

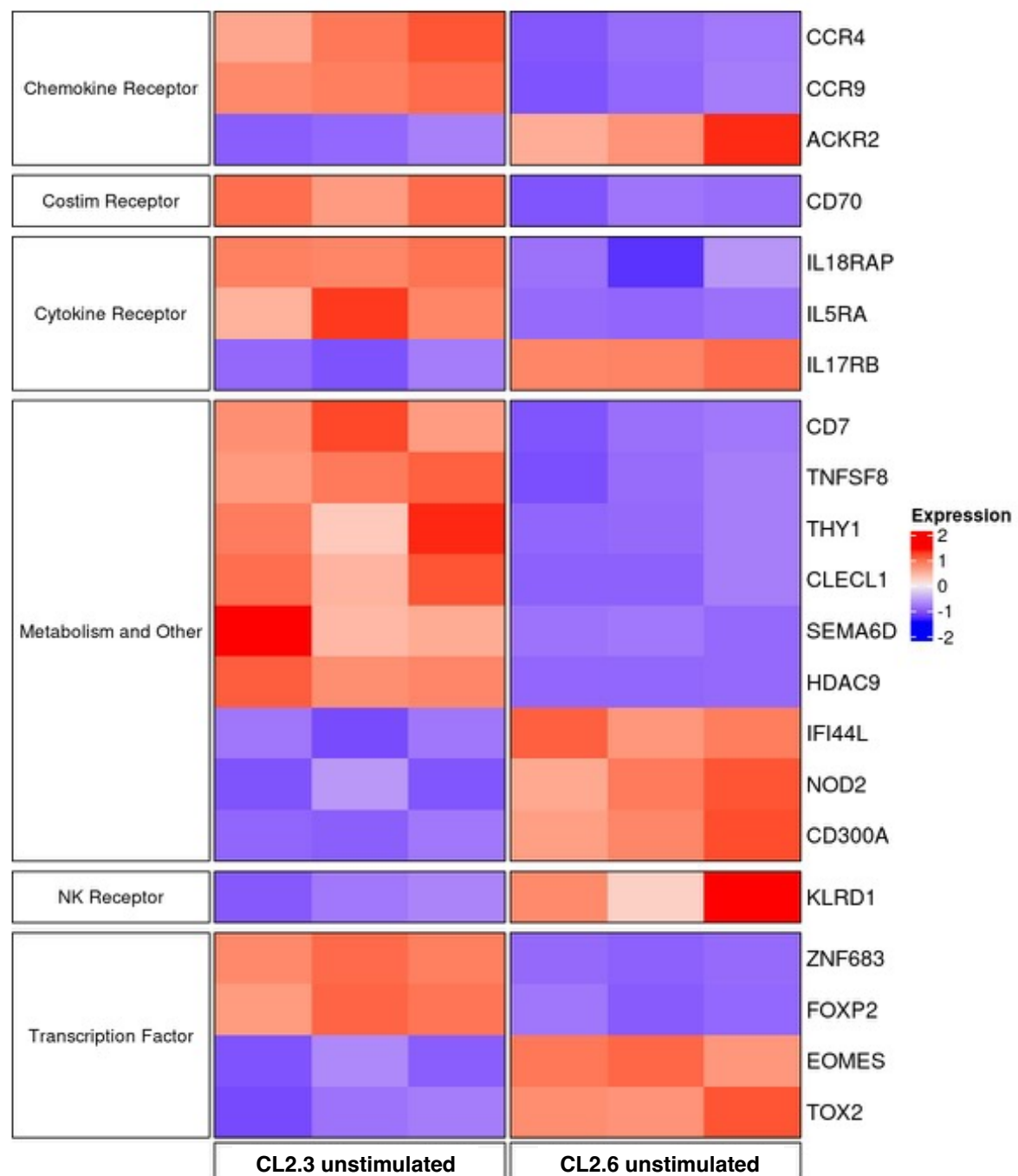


Figure 4.10B: Differentially expressed genes between TCR-2 ORF3a₂₀₇₋₂₁₅-specific T cell clones at baseline. Differential gene expression between unstimulated ORF3a₂₀₇₋₂₁₅-specific T cell clones was performed using DESeq2 with Benjamini–Hochberg method to adjust for multiple testing. LFC shrinkage was performed using the ashhr method. A subset of significantly different genes (adjusted *P* value <0.05 and LFC>1) were included in the heatmap. The heatmap shows normalised and scaled counts. Abbreviations: TCR, T cell receptor; LFC, log₂ fold change; ashhr, adaptive shrinkage.

4.2.3.2 Transcriptomic differences in ORF3a₂₀₇₋₂₁₅-specific CD8⁺ upon stimulation

We next examined transcriptomic changes that occurred upon stimulation of the clones and assessed whether these activation-induced changes differed between clones sharing the same TCR. To do this, we performed differential gene expression analysis comparing unstimulated samples with those stimulated for 2 or 4 hours for each clone. We then determined which genes were significantly differentially expressed upon stimulation and whether these changes were unique to a given clone or shared with the other TCR-matched clone. For the TCR-1 clones, after 2 hours of stimulation, 491 genes were significantly differentially expressed in both clones compared to unstimulated cells while 328 genes were unique to CL1.2 and 269 were unique to CL1.1 (Fig 4.11A). Shared genes upregulated upon stimulation include several cytokines (*IL13*, *IL3*, *IFNG*), activation markers (*PDCD1*, *TIGIT*, *CTLA4*) and signalling molecules including *JUNB* and *FOSB* (Fig 4.11B). After 4 hours of stimulation a greater number of genes were differentially expressed: 1044 shared genes, 477 unique to CL1.2 and 632 unique to CL1.1 (Fig 4.11C). As expected, several cytokines, effector molecules and activation markers were upregulated in both clones following 4 hours of stimulation (Fig 4.11D). We did, however, identify several potentially interesting genes that were only significantly upregulated by the high functional avidity clone CL1.2, including the cytokines *IL2*, *IL4* and *AREG* (Fig 4.11D).

To better visualise the temporal dynamics of genes of interest that exhibited distinct trends between clones, log-normalised counts were plotted at the unstimulated, 2-hour, and 4-hour stimulation timepoints (Fig 4.12). Compared to CL1.1, CL1.2 showed prolonged upregulated expression of several cytokines, including *IL4*, *IL2* and *LIF* (Fig 4.12A-C). As sustained overproduction cytokines has been proposed as a mechanism contributing to tissue damage and immunopathology in COVID-19³⁰⁵, it's possible that the upregulation of these cytokine genes in the highly functional ORF3a₂₀₇₋₂₁₅-specific T cell clones could contribute to the association of this response with severe disease. Additionally, the expression of *GZMB* continues to increase from 2-hours of stimulation to 4-hours of stimulation in the CL1.2 while it levels off for CL1.1 (Fig 4.12D). Although granzyme B is traditionally thought of as a potent anti-viral molecule it has also been

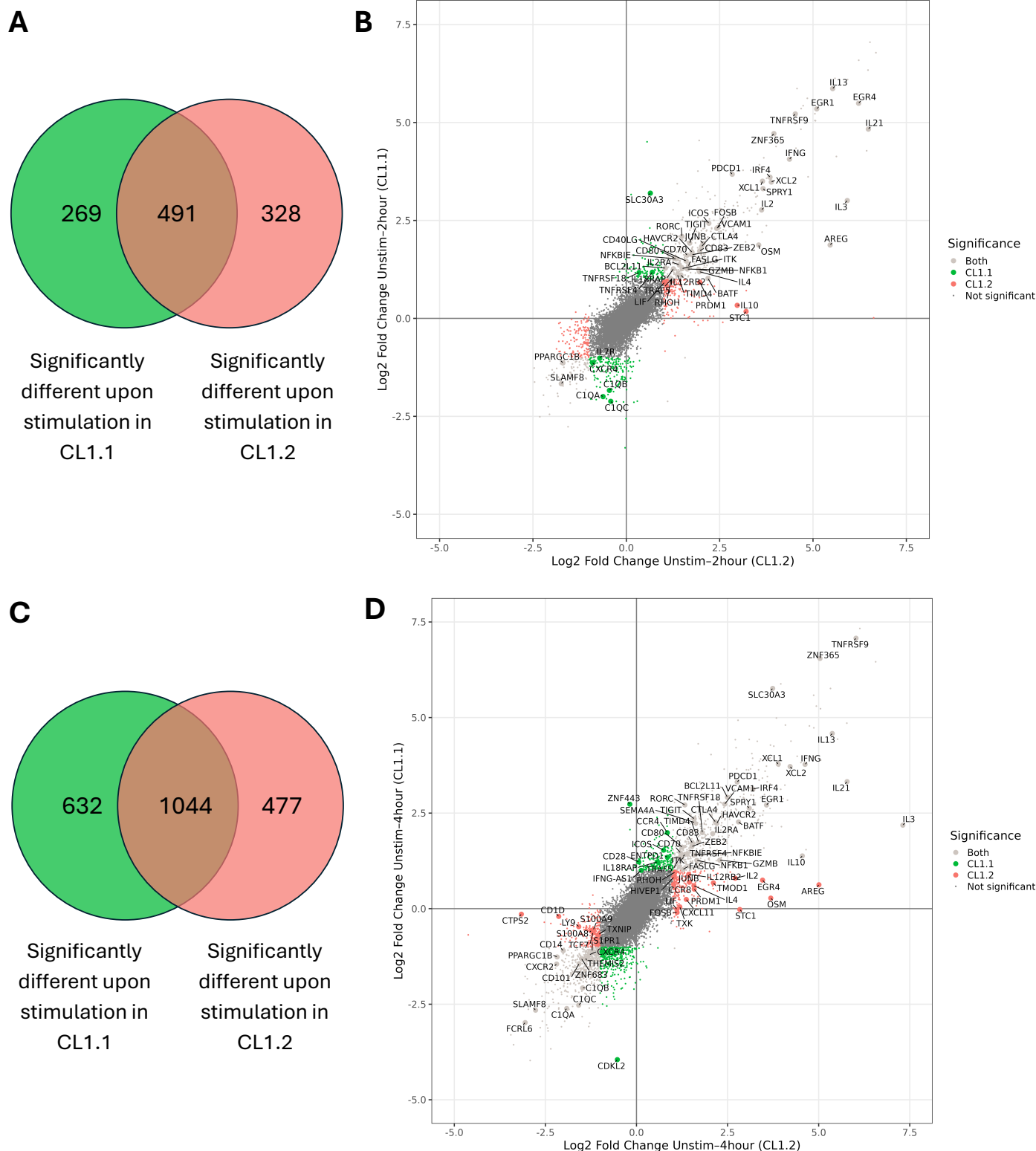


Figure 4.11: Differentially expressed genes between TCR-1 ORF3a₂₀₇₋₂₁₅-specific T cell clones upon stimulation. Differential gene expression between unstimulated and stimulated ORF3a₂₀₇₋₂₁₅-specific T cell clones was performed using DESeq2 with Benjamini–Hochberg method to adjust for multiple testing. LFC shrinkage was performed using the ash method. Genes were considered significant if their adjusted P value < 0.05 and $LFC > 1$. **(A)** Number of unique and shared genes that were significantly differentially expressed between unstimulated and 2-hour stimulated conditions across clones. **(B)** Comparison of LFC of CL1.2 from unstimulated to 2-hour stimulation to LFC of CL1.1 from unstimulated to 2-hour stimulation. Significant genes of interest are labelled. **(C)** Number of unique and shared genes that were significantly differentially expressed between unstimulated and 4-hour stimulated conditions across clones **(D)** Comparison of LFC of CL1.2 from unstimulated to 4-hour stimulation to LFC of CL1.1 from unstimulated to 2-hour stimulation. Abbreviations: TCR, T cell receptor; LFC, log₂ fold change; ash, adaptive shrinkage.

shown to delay wound healing³⁰⁶, suggesting its prolonged expression could contribute to immunopathology. *OSM* and *AREG* also exhibit sustained expression for CL1.2 while levels in CL1.1 decrease back to baseline after 4 hours of stimulation (Fig 4.12E-F). *OSM* is a pleiotropic cytokine that has previously been linked to driving inflammation and tissue damage in the gut³⁰⁷ however a recent study suggested that *OSM* could repair the lung epithelial barrier during inflammatory damage due to viral infection³⁰⁸. *AREG* has been implicated as an immunoregulatory molecule that functions at the interface between T cells and APCs³⁰⁹ and thus its production from these high functional avidity clones may be in an attempt to self-limit damage.

Analysis of TCR-2 clones (Fig 4.13) also revealed shared many genes that were significantly differently expressed upon stimulation at 2 hours (775) and 4 hours (1212). Shared genes that were upregulated upon stimulation included activation markers and several cytokines, as well as the proinflammatory chemokine *CCL4L2*, which has been associated with highly inflammatory T cells and is upregulated in severe COVID-19 patients^{310, 311}. (Fig 4.13B and Fig 4.13D). Again, certain cytokines were exclusively significantly upregulated by the high functional avidity clone in this pair (CL2.3). We also observed significant upregulation of *FASLG* by the high functional avidity clone (Fig 4.13D), which may in part account for the enhanced killing capacity of this clone. Taken together, these results indicate that high functional avidity clones maintain an exaggerated cytokine and effector profile, which may partially explain the association between this T cell response and disease severity.

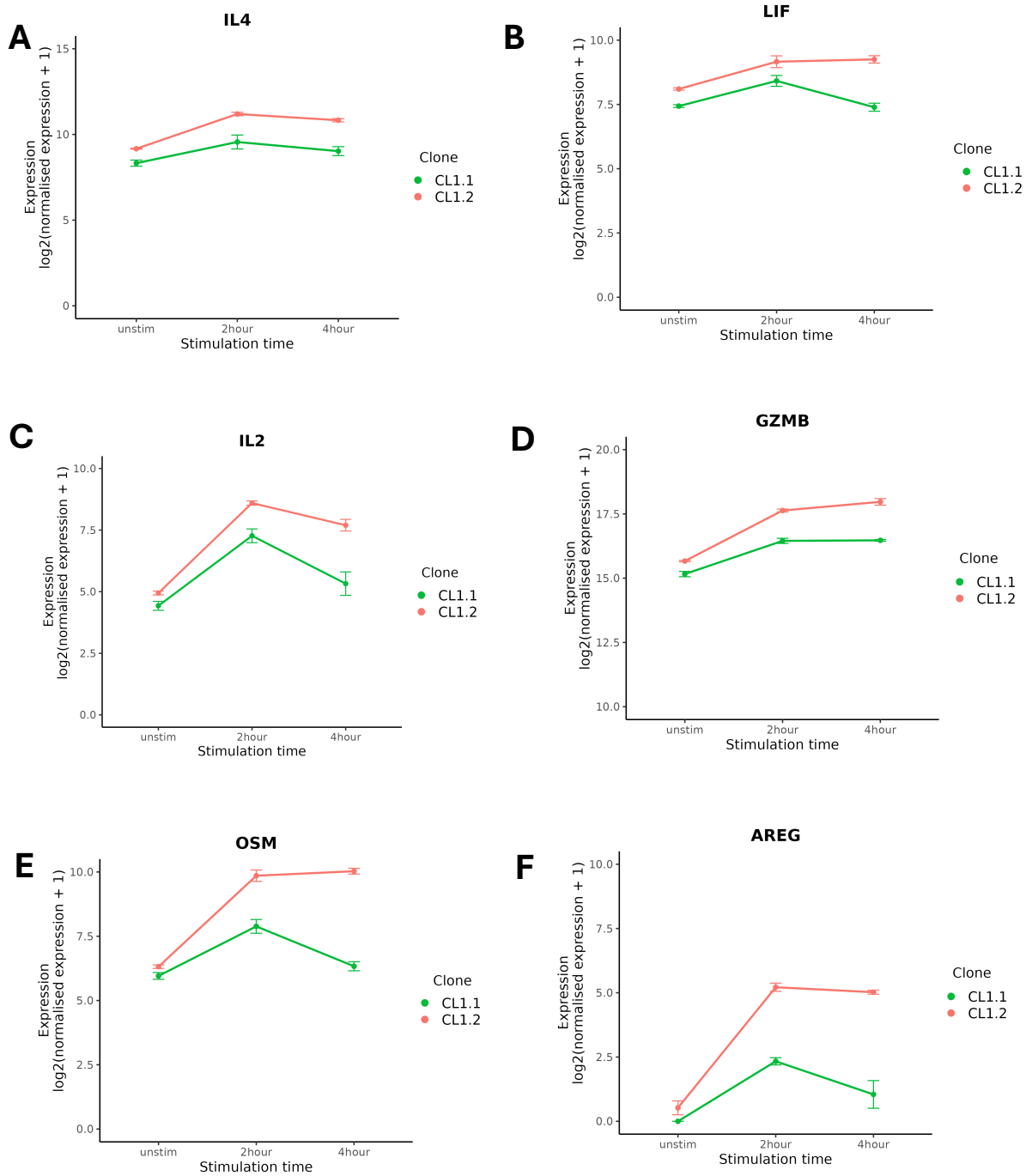


Figure 4.12: Temporal differences in gene expression between TCR-1 ORF3a₂₀₇₋₂₁₅-specific T cell clones upon stimulation. Log normalised counts of (A) IL4, (B) LIF, (C) IL2, (D) GZMB, (E) OSM and (F) AREG at unstimulated, 2-hour and 4-hour stimulated timepoints. Differential gene expression between unstimulated and stimulated ORF3a₂₀₇₋₂₁₅-specific T cell clones was performed using DESeq2. Genes were considered significant if their adjusted *P* value <0.05 and LFC>1. All genes included in this figure were significantly different when comparing unstimulated to 4-hour stimulation for CL1.2. Abbreviations: TCR, T cell receptor; LFC, log2 fold change.

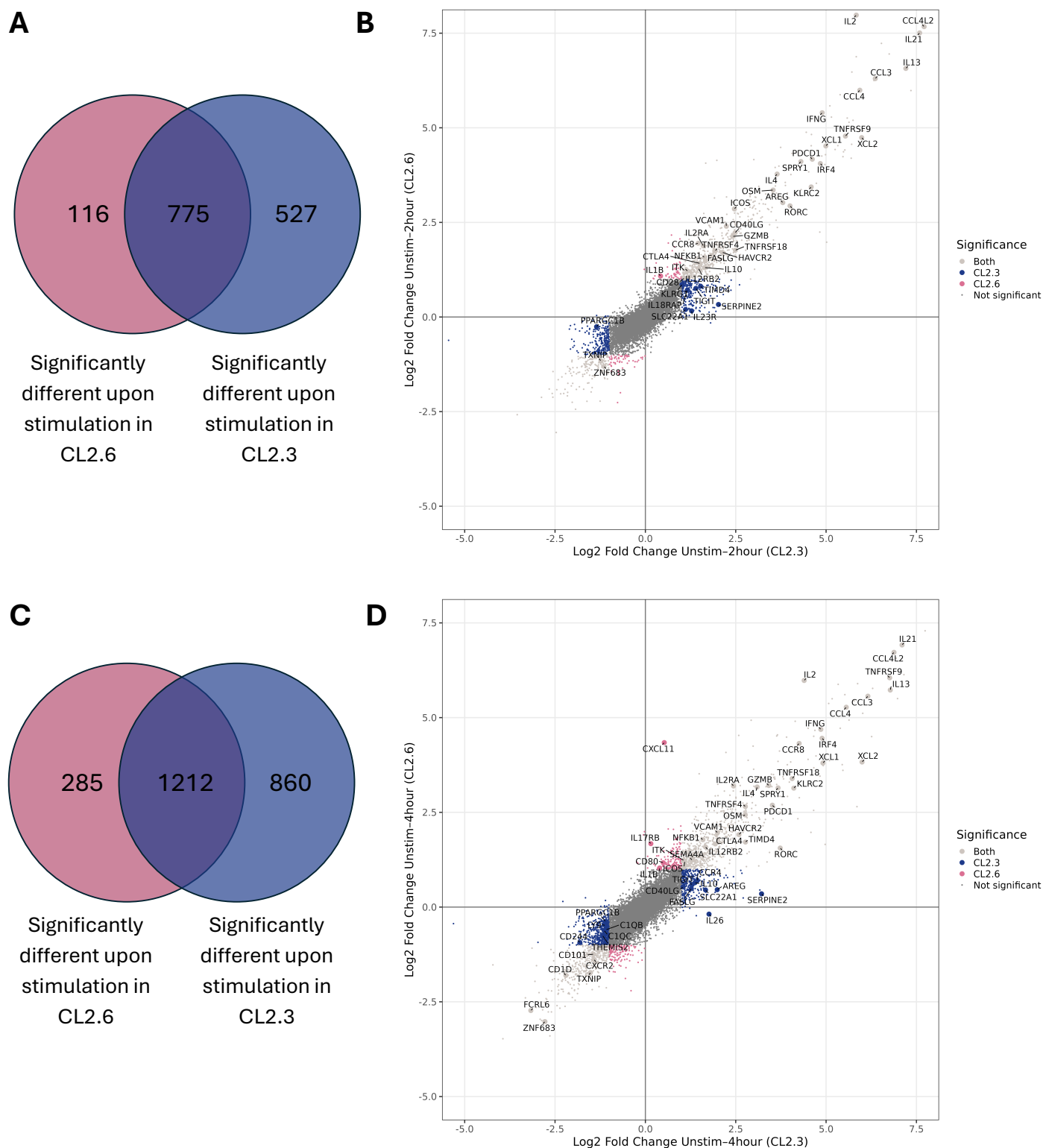


Figure 4.13: Differentially expressed genes between TCR-2 ORF3a₂₀₇₋₂₁₅-specific T cell clones upon stimulation. Differential gene expression between unstimulated and stimulated ORF3a₂₀₇₋₂₁₅-specific T cell clones was performed using DESeq2 with Benjamini–Hochberg method to adjust for multiple testing. LFC shrinkage was performed using the ash method. Genes were considered significant if their adjusted p value <0.05 and LFC>1. **(A)** Number of unique and shared genes that were significantly differentially expressed between unstimulated and 4-hour stimulated conditions across clones **(B)** Comparison of LFC of CL2.3 from unstimulated to 2-hour stimulation to LFC of CL2.6 from unstimulated to 2-hour stimulation. **(C)** Number of unique and shared genes that were significantly differentially expressed between unstimulated and 2-hour stimulated conditions across clones. **(D)** Comparison of LFC of CL2.3 from unstimulated to 4-hour stimulation to LFC of CL2.6 from unstimulated to 2-hour stimulation. Abbreviations: TCR, T cell receptor; LFC, log2 fold change; ash, adaptive shrinkage.

4.2.4 Proteomic differences in ORF3a₂₀₇₋₂₁₅-specific CD8⁺ T cell clones

We then investigated differences between the clones at the protein level. First, we interrogated differences between the clones at baseline, without any stimulation (Fig 4.14A). For the TCR 1 clones a total of 508 proteins were significantly different (LFC>0.25, adjusted *P* value <0.05); 323 were expressed at a higher level by CL1.2 and 185 were expressed at a higher level by CL1.1 (Fig 4.14A). CL1.2 higher expression of several cytotoxic effectors including granzyme B, granulysin, and perforin, suggesting that this clone is primed for efficient release of these cytotoxic molecules upon its stimulation, accounting for its' enhanced killing capacity (Fig 4.14A). We ran pathway enrichment analysis on the proteins that were differentially expressed between the clones (Fig 4.14B-C). CL1.1 exhibited higher expression of several genes involved in interferon signalling (Fig 4.14B) while CL1.2 had enrichment of lipid metabolism pathways and pathways relating to the adhesion molecules integrins and their signalling (Fig 4.14C). In particular, CL1.2 had higher expression of several integrin subunits: integrin α 1, α 2, α 4, α 2B, β 1, β 2 and β 7 (Fig 4.14C).

For TCR 2 clones a total of 480 proteins were significantly different between the clones at baseline; 260 were expressed at a higher level by CL2.3 and 220 were expressed at a higher level by CL2.6 (Fig 4.15A). Again, the high functional avidity clone of this pair had higher expression of the cytotoxic effectors granzyme B and perforin (Fig 4.15A). Pathway analysis revealed an enrichment of genes associated with the phagosome and lysosome in CL2.6 (Fig 4.15B). While CL2.3 showed higher expression of several genes related to the electron transport chain and oxidative phosphorylation as well as cell adhesion molecules (Fig 4.15C). The integrin subunits α 4, β 2 and β 7 (Fig 4.15C) were more highly expressed by the high functional avidity (CL2.3) clone with integrin β 7 being the most significantly different protein between the two clones (Fig 4.15A).

As metabolism underpins T cell function, these data suggest that elevated expression of metabolic pathway-associated proteins could contribute to the enhanced functionality of high functional avidity clones, enabling rapid nutrient metabolism upon activation. Additionally, as we observe higher expression of several integrin molecules on the high functional avidity clones there is a suggestion that these enhance the functionality of the

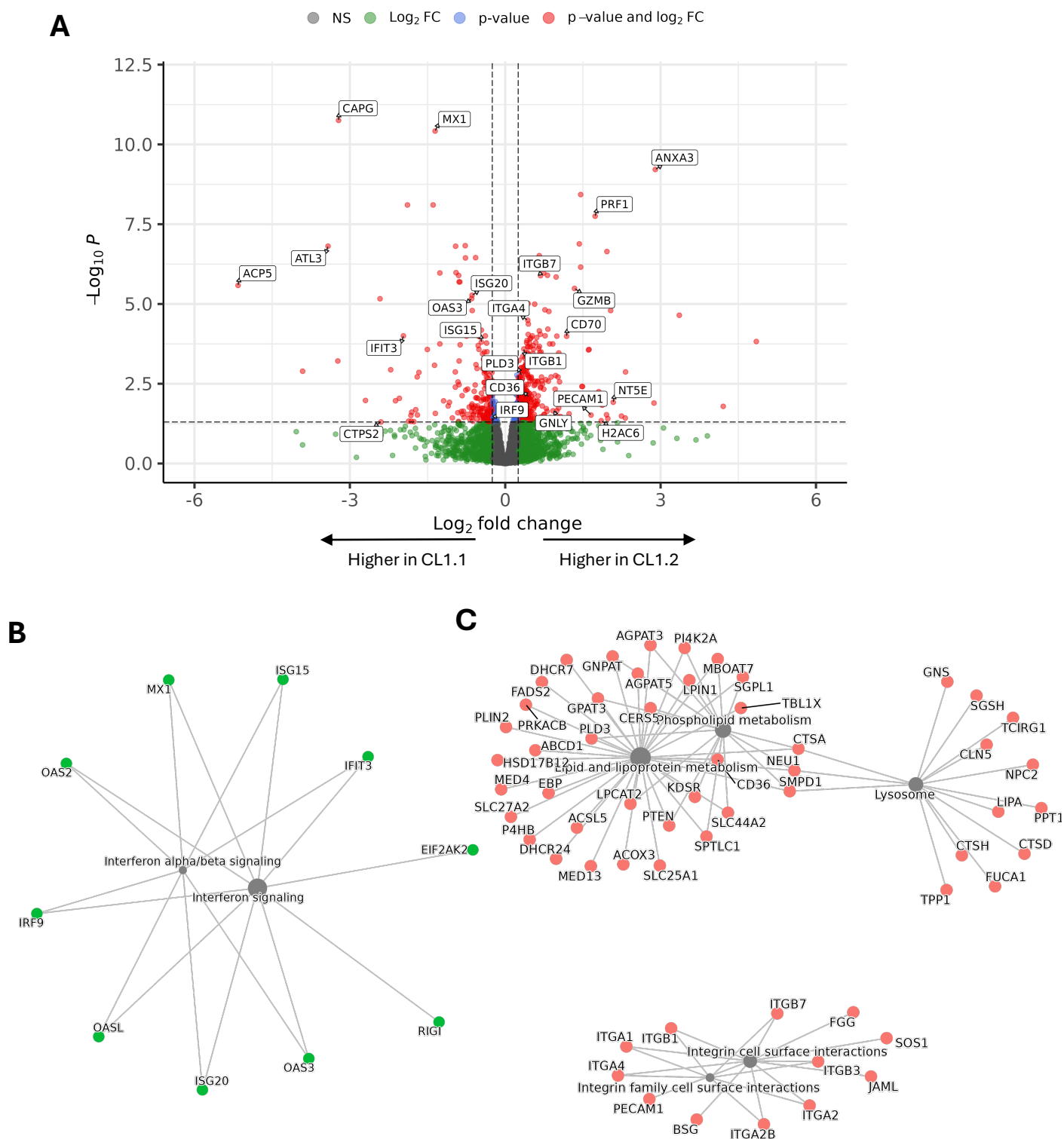


Figure 4.14: Differentially expressed proteins and enriched pathways in unstimulated TCR-1 ORF3a₂₀₇₋₂₁₅-specific T cell clones. (A) Volcano plot highlighting differentially expressed genes between CL1.2 and CL1.1 at baseline. Differential expression testing was performed using DEP2 with the Benjamini–Hochberg method to control for multiple testing. Proteins with an adjusted $p < 0.05$ and a LFC value > 0.25 were classified as significantly differentially expressed. **(B)** Enriched pathways in CL1.1. **(C)** Enriched pathways in CL1.2. Pathway analysis was performed using enrichr and the BioPlanet2019 database to identify enriched pathways. Only pathways of interest with an adjusted p value of < 0.05 are shown. Abbreviations: TCR, T cell receptor; LFC, log₂ fold change.

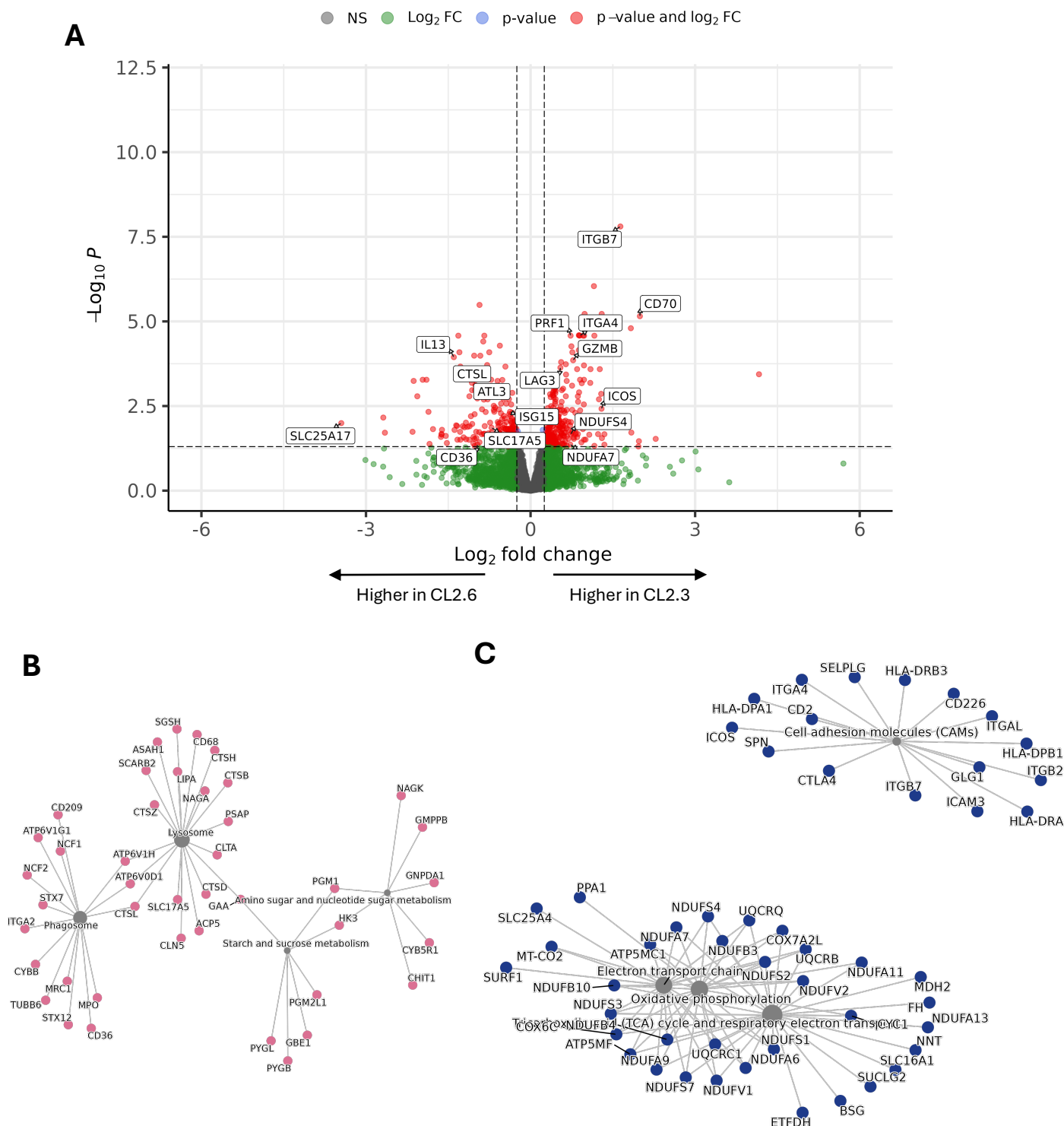


Figure 4.15: Differentially expressed proteins and enriched pathways in unstimulated TCR-2 ORF3a₂₀₇₋₂₁₅-specific T cell clones. (A) Volcano plot highlighting differentially expressed genes between CL2.3 and CL2.6 at baseline. Differential expression testing was performed using DEP2 with the Benjamini–Hochberg procedure to control for multiple testing. Proteins with an adjusted $p < 0.05$ and a LFC value > 0.25 were classified as significantly differentially expressed. Significant proteins of interest are labelled. (B) Enriched pathways in CL2.6. (C) Enriched pathways in CL2.3. Pathway analysis was performed using enrichr and the BioPlanet2019 database to identify enriched pathways. Only pathways of interest with an adjusted P value of < 0.05 are shown. Abbreviations: TCR, T cell receptor; LFC, log₂ fold change.

T cells and could be associated with contributing to disease severity which we will discuss in detail in Chapter 5.

To investigate differences between the clones when they were stimulated, we performed differential gene expression testing between the unstimulated and 2/4-hour stimulated samples for each clone and then analysed whether the proteins were unique to the clone or overlapped with the other TCR-matched clone. At the 2-hour timepoint, 9 proteins were significantly different in both clones, with an additional 32 and 10 proteins uniquely altered in CL1.2 and CL1.1, respectively (Fig 4.16A). After 4 hours of stimulation, 52 proteins were differentially expressed relative to baseline in both clones; however, a further 307 proteins were uniquely differentially expressed in CL1.2, demonstrating a substantially stronger activation-induced response in the high functional avidity clone (Fig 4.16C-D).

We repeated this analysis with TCR-2 clones, although could only do the comparison of 2-hour stimulation to unstimulated as due to limited cell number generate 4-hour stimulation samples for CL2.6 could not be generated. Only two proteins were significantly different for both clones after 2 hours of stimulation, with an additional 11 uniquely altered in CL2.3 (Fig 4.17A). These proteins included the cytokines TNF and IL-13 (Fig 4.17B). This again highlights more profound activation induced changes in the high functional avidity clone. At the 4-hour stimulation timepoint, several cytokines were upregulated by CL2.3, including IL-13, TNF and IL-4 (Fig 4.17C). We also observed decreased expression of the integrins $\alpha 4$ and $\beta 7$ upon stimulation, further suggesting that they could be playing a functional role (Fig 4.17C).

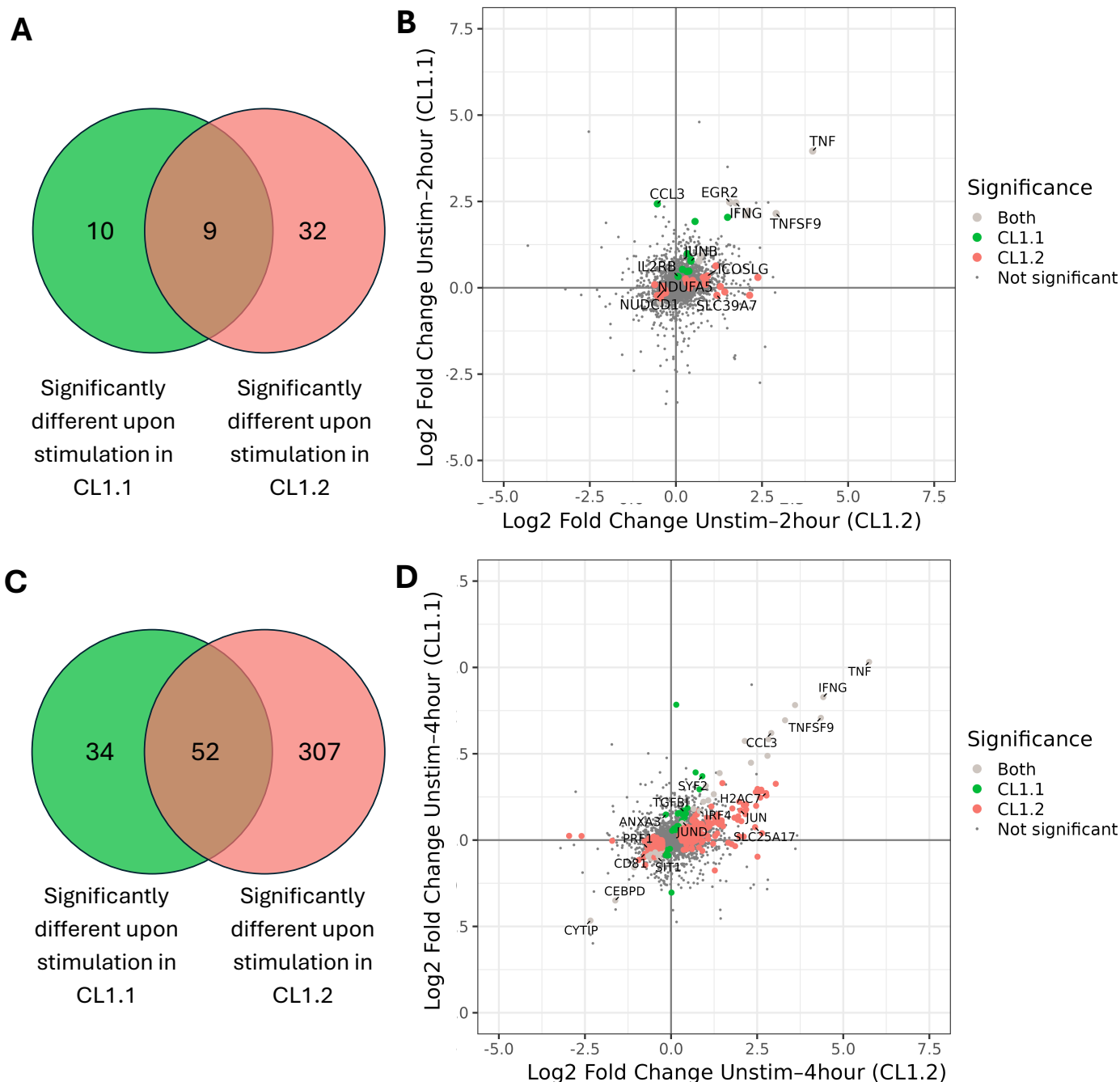


Figure 4.16: Differentially expressed proteins between TCR-1 ORF3a₂₀₇₋₂₁₅-specific T cell clones upon stimulation. Differential protein expression between unstimulated and stimulated ORF3a₂₀₇₋₂₁₅-specific T cell clones was performed using DEP2. Proteins with an adjusted $p < 0.05$ and a LFC > 0.25 were classified as significantly differentially expressed. **(A)** Number of unique and shared proteins that were significantly differentially expressed between unstimulated and 2-hour stimulated conditions across clones. **(B)** Comparison of LFC of CL1.2 from unstimulated to 2-hour stimulation to LFC of CL1.1 from unstimulated to 2-hour stimulation. **(C)** Number of unique and shared proteins that were significantly differentially expressed between unstimulated and 4-hour stimulated conditions across clones. **(D)** Comparison of LFC of CL1.2 from unstimulated to 4-hour stimulation to LFC of CL1.1 from unstimulated to 2-hour stimulation. Abbreviations: TCR, T cell receptor; LFC, log2 fold change.

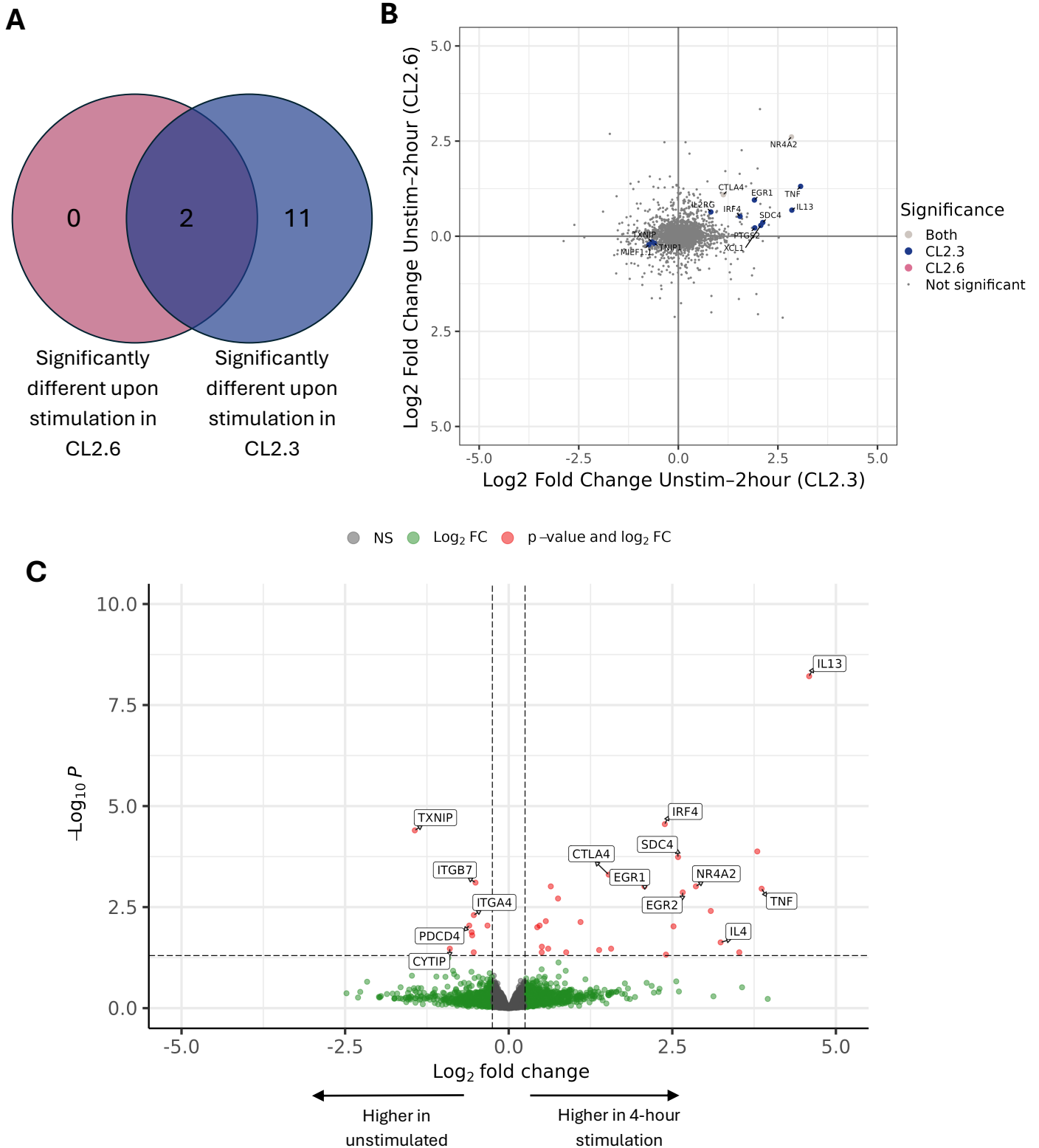


Figure 4.17: Differentially expressed proteins between TCR-2 ORF3a₂₀₇₋₂₁₅-specific T cell clones upon stimulation. Differential protein expression between unstimulated and stimulated ORF3a₂₀₇₋₂₁₅-specific T cell clones was performed using DEP2. Proteins with an adjusted $p < 0.05$ and a LFC value > 0.25 were classified as significantly differentially expressed. **(A)** Number of unique and shared proteins that were significantly differentially expressed between unstimulated and 2-hour stimulated conditions across clones. **(B)** Comparison of LFC of CL2.3 from unstimulated to 2-hour stimulation to LFC of CL2.6 from unstimulated to 2-hour stimulation. **(C)** Volcano plot highlighting differentially expressed genes between CL2.3 at unstimulated and 4-hour stimulation timepoints. Abbreviations: TCR, T cell receptor; LFC, log₂ fold change.

4.3 Discussion

Data from the first wave of the COVID-19 pandemic demonstrated an association between the SARS-CoV-2 ORF3a₂₀₇₋₂₁₅-specific T cell response and disease severity in HLA-A*01:01 positive individuals. In this chapter we utilise ORF3a₂₀₇₋₂₁₅-specific CD8⁺ T cell clones to investigate features of T cells specific for this epitope that could contribute to the association with severe disease.

We identified ORF3a₂₀₇₋₂₁₅-specific CD8⁺ T cell clones that despite bearing the same TCR differ in their functional avidity and functional capabilities, including cytokine production, killing capacity and proliferative capacity. We selected two pairs of clones to perform detailed transcriptomic and proteomic analysis of to identify features associated with functional superiority. We utilise these high functional avidity clones as extreme examples of T cells specific for this response to identify features of them that could explain the association with disease severity.

The transcriptomic profile of the high functional avidity clones phenocopies that of highly differentiated effector CD8⁺ T cells with higher levels of cytokines, genes associated with activation and chemokine receptors at baseline, suggesting they are ready to rapidly respond to antigen stimulation. We also observed temporal differences in transcription of cytokines upon stimulation with the high functional avidity clones exhibiting upregulation of several cytokines for extended periods. At the protein level we observed differences in metabolism; the high functional avidity clones have enrichment of proteins involved in several metabolic pathways. The high functional avidity clones had higher levels of cytotoxic effectors at baseline, providing a potential explanation for their enhanced killing capacity. We also observed higher expression of several integrin molecules on the high functional avidity clones, suggesting that these could enhance the functionality of the T cells. Together, these findings suggest that functional heterogeneity among ORF3a₂₀₇₋₂₁₅-specific clones reflects coordinated differences in activation state, metabolism, and effector capacity.

The high expression of activation markers on T cells and disease severity has been noted in other studies. Several studies observed high levels of CD38⁺HLA-DR⁺ T cells (commonly used as markers for highly activated T cells) in severe COVID-19 patients³¹²,

^{313, 314}. These overactivated CD38⁺CD8⁺ T cells have been associated with tissue damage of several organs in severe COVID-19 patients^{313, 315}. Thus, the high expression of activation markers, including HLA-DR on the high functional avidity clones, is suggestive of the possibility that excessive overactivation of ORF3a₂₀₇₋₂₁₅-specific CD8⁺ T cells may account for their association with disease severity. Spatial transcriptomic analysis of the lungs of deceased COVID-19 patients revealed increased expression of granzymes and perforin in areas of severe alveolar damage, compared to mild alveolar damage¹⁵⁰. Therefore, although important for inducing apoptosis in virally infected cells, the high expression of cytotoxic effectors at baseline in the highly functional ORF3a₂₀₇₋₂₁₅-specific T cells may push the balance from anti-viral immunity to tissue damage and contribute to the association with disease severity.

Severe COVID-19 is often associated with an excessive inflammatory reaction, referred to as a “cytokine storm” which can hinder gas exchange and result in the development of acute respiratory distress syndrome^{305, 316}. Besides contributing to tissue damage through production of cytotoxic effectors, T cells can also contribute to disease severity indirectly through production of cytokines³¹³. A study looking at plasma cytokine levels in hospitalised COVID-19 patients showed increased levels of several T cell-related cytokines, including IL-4, IL-7 and TNF in severe patients compared to mild/moderate patients³¹⁷. In our study the high functional avidity ORF3a₂₀₇₋₂₁₅-specific CD8⁺ T cell clones exhibited higher levels of several cytokines assessed by intracellular cytokine staining and Luminex. Additionally transcriptomic data showed that following activation the high functional avidity clones show prolonged upregulated expression of several cytokines. Thus, by producing high levels of cytokines over an extended period, ORF3a₂₀₇₋₂₁₅-specific T cells could contribute to the cytokine storm that exacerbates tissue damage and disease severity.

Our proteomics data revealed higher expression of several integrin molecules on the high functional avidity ORF3a₂₀₇₋₂₁₅-specific T cell clones. Integrins are typically thought of as cell adhesion molecules that play a fundamental role in T cell migration, but LFA-1 (α L β 2) has also been shown to be a costimulatory molecule that can modulate T cell function. Thus, we hypothesise that the high expression of other integrin family members on the

ORF3a₂₀₇₋₂₁₅-specific T cells could be enhancing their functionality which we will discuss in detail in Chapter 5.

It is important to consider the relationship between the T cell response and disease severity at distinct timepoints during SARS-CoV-2 infection. Having an early virus specific response is likely protective but later in infection overactivated T cells may contribute to tissue damage. Nienhold and colleagues found that patients who died from COVID-19 after a long stay in hospital generally had low viral load but exhibited significant lung tissue damage and infiltration of activated CD8⁺ T cells. The authors observed that the number of CD8⁺ T cells within lung tissue increased with time from the start of infection³¹⁸. Given that the ORF3a protein plays a role in virion assembly and egress³¹⁹ it is plausible to assume that this protein is expressed relatively late in the virus lifecycle and thus in the infection time course. Therefore, T cells specific for the FTS epitope from this protein may be associated with severe disease partially because they are unlikely to have experienced their antigen until relatively late on in the infection.

It is also worth considering the effects of the ORF3a protein itself and its association with disease severity. As well as facilitating viral assembly and release and suppressing anti-viral responses, the ORF3a protein, which is the largest accessory protein of the virus, has been implicated in triggering excessive inflammation³¹⁹. The ORF3a protein can activate the NLRP3 inflammasome which triggers production of many cytokines, contributing to the cytokine storm and an excessive proinflammatory response³²⁰. Importantly studies using transgenic mice have suggested that ORF3a contributes to lung pathology and its deletion reduces the risk of a cytokine storm³²¹. It is therefore plausible that the heightened ORF3a₂₀₇₋₂₁₅-specific T cell responses observed in some patients reflect increased expression of the ORF3a protein, and that the link with disease severity may arise from the inflammatory effects of the protein itself rather than from the T cell response. However, although other studies have not looked directly at the HLA-A*01:01 restricted ORF3a₂₀₇₋₂₁₅-specific T cell response and its association with disease severity, there have been studies investigating HLA genotypes and their effect on disease outcome in COVID-19. In a Russian cohort the presence of HLA-A*01:01 was found to be associated with higher risk of fatal outcome³²², providing further evidence to the idea that the T cells are likely involved in the association with severe disease.

We recognise that our current study into features of ORF3a₂₀₇₋₂₁₅-specific T cells that could contribute to their association with disease severity, has several limitations. The functional assays are limited as they could not be repeated due to limited cell numbers thus statistical testing could not be performed to determine significance. However, although, we couldn't repeat the functional assays, the fact we saw the same observation across several different assays (such as Luminex and ICS) increases our confidence in the findings. All experiments were performed under *in vitro* conditions that do not fully capture the complex inflammatory environment of severe disease. Additionally, the T cell clones we performed omics analysis of were all isolated from one patient so may not be representative of ORF3a₂₀₇₋₂₁₅-specific T cells from other patients. For the omics analysis we could not use peptide loaded BCLs to activate the T cells as the BCLs would contaminant the omics samples and thus we activated the clones using the less physiological method of anti-CD3/CD28 antibody. However, we optimised the concentration and length of stimulation to best mimic stimulation with peptide loaded BCLs. To validate our findings of features associated with severe disease associated T cells we could perform spatial transcriptomics of lung tissue looking for T cells with the features we have identified and see if they are found in areas of immunopathology in lung tissue.

In conclusion this study sheds light on features of ORF3a₂₀₇₋₂₁₅-specific T cells that may drive their association with severe disease in COVID-19. In particular, we identify high baseline expression of cytotoxic effectors, prolonged production of cytokines and high expression of integrin molecules in the highly functional ORF3a₂₀₇₋₂₁₅-specific T cells. These data highlight that there are many factors that influence the functionality of T cells, other than their TCR. This study also highlights the importance of looking at T cells in an antigen-specific manner when looking for associations with disease outcome.

5. Functional effects of $\alpha 4$ integrins on CD8⁺ T cells

5.1 Introduction

The identification of the integrin ligand fibronectin, in the 1970s, proved to be a breakthrough discovery that paved the way for the field of integrin biology^{323, 324, 325}. Research into these cell adhesion molecules began in earnest in the 1980s, with the name “integrin” first proposed in 1986³²⁶. During the 1980s and 1990s, biochemical studies led to the discovery of several integrin family members, and the completion of the human genome sequence in 2000 enabled the identification of all 26 known integrin subunits³²⁷.

Of these 26 subunits, 18 are α subunits and 8 are β subunits which combine to form 24 heterodimers (Fig 1.8A) that have varied binding capabilities. The α and β subunits are both single-pass transmembrane proteins. The integrin subunits have short cytoplasmic tails, which themselves do not possess kinase domains but serve as a hub for protein-protein interactions between integrins and intracellular proteins which enable downstream signalling. Structural studies have revealed that integrin heterodimers exist in several distinct conformations^{328, 329, 330}: a bent conformation which has low affinity for ligands, an extended conformation with closed headpiece which has intermediate affinity for ligands and a high affinity conformation in which the heterodimer is extended and has an open headpiece^{189, 327} (Fig 1.8B).

All cells in the human body, except for mature red blood cells, express at least one integrin heterodimer and these molecules play fundamental roles in cell-cell adhesion and adhesion to the extracellular matrix. Of particular interest to this thesis chapter, is the subset of integrin heterodimers that are expressed exclusively by leukocytes³³¹. Of these, LFA-1 ($\alpha L\beta 2$), which was first found to be expressed by T cells in the 1980s³³², is the best characterised. The importance of these integrins on immune cells was highlighted by the observation that mutations in the gene for the $\beta 2$ integrin subunit resulted in leukocyte adhesion deficiency¹⁹⁰ (now known as LAD-I); a rare genetic condition in which patients suffer from recurrent infections. Mortality for severe LAD-I is as high as 75% by the age of 2 in patients who do not receive stem cell transplants^{333, 334}.

Much of the research on the integrins expressed by T cells has focussed on their effects on lymphocyte migration, adhesion and homing (see section 1.7.4). Recently, other effects of integrins on T cells, aside from their effects on migration, have been studied in greater detail. Signalling from immune cell integrins is tightly intertwined with that of other receptors, via inside out and outside in signalling (see section 1.7.4). LFA-1 plays a fundamental role in the immune synapse – the interface between a T cell and an APC or target cell^{51, 52, 184, 335}. LFA-1 is primarily located in the pSMAC of the immune synapse, where it functions not only as an adhesion molecule but also to provide costimulation; its engagement with ICAM-1 increases TCR-induced tyrosine phosphorylation and activation of downstream TCR signalling molecules, including PLC γ 1 and PI3K^{53, 336, 337, 338}. Through this mechanism, LFA-1 can decrease the threshold of pMHC needed for T cell activation and thus increase T cell functionality. Additionally, a recent study demonstrated that ligand-bound LFA-1 plays a direct role in facilitating T cell cytotoxicity by functioning as a spatial cue to attract granules containing perforin and granzyme and induce their fusion with the plasma membrane⁵⁷. Aside from the well-characterised LFA-1, the unconventional integrin pairing α E β 3, has recently also been shown to promote TCR signalling³⁰¹. However, the functional effects of other integrin heterodimers are not well characterised on T cells.

Having identified differential expression of integrin subunits on ORF3a₂₀₇₋₂₁₅-specific CD8⁺ T cell clones in Chapter 4, in this chapter we explore whether the integrin heterodimers α 4 β 1 and α 4 β 7 influence CD8⁺ T cell functionality. The ligands for these integrins include the immunoglobulin superfamily members VCAM-1 and MAdCAM-1 and the extracellular matrix protein fibronectin¹⁸⁴. In this chapter we focus on the interactions of the integrins with VCAM-1 which is a ligand for α 4 β 1 and α 4 β 7 and MAdCAM-1 which is bound by α 4 β 7. The expression of these integrins on immune cells has been implicated in the pathogenesis of inflammatory conditions including Crohn's disease (CD) and multiple sclerosis (MS). CD is a form of inflammatory bowel disease^{339, 340} characterised by the interplay of genetic and environmental factors and a dysregulated immune system, including a proinflammatory T cell response^{340, 341} that expresses high levels of α 4 β 1 and α 4 β 7³⁴², all of which contribute to disease pathogenesis. The ligands for these integrins are also implicated in CD pathogenesis;

MAdCAM-1 expression is increased in areas of active inflammation in CD³⁴³ and levels of VCAM-1 are higher in the large intestine³³⁹.

Together this data provided the rational for the development of monoclonal antibodies (mAb) targeting the integrins $\alpha 4\beta 1$ and $\alpha 4\beta 7$ for the treatment of CD and other inflammatory conditions. Natalizumab is a humanised IgG4 monoclonal antibody targeting the $\alpha 4$ chain of both $\alpha 4\beta 1$ and $\alpha 4\beta 7$ integrins, thereby inhibiting their binding to VCAM-1 and MAdCAM-1^{344, 345}, and was shown in early clinical trials to induce remission in CD³⁴⁶ and to limit immune cell trafficking to the central nervous system in MS²⁰⁰. However, its use is now restricted due to an increased risk of progressive multifocal leukoencephalopathy caused by John Cunningham virus infection³⁴⁷.

Vedolizumab is a humanised IgG1 mAb that targets $\alpha 4\beta 7$ by binding to an epitope within the $\beta 7$ -chain of the heterodimer³⁴⁸ and was approved for the treatment of CD in 2014³⁴⁹. Due to the epitope it binds, vedolizumab prevents the binding of $\alpha 4\beta 7$ to MAdCAM-1 but not to VCAM-1³⁵⁰. However, vedolizumab does induce internalisation of $\alpha 4\beta 7$ ³⁵¹, thereby reducing its expressing on cells treated with this mAb. Preclinical studies in animal models found a reduction in $\alpha 4\beta 7$ T cells in the intestinal lamina propria of animals treated with vedolizumab³⁵² so inhibition of the migration of proinflammatory cells to intestinal tissue was assumed to be the mechanism of action. However, there is a growing body of evidence that vedolizumab does not reduce lymphocyte trafficking to the gut in humans; Zeissig and colleagues found no reduction in the abundance and clonal diversity of lamina propria T cells following vedolizumab treatment³⁵³ and DeBerg and colleagues found no depletion of CD4 T cells from the colonic mucosa following vedolizumab treatment¹⁹⁹. Despite this, vedolizumab is effective in many patients, thus there is a need in the field to identify the mechanism of action of this drug.

Previous studies have shown that other integrin heterodimers can influence T cell function. In Chapter 4, we observed higher expression of $\alpha 4$ -containing integrins in ORF3a₂₀₇₋₂₁₅-specific CD8⁺ T cell clones with enhanced functionality, suggesting that these integrins may contribute to their heightened responses. In this chapter, we first validate these differences in integrin expression and then use *in vitro* functional assays

and immune synapse imaging to investigate how $\alpha 4$ -containing integrins modulate CD8⁺ T cell functionality.

5.2 Results

5.2.1 Differences in integrin expression between functionally distinct CD8⁺ T cell clones

In the previous chapter, proteomic analysis revealed differences in integrin expression between functionally distinct ORF3a₂₀₇₋₂₁₅-specific CD8⁺ T cell clones at baseline. To explore this observation further, we reanalysed the proteomic data to examine all integrin molecules and compare their expression between the clones at the unstimulated, 2-hour, and 4-hour stimulation timepoints (Fig 5.1A-B). Integrins that were significantly different between the clones in at least one of the three timepoint comparisons, were included in the heatmaps.

For the TCR-1 clones, the high functional avidity clone (CL1.2) had higher expression of several α subunits at baseline, including $\alpha 1$, $\alpha 2$, $\alpha 4$ and $\alpha 5$, while CL1.1 had higher expression of αM (Fig 5.1A). CL1.2 had higher expression of the β subunits $\beta 1$ and $\beta 7$ at baseline. Both clones exhibited an upregulation of $\beta 3$ with stimulation, although this was more profound for CL1.2. Integrin $\beta 3$ has recently been shown to promote cytotoxicity and increase TCR signalling³⁰¹. We also observed several differences between TCR-2 clones; CL2.3 had higher expression of both subunits of the well characterised LFA-1 (αL and $\beta 2$), and higher expression of $\alpha 4$ and $\beta 7$ at baseline (Fig 5.1B). Interestingly, for both CL1.2 and CL2.3 we observed decreased levels of the integrins $\alpha 4$ and $\beta 7$ upon stimulation, suggesting that these integrins could be playing a functional role.

As the integrins $\alpha 4$ and $\beta 7$ were expressed at higher levels in the high functional avidity clones of both TCR-matched pairs and given that these integrins are clinically targeted but their mechanisms of action remain unclear, we decided to investigate them in greater detail. The integrin subunits $\alpha 4$ and $\beta 7$ can pair with each other and often do so on T cells, however $\alpha 4$ can also pair with $\beta 1$ which was also expressed at a higher level by CL1.2 and thus we also investigated integrin $\beta 1$ in more detail. In addition to $\alpha 4$, $\beta 7$ can pair with αE

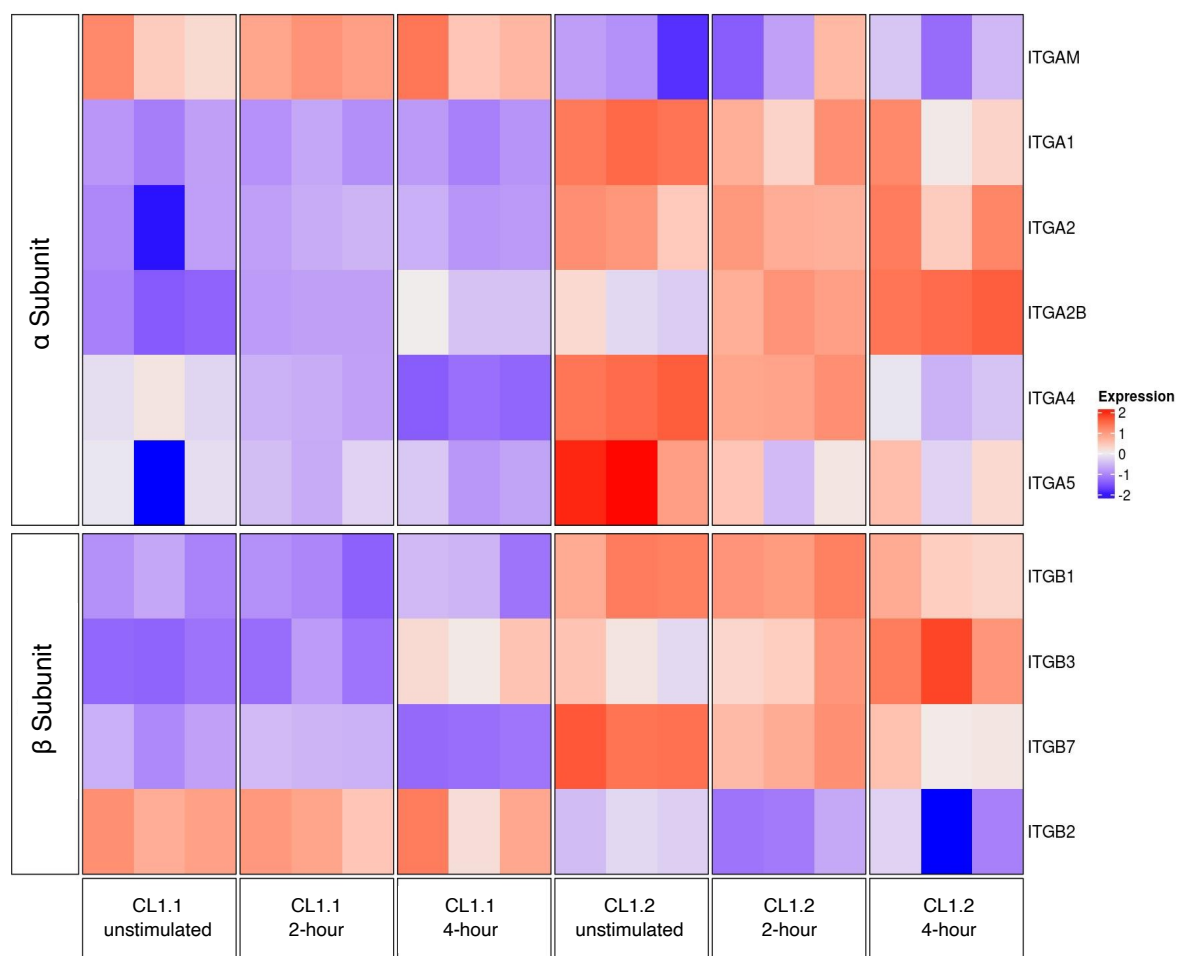


Figure 5.1A: Integrin expression of TCR-1 clones at baseline and upon stimulation. Differential protein expression of ORF3a₂₀₅₋₂₁₇-specific T cell clones at unstimulated, 2-hour and 4-hour stimulation samples was performed using DEP2. Proteins with an adjusted $p < 0.05$ were classified as significantly differentially expressed. Integrins that were significantly different between CL1.1 and CL1.2 at any of the timepoints were included in the heatmap. Colours represent row-wise z-scored protein expression values. Each protein was scaled independently prior to plotting.

(CD103), however we did not identify this protein in our proteomics dataset and confirmed via flow cytometry that the ORF3a₂₀₇₋₂₁₅-specific CD8⁺ T cell clones were negative for α E (data not shown).

We first validated the differences in integrin expression between the clones using flow cytometry. To account for variation between experiments performed with different antibodies and flow cytometers, we calculated the mean fluorescence intensity (MFI) of the high functional avidity clone relative to that of the low functional avidity clone in each independent experiment. Compared with CL1.1, the relative MFIs of α 4, β 1, and β 7 of CL1.2 were increased by approximately 1.7-, 1.5-, and 1.9-fold, respectively (Fig. 5.2D–

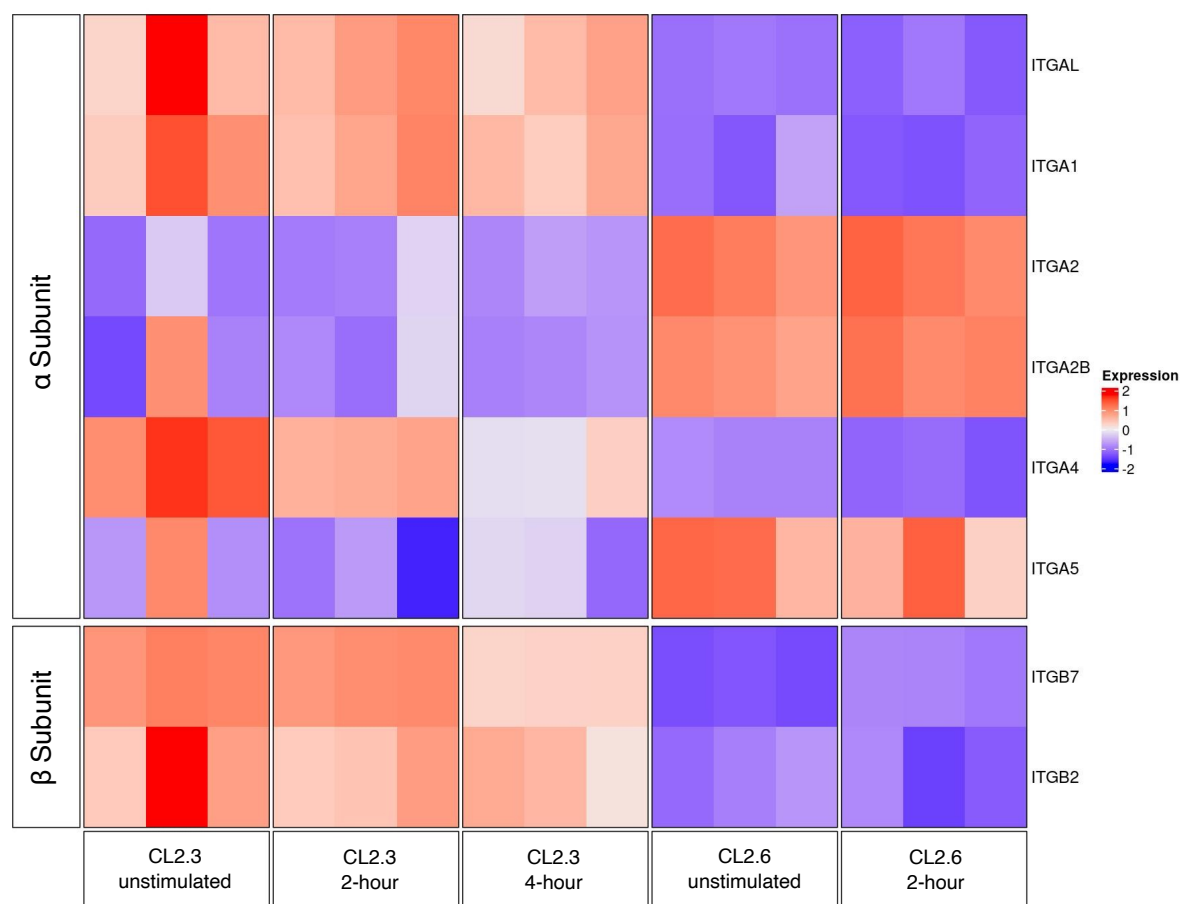


Figure 5.1B: Integrin expression of TCR-2 clones at baseline and upon stimulation. Differential protein expression of ORF3a₂₀₅₋₂₁₇-specific T cell clones at unstimulated, 2-hour and 4-hour stimulation samples was performed using DEP2. Proteins with an adjusted $p < 0.05$ were classified as significantly differentially expressed. Integrins that were significantly different between CL2.3 and CL2.6 at any of the timepoints were included in the heatmap. Colours represent row-wise z-scored protein expression values. Each protein was scaled independently prior to plotting.

F), although $\beta 1$ did not reach significance (Fig 5.2E). Compared with CL2.6, the relative MFIs of $\alpha 4$, $\beta 1$, and $\beta 7$ of CL2.3 were increased by approximately 1.5-, 1.3-, and 2.5-fold, respectively (Fig 5.2G-I). These data confirmed our observations from the proteomics data and demonstrated that these differences in integrin expression were stable as these flow cytometry experiments were repeated over a period of 6 months.

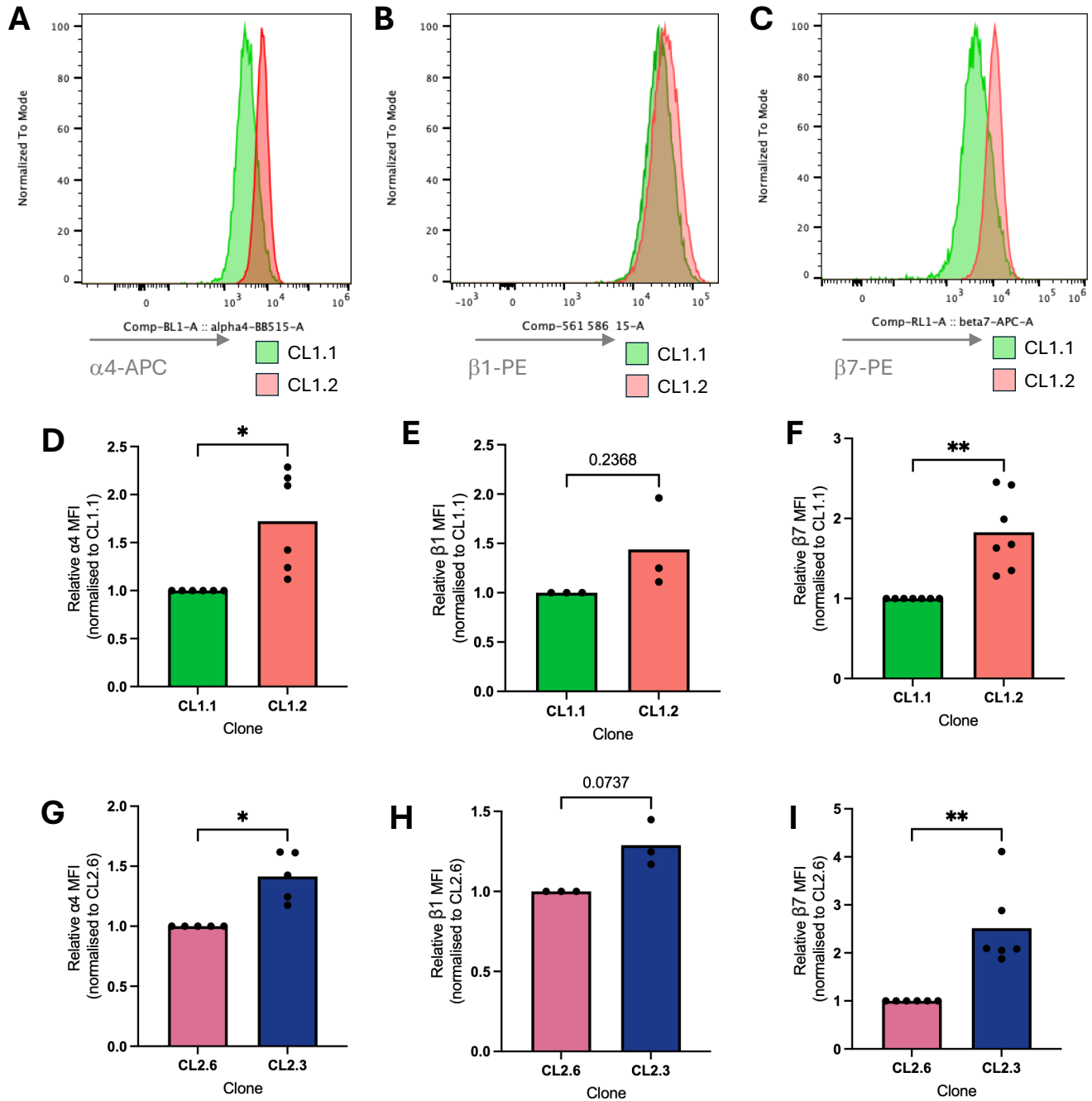


Figure 5.2: Differences in integrin expression between ORF3a₂₀₇₋₂₁₅-specific T cell clones. T cell clones were stained with fluorescently conjugated antibodies targeting the integrins $\alpha 4$, $\beta 1$ and $\beta 7$ and levels were assessed via flow cytometry. Representative staining of (A) $\alpha 4$, (B) $\beta 1$ and (C) $\beta 7$ for TCR-1 clones. CL1.1 is shown in green, CL1.2 in red. (D) Relative $\alpha 4$ MFI of TCR-1 clones, normalised to CL1.1. n = 6 independent experiments. (E) Relative $\beta 1$ MFI of TCR-1 clones, normalised to CL1.1. n = 3 independent experiments. (F) Relative $\beta 7$ MFI of TCR-1 clones, normalised to CL1.1. n = 7 independent experiments. (G) Relative $\alpha 4$ MFI of TCR-2 clones, normalised to CL2.6. n = 5 independent experiments. (H) Relative $\beta 1$ MFI of TCR-2 clones, normalised to CL2.6. n = 3 independent experiments. (I) Relative $\beta 7$ MFI of TCR-2 clones, normalised to CL2.6. n = 6 independent experiments. Significance was assessed by paired T test. **, $p \leq 0.01$; *, $p \leq 0.05$. Abbreviations: MFI, mean fluorescence intensity; TCR, T cell receptor.

We also aimed to validate the decreased expression of the integrins upon stimulation that we observed in the proteomics data. We cocultured FTS-peptide loaded BCLs or control BCLs with the ORF3a₂₀₇₋₂₁₅-specific T cells for 4 hours and measured $\beta 7$ expression via flow cytometry. For all four clones we observed decreased expression of $\beta 7$ when the clones were stimulated with peptide-loaded BCLs (Fig 5.3B), compared to when they were unstimulated.

Together these data show higher expression of the integrins $\alpha 4$, $\beta 7$ and to a lesser extent $\beta 1$, on functionally superior CD8⁺ T cell clones, compared to their TCR-matched counterparts. We also observe decreased expression of these integrins upon stimulation, which is suggestive of a functional role.

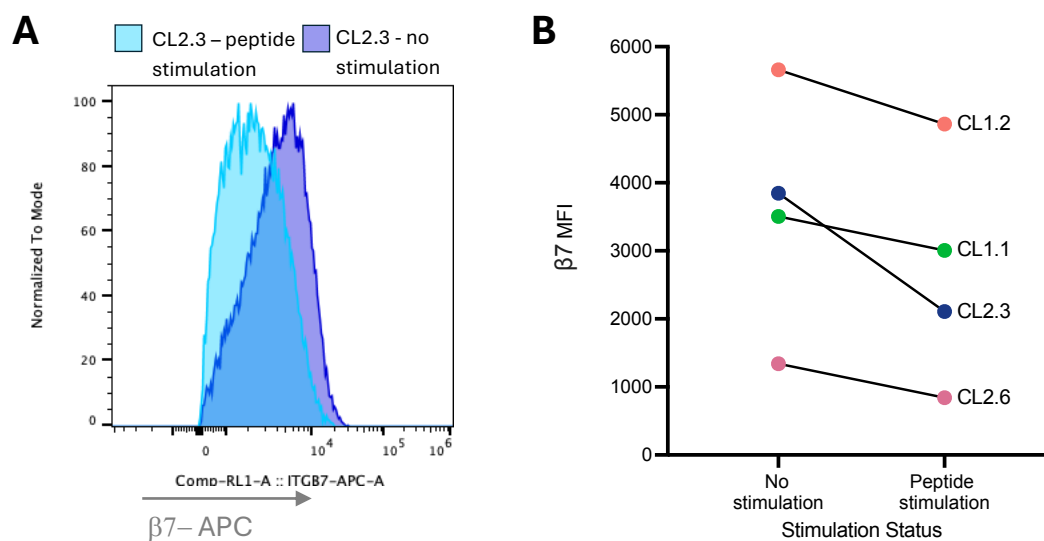


Figure 5.3: Decreased integrin $\beta 7$ expression upon T cell stimulation. T cell clones were cocultured for 4 hours with peptide loaded BCLs or negative control-BCLs. T cell clones were stained with fluorescently conjugated antibodies targeting the integrin $\beta 7$ and levels were assessed via flow cytometry. **(A)** Representative flow cytometry staining of CL2.3, with peptide-loaded BCLs and with control BCLs. **(B)** $\beta 7$ MFIs for clones with and without peptide stimulation. Abbreviations: BCL, B cell line; MFI; mean fluorescence intensity.

5.2.2 The effect of $\alpha 4\beta 7$ and $\alpha 4\beta 1$ expression on cytokine production, degranulation and killing capacity of CD8⁺ T cells

The integrin ligand VCAM-1 has previously been shown to increase IL-2 production from CD4⁺ T cells, which was abrogated by $\alpha 4\beta 1$ -blocking antibodies¹⁹⁸, suggesting that this integrin may act as a costimulatory receptor on T cells. Recently, DeBerg and colleagues observed that costimulation via MAdCAM-1 can induce transcriptomic changes that resemble that of CD28 costimulation in CD4⁺ T cells¹⁹⁹. Another recent study demonstrated that in the presence of TGF- β and retinoic acid, MAdCAM-1 can induce phenotypic changes in CD8⁺ T cells³⁵⁴.

To confirm previous findings suggesting that $\alpha 4\beta 7$ and $\alpha 4\beta 1$ can influence T cell function, and to extend these observations to other cytokines and functions, we designed a plate-bound assay to assess the costimulatory potential of VCAM-1 and MAdCAM-1. Plates were coated anti-CD3 alone, or in combination with either anti-CD28, VCAM-1 or MAdCAM-1 (Fig 5.4A). CD8⁺ T cell clones, of a range of specificities, were added to coated plates for 4 hours, before permeabilisation and staining for the effector cytokines TNF, IFN γ and the degranulation marker CD107a (Fig 5.4B). Prior to use in functional assays all CD8⁺ T cell clones were screened for expression of $\alpha 4$, $\beta 1$ and $\beta 7$ (data not shown). CD3+VCAM-1 stimulation induced a significantly higher proportion of TNF⁺IFN γ ⁺ and CD107a⁺ cells than CD3 stimulation alone (Fig 5.4C-E). The proportion of TNF⁺IFN γ ⁺ cells induced by VCAM-1 costimulation was greater than induced by anti-CD28 costimulation, suggesting that $\alpha 4$ -containing integrins could be more potent costimulators than the “classical” costimulatory molecule CD28. CD3+MAdCAM-1 also induced a significantly higher proportion of CD107a⁺ than CD3 stimulation alone (Fig 5.4C & Fig 5.4E) and a higher proportion of TNF⁺IFN γ ⁺ than CD3 stimulation alone, although significance was not reached (Fig 5.4B & Fig 5.4D). The proportion of TNF⁺IFN γ ⁺ and CD107a⁺ cells induced by these stimulation methods varied from clone to clone, likely reflecting variation in the maximal functionality between the clones and variation in their expression of $\alpha 4$ integrins. VCAM-1 or MAdCAM-1 stimulation alone, without anti-CD3, did not induce cytokine production or degranulation (data not shown).

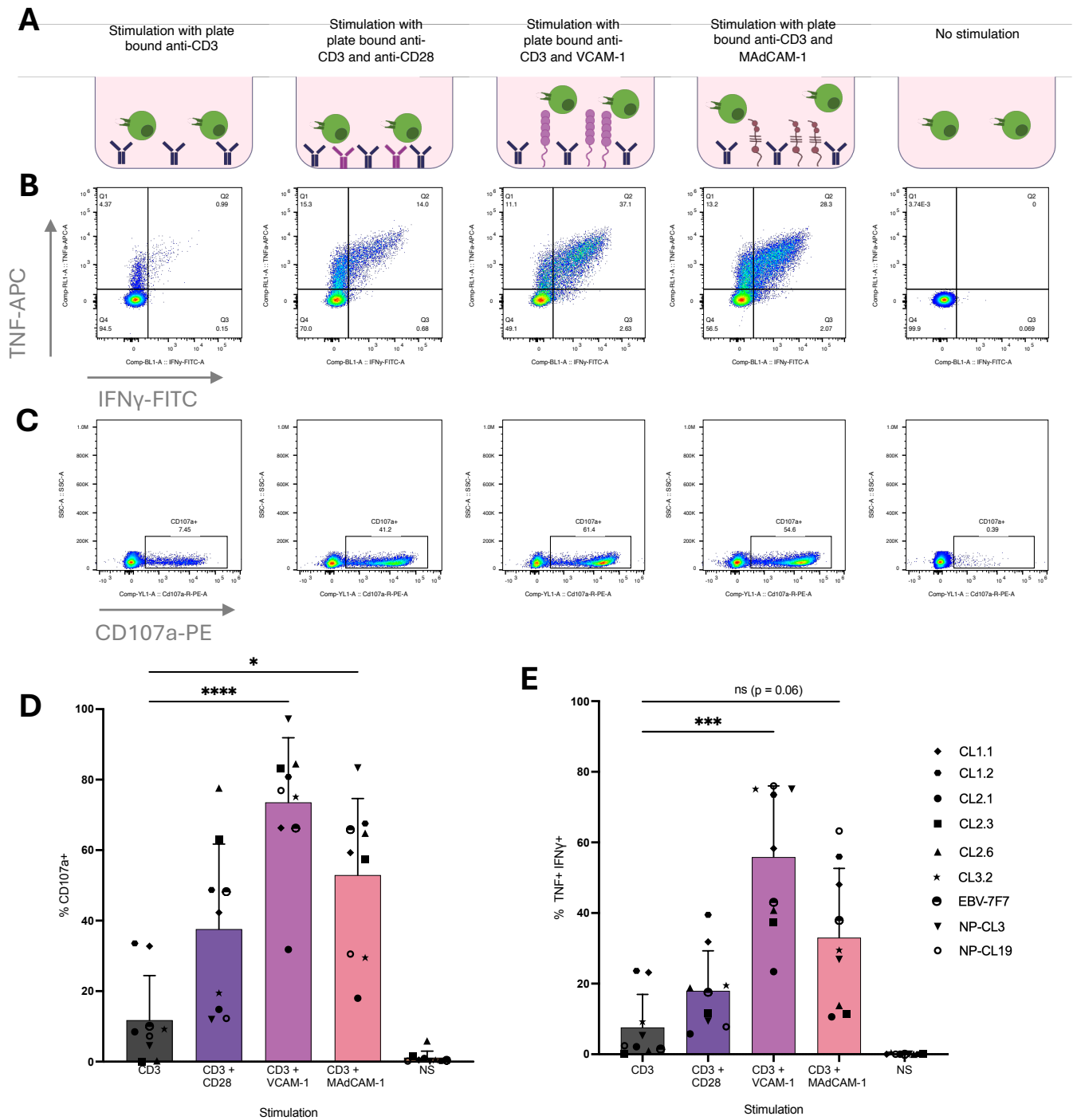


Figure 5.4: Cytokine production and degranulation of T cell clones stimulated via VCAM-1 and MAdCAM-1. (A) Diagram of experimental set up. Plates were coated with anti-CD3 and anti-CD28, VCAM-1 or MAdCAM-1. T cells were added to the wells for 4 hours then permeabilized and stained for CD107a, TNF and IFN γ . (B) Representative staining of TNF and IFN γ for a T cell clone across stimulation conditions: anti-CD3, anti-CD3+anti-CD28, anti-CD3+VCAM-1, anti-CD3+MAdCAM-1 and no stimulation. (C) Representative staining of CD107a for a T cell clone across stimulation conditions. (D) Percentage TNF+IFN γ + cells in each stimulation condition. (E) Percentage CD107a+ cells in each stimulation condition. (n=8 clones, CL1.1-CL3.2, ORF3a₂₀₇₋₂₁₅-specific; EBV-7F7, EBV BMLF₂₈₀₋₂₈₈-specific NP-CL3 & NP-CL19, SARS-CoV-2 NP₁₀₅₋₁₁₃-specific). Mean of technical replicates for each clone were plotted. Friedman test with Dunn's multiple comparisons test was used to determine significance. ****, $p < 0.0001$; ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$; ns, not significant. Abbreviations: TNF; tumour necrosis factor; IFN γ , interferon gamma; VCAM-1, vascular cell adhesion molecule 1; MAdCAM-1, mucosal addressin cell adhesion molecule 1.

To test whether the costimulatory effects of VCAM-1 and MAdCAM-1 were occurring through the $\alpha 4$ integrins we then repeated these experiments with the addition of blocking antibodies targeting the integrins. The addition of natalizumab, which blocks $\alpha 4\beta 7$ and $\alpha 4\beta 1$, abrogated the costimulatory effect of VCAM-1 (Fig 5.5A & Fig 5.5B). Both Natalizumab and Vedolizumab (which blocks $\alpha 4\beta 7$ at an epitope that prevents MAdCAM-1 binding) abrogated the costimulatory effect of MAdCAM-1, although only vedolizumab treatment reached statistical significance when compared to the no drug control (Fig 5.5A & Fig 5.5B). Corresponding isotype controls did not significantly alter the response compared to the no drug condition.

To allow us to investigate the functional effects of these integrins in a more physiological setting we aimed to identify a target cell line that expressed the integrin ligands VCAM-1 and MAdCAM-1^{355,356}. In the human body these ligands are primarily expressed by endothelial cells^{357, 358, 359} so we initially screened several primary human umbilical vein endothelial cells (HUVECs) for expression of these ligands. At baseline there was little to no expression of the ligands but upon TNF stimulation of these cells we observed strong upregulation of VCAM-1 (data not shown). To use these HUVEC cells as target cells for the T cell clones, they needed to express HLA-A*01:01 or HLA-A*02:01, corresponding to the specificities of the ORF3a₂₀₇₋₂₁₅-specific T cell clones and a EBV BMLF₂₈₀₋₂₈₈-specific clone (7F7) which was used for subsequent functional assays. However, none of the four HUVEC cell lines we screened expressed either of these HLA molecules (data not shown). Although primarily expressed by endothelial cells, VCAM-1 expression has also been identified on some immune cell populations including B cells^{360, 361}. We therefore screened several HLA-A*01:01⁺ BCLs and HLA-A*02:01⁺ BCLs for expression of VCAM-1 and MAdCAM-1. We identified two BCLs that were between 20-40% positive for VCAM-1 (Fig 5.6A-B) that could be used as target cells in our subsequent experiments. We did not identify any BCLs or HUVECs that expressed MAdCAM-1, although as we did not have a positive control, it is not possible for us to rule out issues with the antibodies.

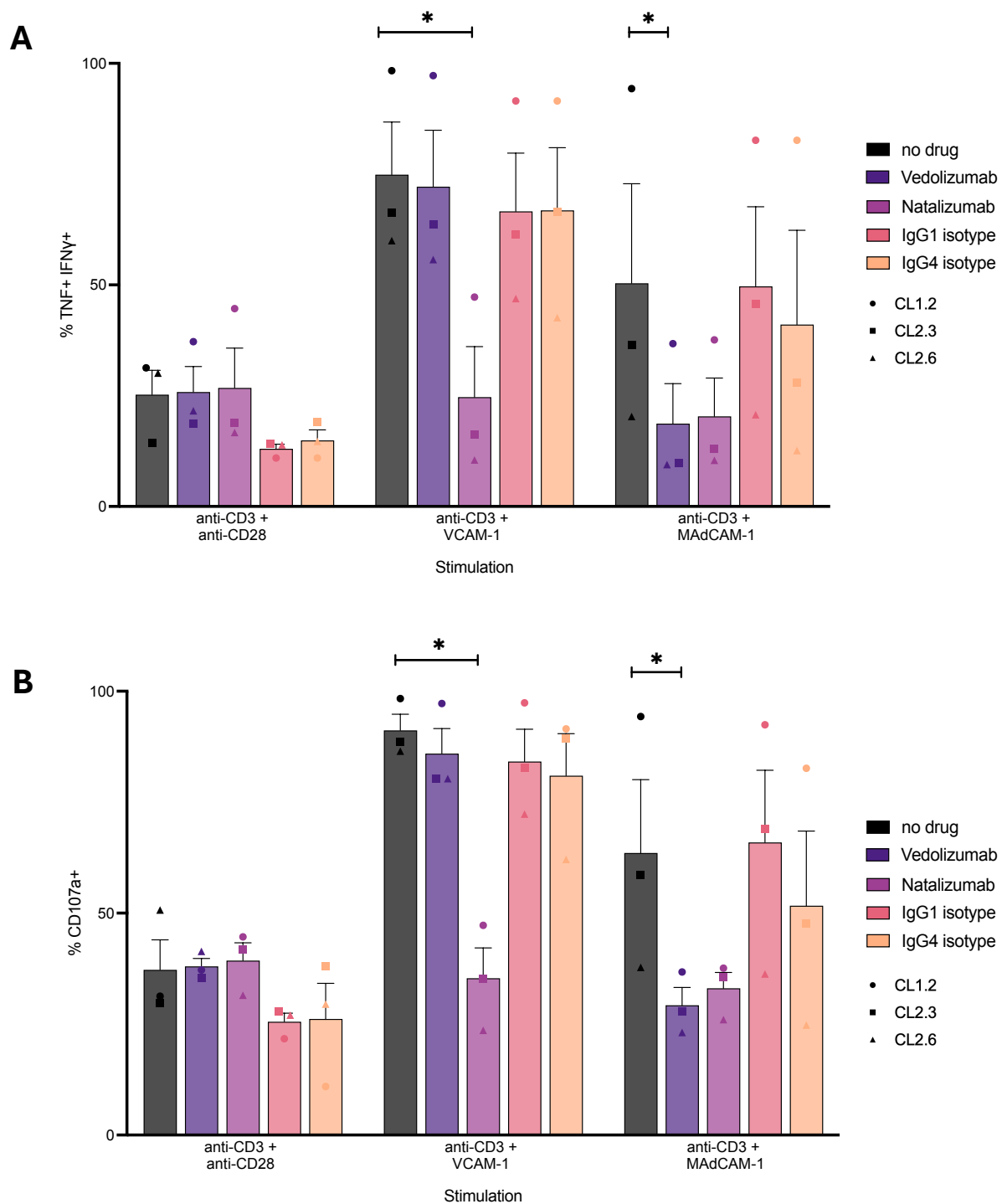


Figure 5.5: Blocking integrins decreases functionality of T cell clones stimulated via VCAM-1 and MAdCAM-1. Plates were coated with anti-CD3 and anti-CD28, VCAM-1 or MAdCAM-1. T cells pretreated with integrin blocking antibodies and isotype controls, stained for CD107a, stimulated for 4 hours and then permeabilised and stained for TNF and IFN γ . **(A)** Percentage TNF+IFN γ + cells in each stimulation condition. **(B)** Percentage CD107a+ cells in each stimulation condition. Each shape represents a different T cell clone (n=3 clones, ORF3a₂₀₇₋₂₁₅-specific). Mean of technical replicates for each clone were plotted. Friedman test with Dunn's multiple comparisons test was used to determine significance. *, $p < 0.05$; non-significant comparisons are not indicated. Abbreviations: TNF, tumour necrosis factor; IFN γ , interferon gamma; VCAM-1, vascular cell adhesion molecule 1; MAdCAM-1, mucosal addressin cell adhesion molecule 1.

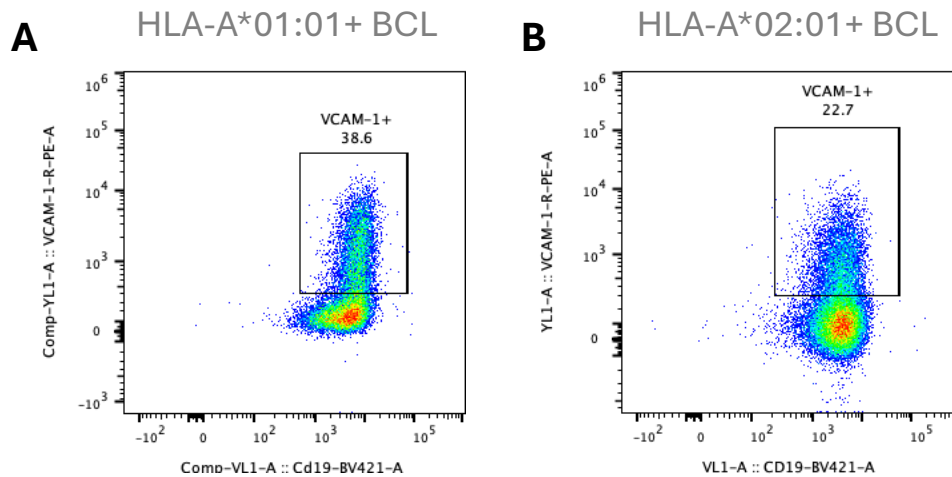


Figure 5.6: Identification of VCAM-1 expressing BCLs. BCLs were stained with fluorescently conjugated antibodies targeting CD19-BV421 and VCAM-1-PE and levels were assessed via flow cytometry. **(A)** VCAM-1 staining for HLA-A*01:01+ BCL. **(B)** VCAM-1 staining for HLA-A*02:01+ BCL. Abbreviations: BCL, B cell line; VCAM-1, vascular cell adhesion molecule 1.

We then cocultured T cell clones pretreated with the integrin-blocking antibodies with VCAM-1⁺ BCLs (Fig 5.6A-B) that had been preloaded with peptide. In addition to Natalizumab and Vedolizumab, we also used Abrilumab, a human IgG2 mAb targeting $\alpha 4\beta 7$, blocking both its interaction with VCAM-1 and MAdCAM-1, which is in clinical development for the treatment of CD and ulcerative colitis^{362, 363}. Pretreatment with these integrin-blocking antibodies decreased the proportion of TNF⁺IFN γ ⁺ and CD107a⁺ cells compared to the no-drug control, although only natalizumab treatment induced a statistically significant reduction (Fig 5.7A-B). Variation between clones likely reflects variation in the maximal functional capacity of each clone.

To assess another functional output of T cells, and to determine whether this was modulated by integrin blockade, we performed a killing assay. The T cell clone was pretreated with integrin-blocking antibodies before coculture with peptide-loaded BCLs at an effector: target ratio of 2:1 and 1:1. The proportion of live BCLs was measured and used to determine the killing capacity of the T cell clone, with and without integrin blockade. As some of the conditions were only repeated once or twice, statistical testing was not performed, however we observed a marked decrease in killing capacity when T cells were pretreated with integrin-blocking antibodies (Fig 5.8A-B). At an E:T ratio of 2:1,

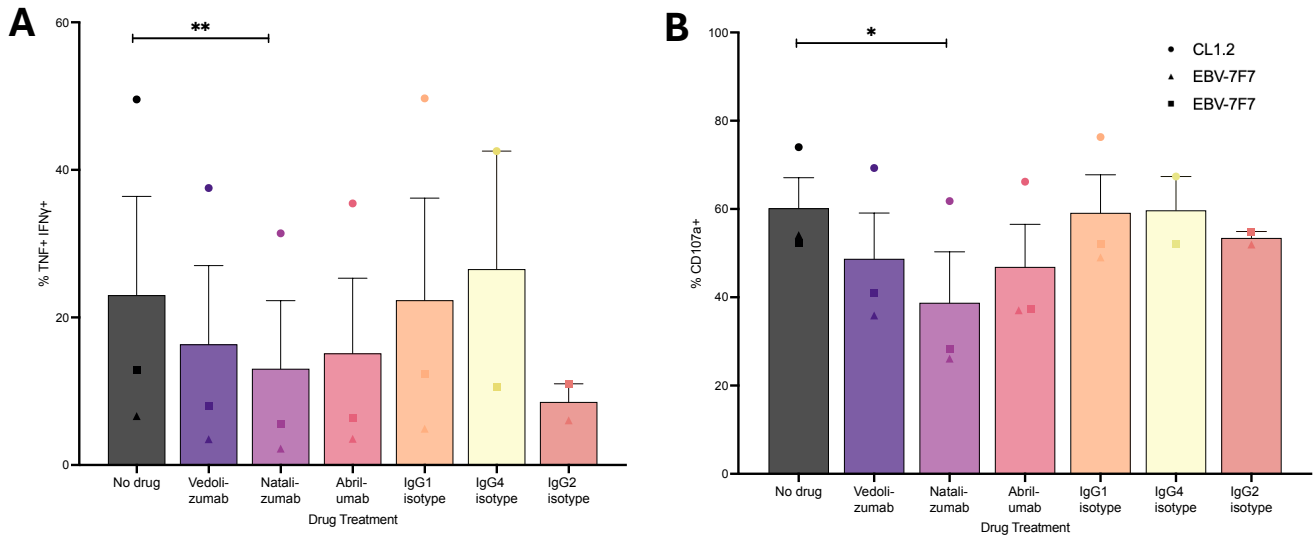


Figure 5.7: Blocking integrins decreases functionality of T cell clones co-cultured with VCAM-1⁺ BCLs. T cells pretreated with integrin blocking antibodies and isotype controls, stained for CD107a, stimulated for 4 hours and then permeabilised and stained for TNF and IFN γ . **(A)** Percentage TNF+IFN γ + cells in each of the drug treatments **(B)** Percentage CD107a+ cells in each of the drug treatments Each shape represents an independent experiment (n=3, CL1.2, ORF3a₂₀₇₋₂₁₅-specific; EBV-7F7, EBV BMLF₂₈₀₋₂₈₈-specific). Mean of technical replicates for each experiment were plotted. Friedman test with Dunn's multiple comparisons test was used to determine significance. **, $p < 0.01$; *, $p < 0.05$; non-significant comparisons are not indicated. Abbreviations: BCL, B cell line; VCAM-1, vascular cell adhesion molecule 1; TNF, tumour necrosis factor; IFN γ , interferon gamma.

integrin blocking decreased killing capacity from an average of ~62% to ~30-40% (Fig 5.8A). At an E:T ratio of 1:1 the effect was less marked, with a decrease in killing capacity from ~45% to ~30%.

Together, these data suggest that the integrins $\alpha 4\beta 7$ and $\alpha 4\beta 1$ have direct functional effects on CD8⁺ T cells, as pharmacological blockade of these integrins reduces the functionality of T cells in the presence of their ligands. Not only does inhibition of these integrins limit the cytokine production of CD8⁺ T cells but also reduces their ability to kill target cells.

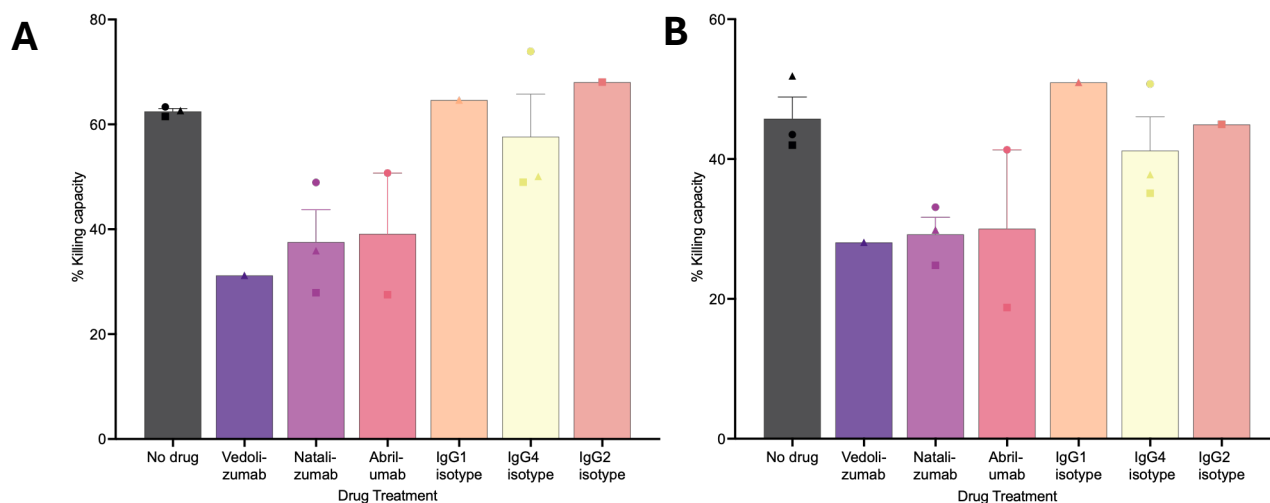


Figure 5.8: Blocking integrins decreases killing capacity of T cell clones co-cultured with VCAM-1⁺ BCLs. T cells were pretreated with integrin blocking antibodies and isotype controls. CFSE stained peptide-loaded BCLs (or control BCLs) were cocultured with T cell clones for 4 hours at the following E:T ratios: 2:1, 1:1. Live/Dead staining (7-AAD) was used to determine what proportion of live BCLs. The killing capacity was determined by normalizing the % of live peptide-loaded BCLs at each E:T ratio to the % of live control BCLs at the corresponding E:T ratio. **(A)** Killing capacity at E:T ratio 2:1. **(B)** Killing capacity at E:T ratio 1:1. Three independent experiments were performed (n=3, 7F7 clone, EBV BMLF₂₈₀₋₂₈₈-specific). Mean of technical replicates for independent experiment were plotted. Abbreviations: BCL, B cell line; VCAM-1, vascular cell adhesion molecule 1; E:T, effector: target.

5.2.3 Generation of integrin knockout T cell clones and assessment of their functionality

To allow us to investigate the functional effect of $\alpha 4$ integrins on CD8⁺ T cells we generated CD8⁺ T cell clones with the integrins $\alpha 4$, $\beta 7$ and $\beta 1$ knocked out. Due to limited cell numbers and difficulty growing the ORF3a₂₀₇₋₂₁₅-specific clones, it was not possible to generate KOs from these clones. Therefore, we screened several other virus-specific CD8⁺ T cell clones to identify a well growing clone that expressed our integrins of interest. We identified the clone EBV-7F7, an HLA-A*02:01 restricted CD8⁺ T cell clone, specific for the EBV BMLF₂₈₀₋₂₈₈ epitope (GLCTLVAML) that grew extremely well and stably expressed our integrins of interest (Fig 5.9A-C).

We selected predesigned CRISPR guides for each of the integrins (Table 2.3) which we chose based on their optimal predicted on/off-target scores and binding sites within the

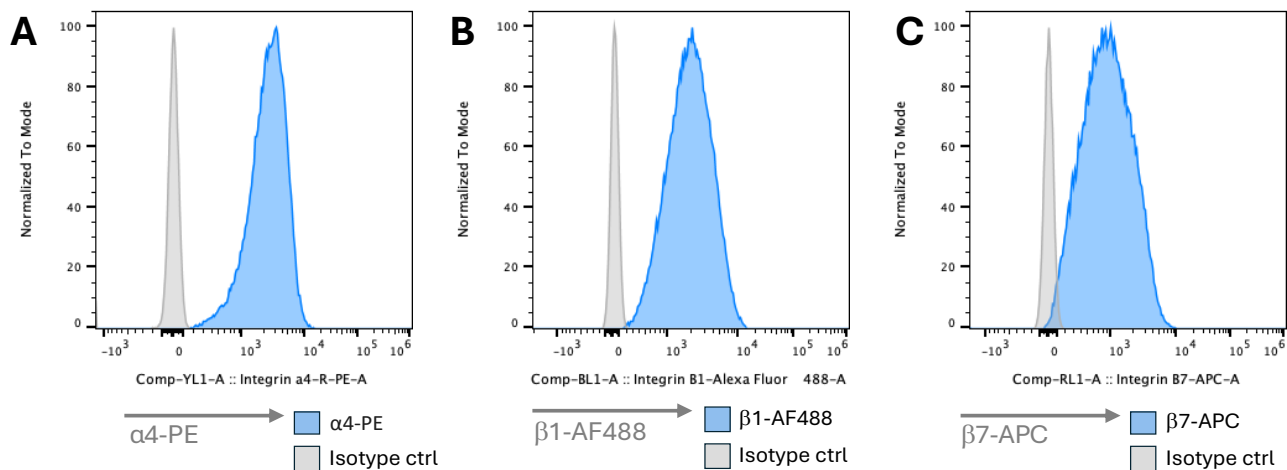


Figure 5.9: Integrin expression in EBV-7F7 T cell clone. To assess surface levels of the integrins the T cell clone was stained with $\alpha 4$ -PE, $\beta 1$ -AF488 and $\beta 7$ -APC (and isotype controls) and measured via flow cytometry. **(A)** Integrin $\alpha 4$ staining. **(B)** Integrin $\beta 1$ staining. **(C)** Integrin $\beta 7$ staining.

gene, prioritising those targeting early exons to minimize the likelihood of truncated protein expression. CRISPR ribonucleoprotein (RNP) complexes were generated for each guide and added to the activated T cell clone for electroporation. Following electroporation, the KO cells were expanded with feeder cells for 11 days before integrin expression was assessed via flow cytometry (Fig 5.10). The *ITGA4* KO was highly efficient, with 97% of cells lacking surface $\alpha 4$ expression (Fig. 5.10B). Interestingly, *ITGA4* KO also resulted in 99% of cells lacking surface $\beta 7$ expression, although these cells likely still expressed $\beta 7$ intracellularly, but it could not be trafficked to the surface without pairing with $\alpha 4$. *ITGB1* KO was less efficient but still resulted in a clear positive and negative population with 53% of the cells lacking surface $\beta 1$ expression (Fig 5.10C). *ITGB1* KO resulted in a slight increase in the percentage of cells with surface expression of $\beta 7$, from 90% in the WT clone (Fig 5.10A) to 99% in the *ITGB1* KO clone. This likely reflects increased availability of $\alpha 4$ to pair with $\beta 7$ due to decreased expression of its other partner. *ITGB7* KO also resulted in 71% of the cells lacking surface expression of $\beta 7$ but did not result in any shift in $\alpha 4$ expression as $\beta 1$ was still available for pairing (Fig 5.10D). Double KOs also efficiently resulted in reduced surface expression of the integrins of interest (Fig 5.10E-F).

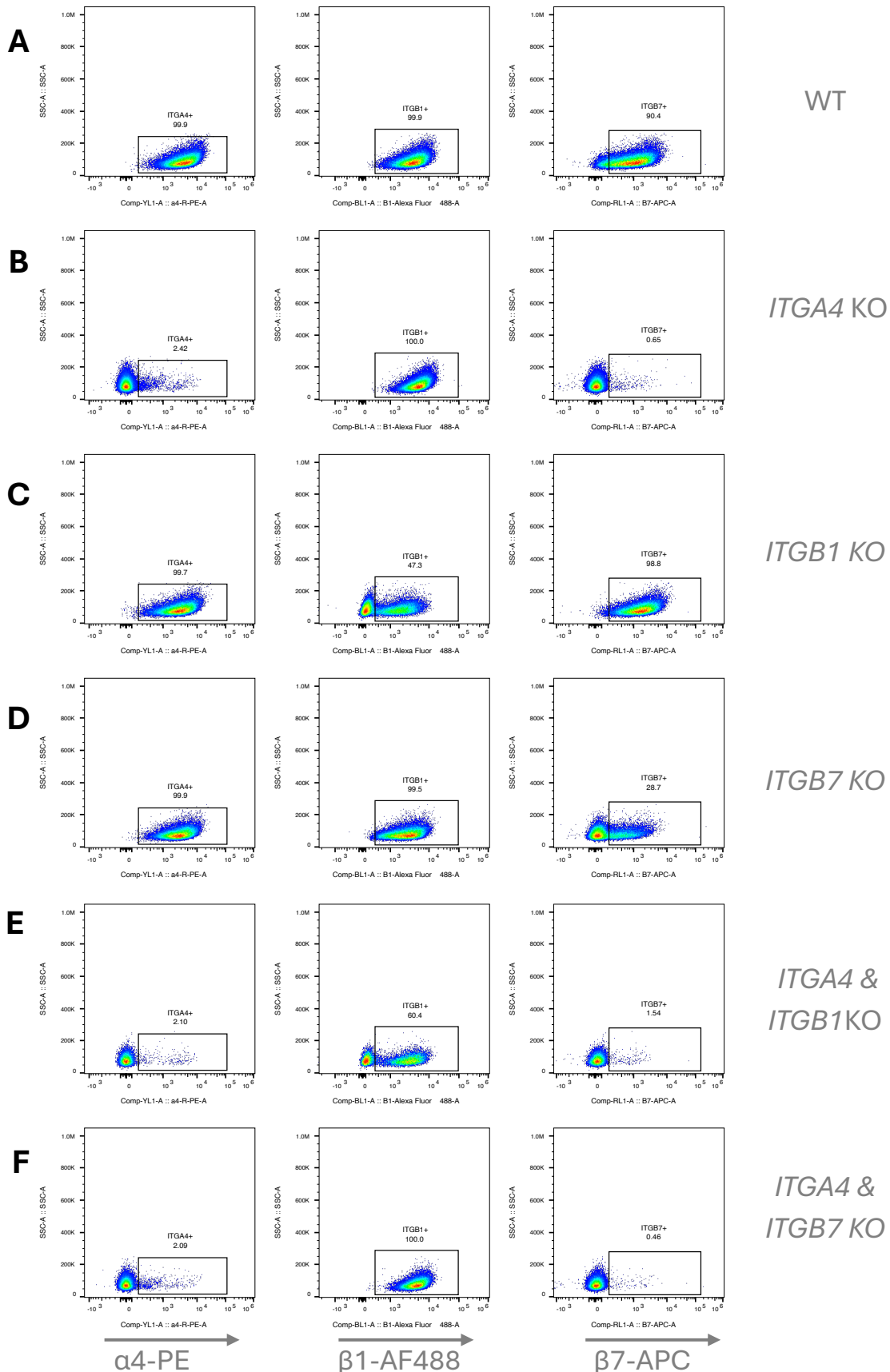


Figure 5.10: Surface integrin expression of integrin knockout T cell clones. EBV-7F7 T cell clone was subjected to CRISPR with guides targeting *ITGA4*, *ITGB1* and *ITGB7*, either alone or in combination. KO T cell clones were expanded via feeder cell stimulation. 11 days post-CRISPR integrin KO T cell clones were stained with $\alpha 4$ -PE, $\beta 1$ -AF488 and $\beta 7$ -APC and surface integrin levels were measured via flow cytometry. Cells were gated on Live⁺CD8⁺. **(A)** WT, **(B)** *ITGA4* KO, **(C)** *ITGB1* KO, **(D)** *ITGB7* KO, **(E)** *ITGA4* and *ITGB1* KO, **(F)** *ITGA4* and *ITGB7* KO. Abbreviations: KO, knockout; WT, wild type.

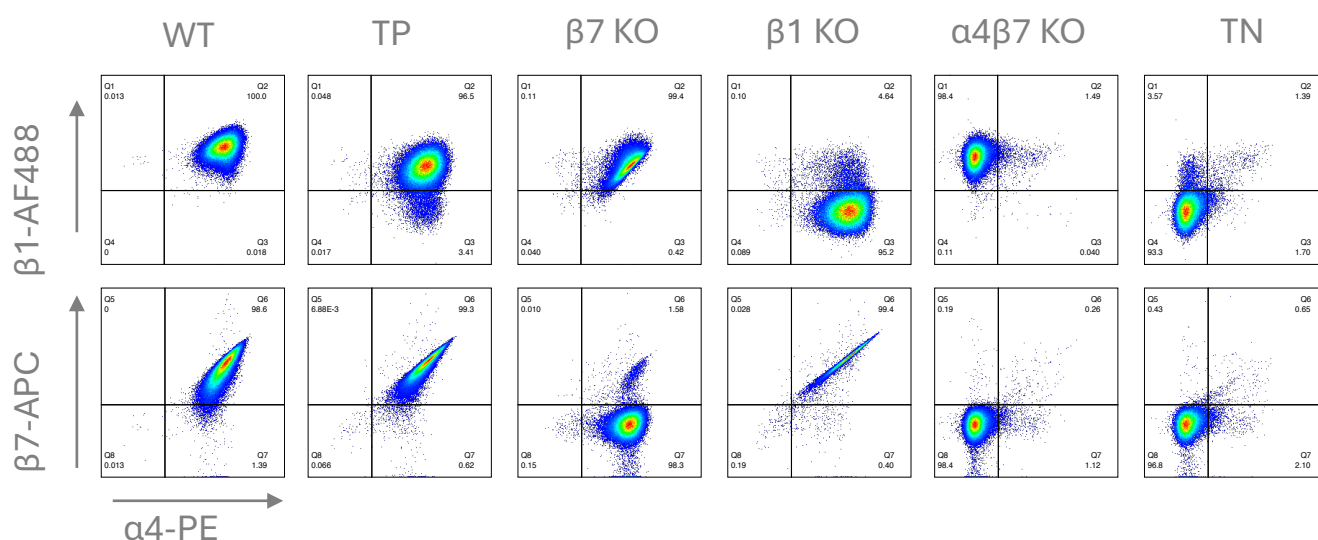


Figure 5.11: Surface integrin expression of integrin knockout T cell clones post sorting. 7F7 T cell clone was subjected to CRISPR with guides targeting the integrins $\alpha 4$, $\beta 1$ and $\beta 7$, either alone or in combination. KO T cell clones were expanded via feeder cell stimulation, FACS sorted and re-expanded via feeder cell stimulation. To assess surface levels of the integrins KO T cell clones were stained with $\alpha 4$ -PE, $\beta 1$ -AF488 and $\beta 7$ -APC and measured via flow cytometry. Abbreviations: FACS, fluorescent activated cell sorting; KO, knockout.

To generate purer KO T cell clones we then sorted these KO cells, to obtain five distinct populations: triple positive (TP: $\alpha 4 + \beta 1 + \beta 7 +$), $\beta 7$ KO ($\alpha 4 + \beta 1 + \beta 7 -$), $\beta 1$ KO ($\alpha 4 + \beta 1 - \beta 7 +$), $\alpha 4 \beta 7$ KO ($\alpha 4 - \beta 1 + \beta 7 -$), and triple negative (TN: $\alpha 4 - \beta 1 - \beta 7 -$). TP cells were sorted from CRISPR-treated T cell clones in which integrin expression was retained and used as controls in subsequent experiments, allowing the effects of integrin knockout to be assessed independently of any effects of CRISPR manipulation. It was not possible to generate an $\alpha 4$ single KO T cell clone at the protein level as the *ITGA4* KO inhibits surface expression of $\beta 7$ protein. Following sorting these KO T cell clones were again expanded with feeder cells and after 11 days we assessed their integrin expression via flow cytometry (Fig 5.11). The KO populations were between 93 and 98.5% pure (Fig 5.11) which we deemed acceptable for assessing functional effects of the integrins. Integrin expression was confirmed again after subsequent rounds of expansion and the KOs were confirmed to be stable (data not shown). It is interesting to note that when $\beta 1$ is knocked out there is a near perfect correlation between $\alpha 4$ and $\beta 7$ (Fig 5.11), as all $\alpha 4$ is paired with $\beta 7$ in the absence of $\beta 1$.

We then repeated the plate-bound costimulation assay using the integrin KO T cell clones. The TP cells exhibited an increased proportion of $\text{TNF}^+\text{IFN}\gamma^+$ and CD107a^+ cells when stimulated with VCAM-1 or MAdCAM-1 in combination with anti-CD3 (Fig 5.12A & Fig 5.12B). This effect was completely abrogated in the TN cells and $\alpha 4\beta 7$ KO cells, confirming that stimulation through $\alpha 4\beta 7$ and $\alpha 4\beta 1$ was responsible for the increased functionality of the T cells in the presence of VCAM-1 and MAdCAM-1. As expected, $\beta 7$ KO reduced the costimulatory effect of MAdCAM-1. Interestingly, $\beta 1$ and $\beta 7$ KO cells were still costimulated by VCAM-1, suggesting that in the absence of $\alpha 4\beta 7$ or $\alpha 4\beta 1$, the other integrin pair could facilitate costimulation (Fig 5.12A-B). Together, these data confirm our observation that the integrins $\alpha 4\beta 7$ and $\alpha 4\beta 1$ have direct functional effects on CD8^+ T cells, as the genetic ablation of these integrins reduces the functionality of T cells in the presence of their ligands.

5.2.4 $\alpha 4\beta 1$ and $\alpha 4\beta 7$ are located in the immune synapse

To gain further insight into how the integrins could be affecting T cell function we investigated their localisation in the immune synapse - the contact that a T cell makes with an APC or target cell that provides a structural framework for direct interactions between the cells^{50, 364}.

To study $\alpha 4$ integrins in the immune synapse we generated supported lipid bilayers (SLBs) with lipids, anti-CD3 and AF405-ICAM-1. T cells were added to the bilayers and stained with fluorescently conjugated antibodies targeting $\alpha 4$, $\beta 7$ and $\beta 1$. Synapses were then imaged using total internal reflection microscopy (TIRF) microscopy. We confirmed localisation of the integrins within the synapse (Fig. 5.13A), including $\alpha 4$ and $\beta 7$ in the central supramolecular activation complex (cSMAC), a region where integrins are not generally thought to localise³³⁵. While recombinant ICAM-1 was located primarily in the peripheral supramolecular activation complex (pSMAC) (Fig 5.13A), suggesting it is bound to LFA-1 on the T cells. These data extend our previous proteomics and flow cytometry findings by demonstrating that the differences in integrin expression between the clones are evident at the level of the immune synapse, with CL1.2 exhibiting significantly higher levels of $\alpha 4$, $\beta 1$ and $\beta 7$ than CL1.1 (Fig 5.13B-D).

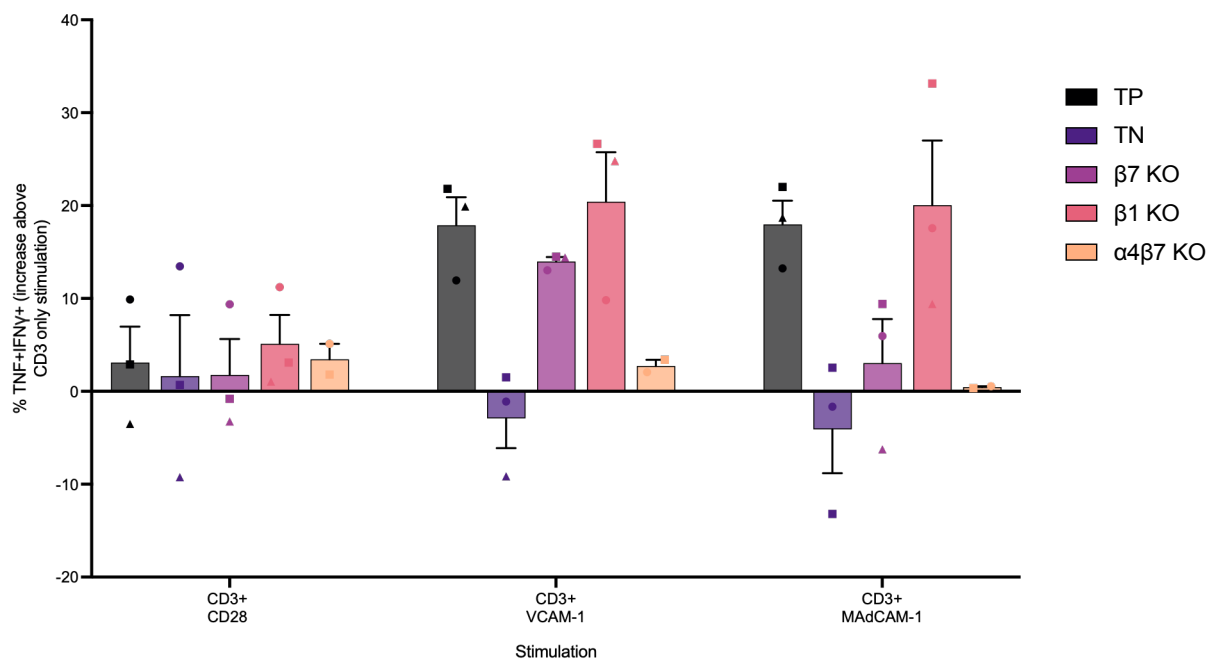
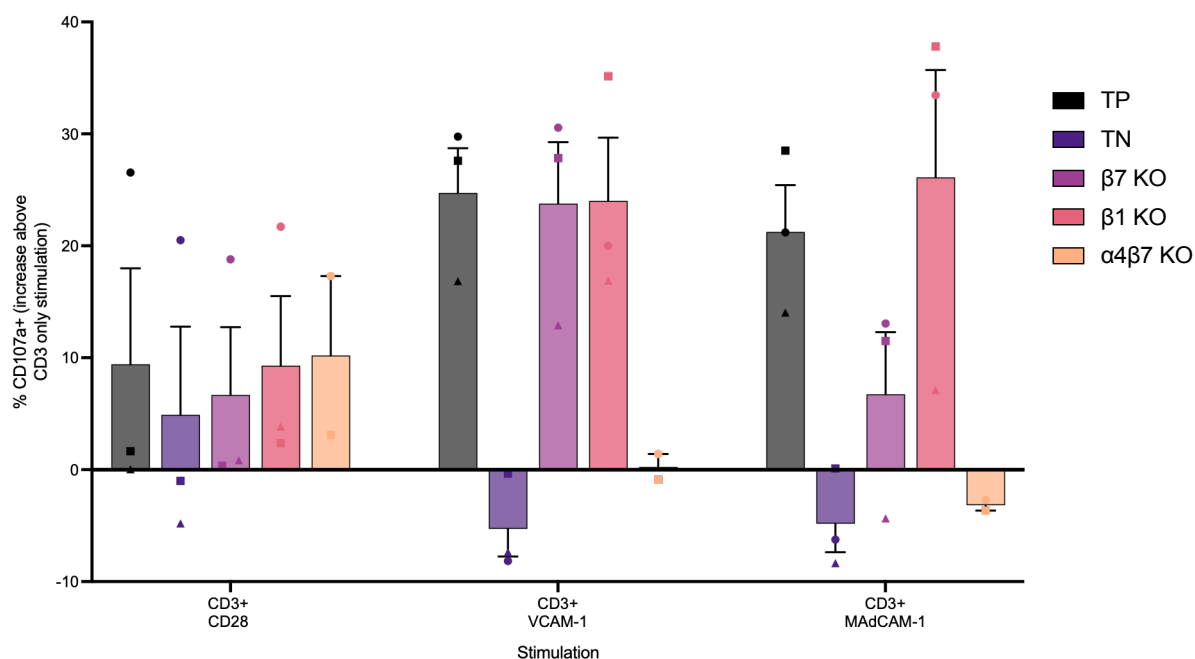
A**B**

Figure 5.12: Cytokine production and degranulation of integrin KO T cell clones stimulated via VCAM-1 and MAdCAM-1. Plates were coated with anti-CD3 and anti-CD28, VCAM-1 or MAdCAM-1. T cells were stained for CD107a, stimulated for 4 hours and then permeabilised and stained for TNF and IFNγ. **(A)** Percentage TNF+IFNγ+ cells (above that of CD3 only stimulation, CD3 only stimulation for each KO T cell clone was subtracted from CD3+ligand stimulation) in each stimulation condition. **(B)** Percentage CD107a+ cells (above that of CD3 only stimulation, CD3 only stimulation for each KO T cell clone was subtracted from CD3+ligand stimulation) in each stimulation condition. Each shape represents an independent experiment (n=3). Mean of technical replicates for each experiment were plotted. Friedman test with Dunn's multiple comparisons test was used to determine significance. *Non-significant comparisons are not indicated.* Abbreviations: TNF; tumour necrosis factor; IFNγ, interferon gamma; VCAM-1, vascular cell adhesion molecule 1; MAdCAM-1, mucosal addressin cell adhesion molecule 1.

Having confirmed the presence of the integrins in the synapse we then aimed to determine how their levels and localisation in the synapse differed in the presence of their ligands VCAM-1 and MAdCAM-1. To determine the concentration of the VCAM-1 and MAdCAM-1 proteins needed to give the desired density of 100 molecules/ μm^2 on the SLBs we first generated bead supported lipid bilayers with a range of concentrations of VCAM-1 and MAdCAM-1 (Fig 5.14A & Fig 5.14B). Regression analysis was performed to correlate the concentration of recombinant protein added to each bead population with MFI, in order to determine protein density on the beads. For VCAM-1 and MAdCAM-1 a concentration of 0.25 $\mu\text{g}/\text{ml}$ and 1.69 $\mu\text{g}/\text{ml}$, respectively, gave the desired density of 100

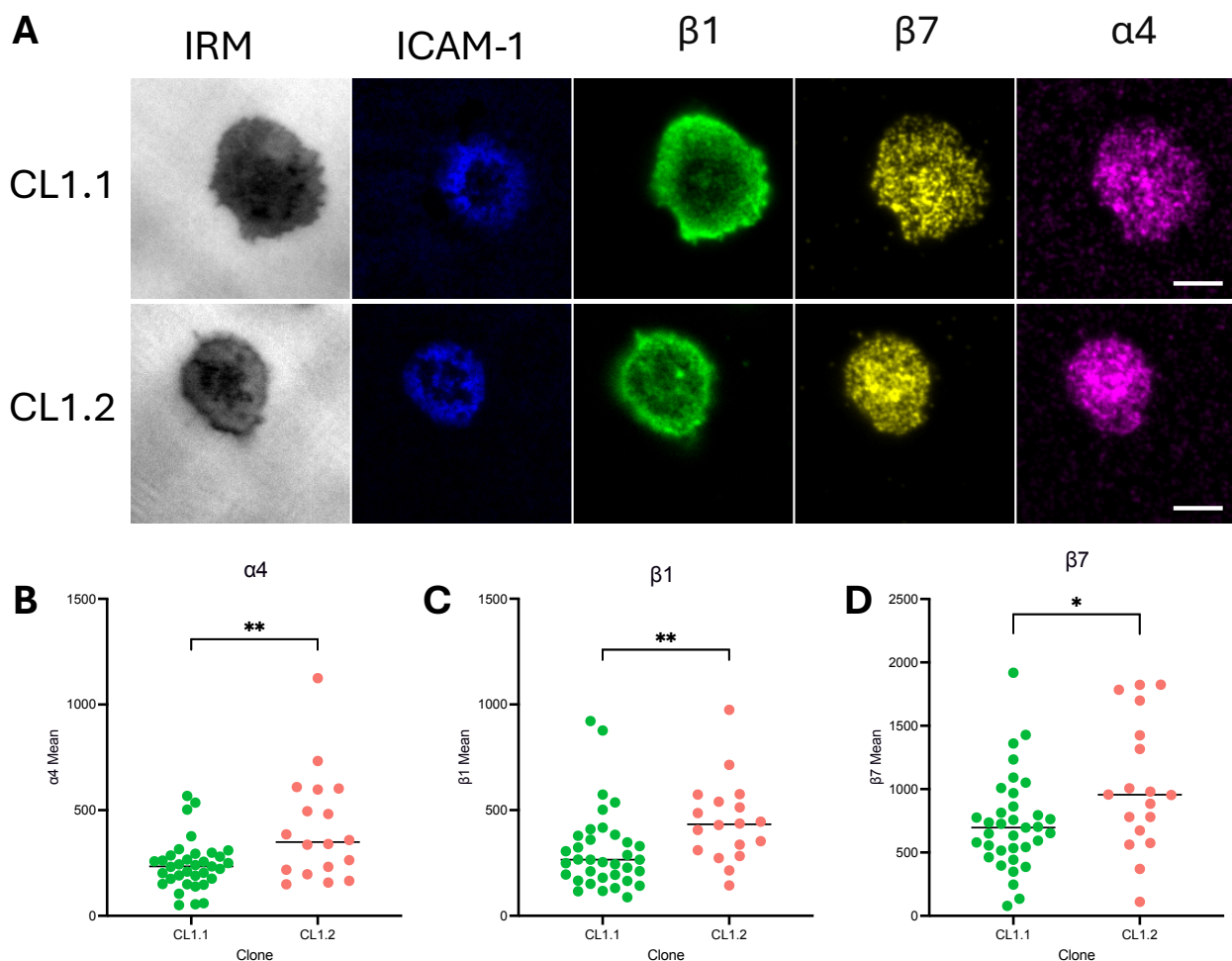


Figure 5.13: Localisation of integrins in the immune synapse. T cells clones were added to supported lipid bilayers containing lipids, anti-CD3 and ICAM-1-AF405. Cells were fixed and stained with anti- $\beta 1$ -AF488, anti- $\beta 7$ -PE and anti- $\alpha 4$ -AF647. Immune synapses were imaged using TIRFM (**A**) Representative images of synapses for CL1.1 and CL1.2. Scale bar, 5 μm . (**B**, **C**, **D**) Mean fluorescence intensity of (**B**) $\alpha 4$, (**C**) $\beta 1$ and (**D**) $\beta 7$ in each synapse. Each dot represents an individually imaged synapse. Black line indicates the median. Mann-Whitney test was used to determine significance. **, $p < 0.01$; *, $p < 0.05$. Abbreviations: IRM, interference reflection microscopy; TIRFM, total internal reflection fluorescence microscopy.

molecules/ μm^2 . In subsequent experiments, we compared synapses formed on SLBs generated under three conditions: anti-CD3 + ICAM-1 (labelled as no ligand), anti-CD3 + ICAM-1 + VCAM-1, and anti-CD3 + MAdCAM-1 (Fig. 5.14C).

When comparing the levels of the integrins in the synapses between these SLB conditions we observed significant differences. Compared to the no ligand condition, the presence of VCAM-1 in the SLB resulted in higher levels of $\alpha 4$, $\beta 7$ and $\beta 1$ in the immune synapse (Fig 5.15A-F), although the increase in $\beta 1$ only reached significance for CL1.2 (Fig 5.15E). When MAdCAM-1 was included in the synapse we observed significantly higher levels of $\alpha 4$ and $\beta 7$ in the synapse for both clones (Fig 5.15A, 5.15C-D & 5.15F). The increased localisation of these integrins in the immune synapse suggests that they

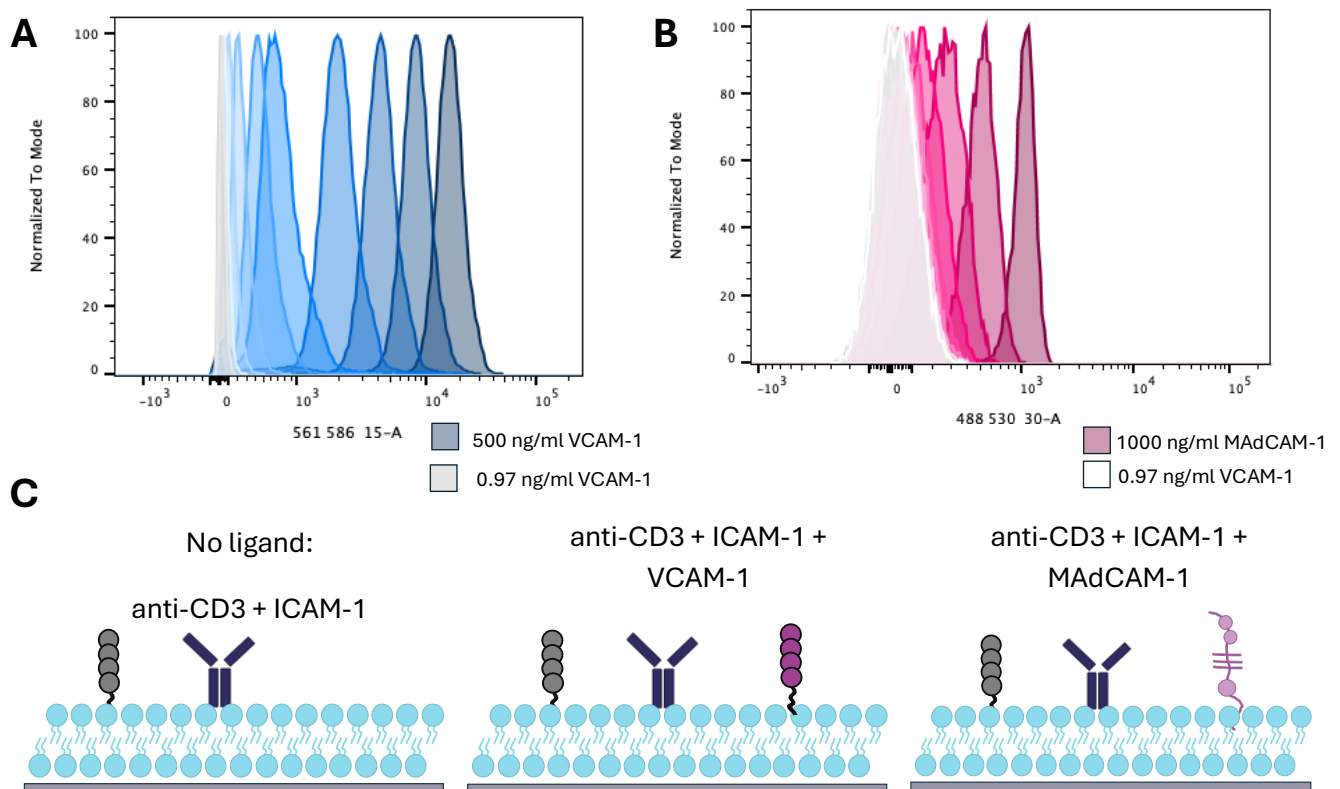


Figure 5.14: Optimisation of VCAM-1 and MAdCAM-1 concentrations for supported lipid bilayers. (A) Flow cytometry analysis of BSLBs reconstituted with a titration series of recombinant VCAM-1 (500 ng/ml top concentration with 2-fold serial dilutions to 0.97 ng/ml). BSLBs were stained with anti-VCAM-1-PE, acquired on the flow cytometer, and gated on singlets. (B) Flow cytometry analysis of BSLBs reconstituted with a titration series of recombinant MAdCAM-1-AF488 (1000 ng/ml top concentration with 2-fold serial dilutions to 0.97 ng/ml). BSLBs were acquired on the flow cytometer and gated on singlets. (C) Experimental setup of supported lipid bilayers with anti-CD3, and ICAM-1 with VCAM-1 or MAdCAM-1 added in some conditions.

could play a role in stabilising the immune synapse. Visually, we observed higher levels of the integrins in the cSMAC of the immune synapse in the presence of their ligands, suggesting that these integrins may be directly interacting with the TCR or its downstream signalling and influencing T cell functionality through this mechanism. To ensure the reproducibility of our findings this experiment was repeated several times with CL1.2 and we consistently observed higher levels of $\alpha 4$ and $\beta 7$ in the synapse in the presence of VCAM-1 and MAdCAM-1, when the average for the experiment was normalised to the no ligand condition (Fig 5.16A & Fig 5.16C). For $\beta 1$ we also observed a trend of increased localisation in the synapse in the presence of VCAM-1, although significance was not reached. This is unsurprising given that $\beta 1$ forms heterodimers with numerous α subunits and only the fraction of $\beta 1$ that is paired with $\alpha 4$ would be expected to engage VCAM-1 and therefore show altered localisation.

To further dissect whether $\alpha 4\beta 1$ and $\alpha 4\beta 7$ exhibit the same or distinct effects in the immune synapse in the presence of their ligands we repeated these SLB experiments using the integrin KO cells (Fig 5.6) and staining for $\alpha 4$. As expected, the TP cells exhibited the same trends as WT clones with increased localisation of $\alpha 4$ in the synapse when VCAM-1 and MAdCAM-1 were included in the bilayer (Fig 5.17A & Fig 5.17D). In $\beta 7$ KO cells which are positive for $\alpha 4\beta 1$, we did not see increased localisation of $\alpha 4$ in the synapse in presence of MAdCAM-1, confirming that it is entirely driven by the $\alpha 4\beta 7$ heterodimer, but we observed increased localisation of $\alpha 4$ in presence of VCAM-1, suggesting that this is at least in part mediated by $\alpha 4\beta 1$ (Fig 5.17B & Fig 5.17E). In $\beta 1$ KO cells which express $\alpha 4\beta 7$, we observed increased localisation of $\alpha 4$ in the synapse in the presence of both VCAM-1 and MAdCAM-1 (Fig 5.17C & Fig 5.17F). This confirms that $\alpha 4\beta 7$, as well as $\alpha 4\beta 1$, can drive increased localisation of $\alpha 4$ in the synapse in the presence of VCAM-1.

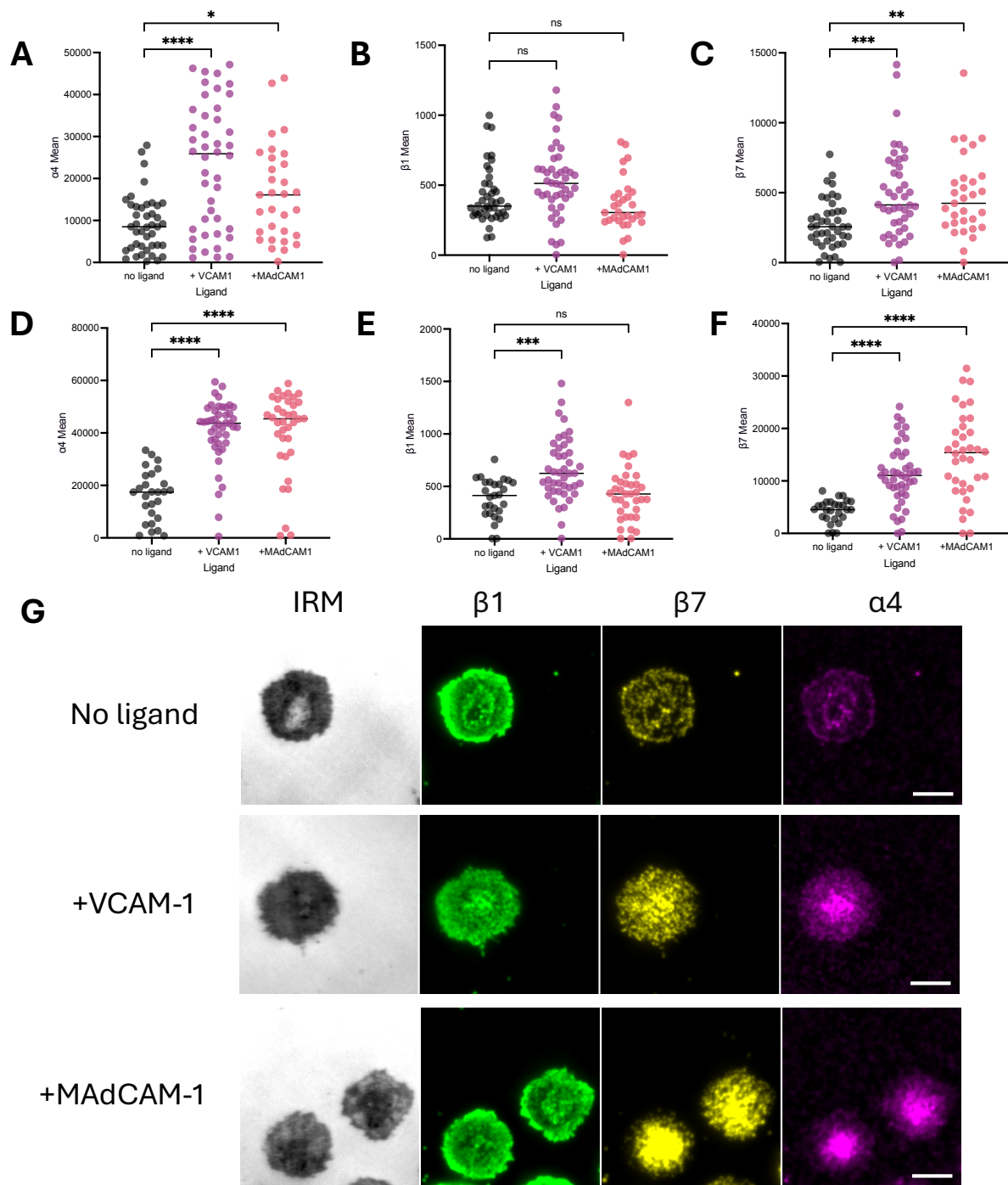


Figure 5.15: Increased localisation of integrins in the immune synapse in presence of VCAM-1 and MAdCAM-1. T cells clones were added to SLBs containing anti-CD3 and ICAM-1 with VCAM-1 or MAdCAM-1 added in some conditions. Cells were fixed and stained with anti-β1-AF488, anti-β7-PE and anti-α4-AF647. Immune synapses were imaged using TIRFM. **(A, B, C)** Mean fluorescence intensity of **(A)** α4, **(B)** β1 and **(C)** β7 in each synapse for CL1.1 (ORF3a₂₀₇₋₂₁₅-specific). **(D, E, F)** Mean fluorescence intensity of **(D)** α4, **(E)** β1 and **(F)** β7 in each synapse for CL1.2 (ORF3a₂₀₇₋₂₁₅-specific). Each dot represents an individually imaged synapse. Black line indicates the median. Kruskal-Wallis with Dunn's multiple comparisons test was used to determine significance. ****, $p < 0.0001$; ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$; ns, not significant. **(G)** Representative images of synapses for CL1.2 with anti-CD3+ICAM-1, + VCAM-1 and +MAdCAM-1 in the SLBs. Scale bar, 5 μm. Abbreviations: SLB, supported lipid bilayer; ICAM-1, intracellular cell adhesion molecule 1; vascular cell adhesion molecule 1; MAdCAM-1, mucosal addressin cell adhesion molecule 1; IRM, interference reflection microscopy; TIRFM, total internal reflection fluorescence microscopy.

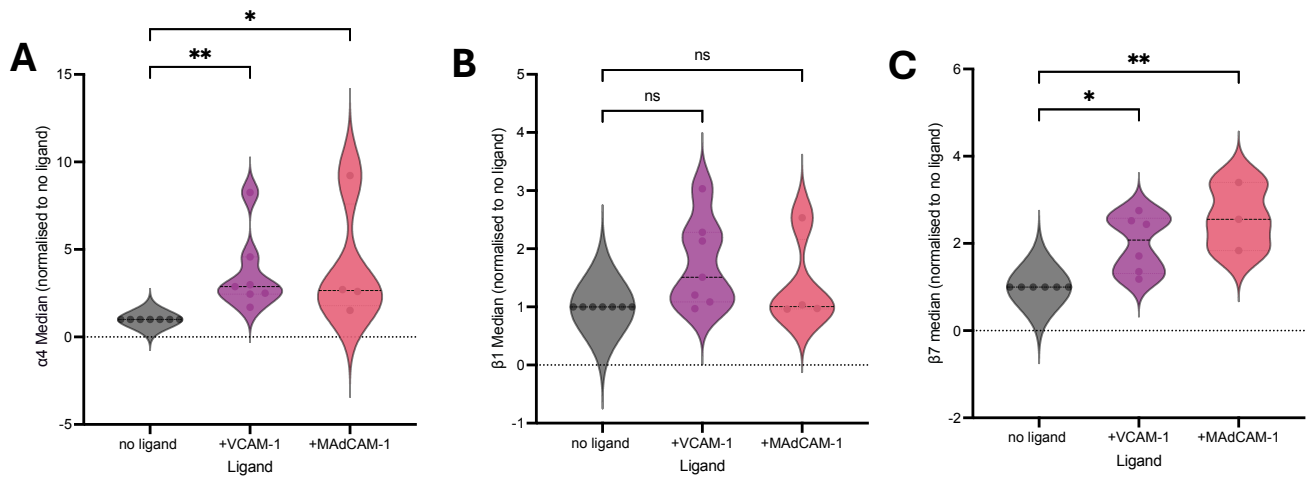


Figure 5.16: Increased localisation of integrins in synapse in presence of VCAM-1 and MAdCAM-1 across experiments. CL1.2 was added to SLBs containing anti-CD3 and ICAM-1 with VCAM-1 or MAdCAM-1 added in some conditions. Cells were fixed and stained with anti- $\beta 1$, anti- $\beta 7$ and anti- $\alpha 4$. Immune synapses were imaged using TIRFM. Median values for each experiment were calculated. Median values were normalised to the no ligand condition. Each dot represents the median value for an independent experiment. Kruskal-Wallis with Dunn's multiple comparisons test was used to determine significance. **, $p < 0.01$; *, $p < 0.05$; ns, not significant. Abbreviations: SLB, supported lipid bilayer; VCAM-1, vascular cell adhesion molecule 1; MAdCAM-1, mucosal addressin cell adhesion molecule 1; IRM, interference reflection microscopy; TIRFM, total internal reflection fluorescence microscopy.

To gain better insight into the potential mechanism through which these integrins could be enhancing T cell functionality we stained for phosphorylated tyrosine (phY) in the immune synapse. Phosphorylation of tyrosine is a key marker of intracellular signalling and thus, we investigated changes in phosphotyrosine levels in the presence versus absence of integrin ligands in the SLBs. In the TP cells we observed increased phY signalling when VCAM-1 was present in the bilayer (Fig 5.18A & Fig 5.18E). This could be suggestive of increased TCR signalling or increased signalling downstream of the integrin molecules. To determine whether this was due to $\alpha 4\beta 7$ and/or $\alpha 4\beta 1$ we also stained for phY in the integrin KO cells. As expected, the TN cells exhibited no differences in phY levels in the presence of the integrin ligands (Fig 5.18B). In $\beta 7$ KO cells we still observed increased phY levels in the presence of VCAM-1 (Fig 5.18C) but this was not the case for $\beta 1$ KO cells (Fig 5.18D). This suggests that $\alpha 4\beta 1$ but not $\alpha 4\beta 7$ is responsible for increased phY signalling in the presence of VCAM-1 in the immune synapse. These data suggest that the integrin heterodimers $\alpha 4\beta 1$ and $\alpha 4\beta 7$ may affect T cell functionality via distinct mechanisms. No increases in phY were observed for any of the T cell lines when MAdCAM-1 was present in the bilayer (Fig 5.18A-E).

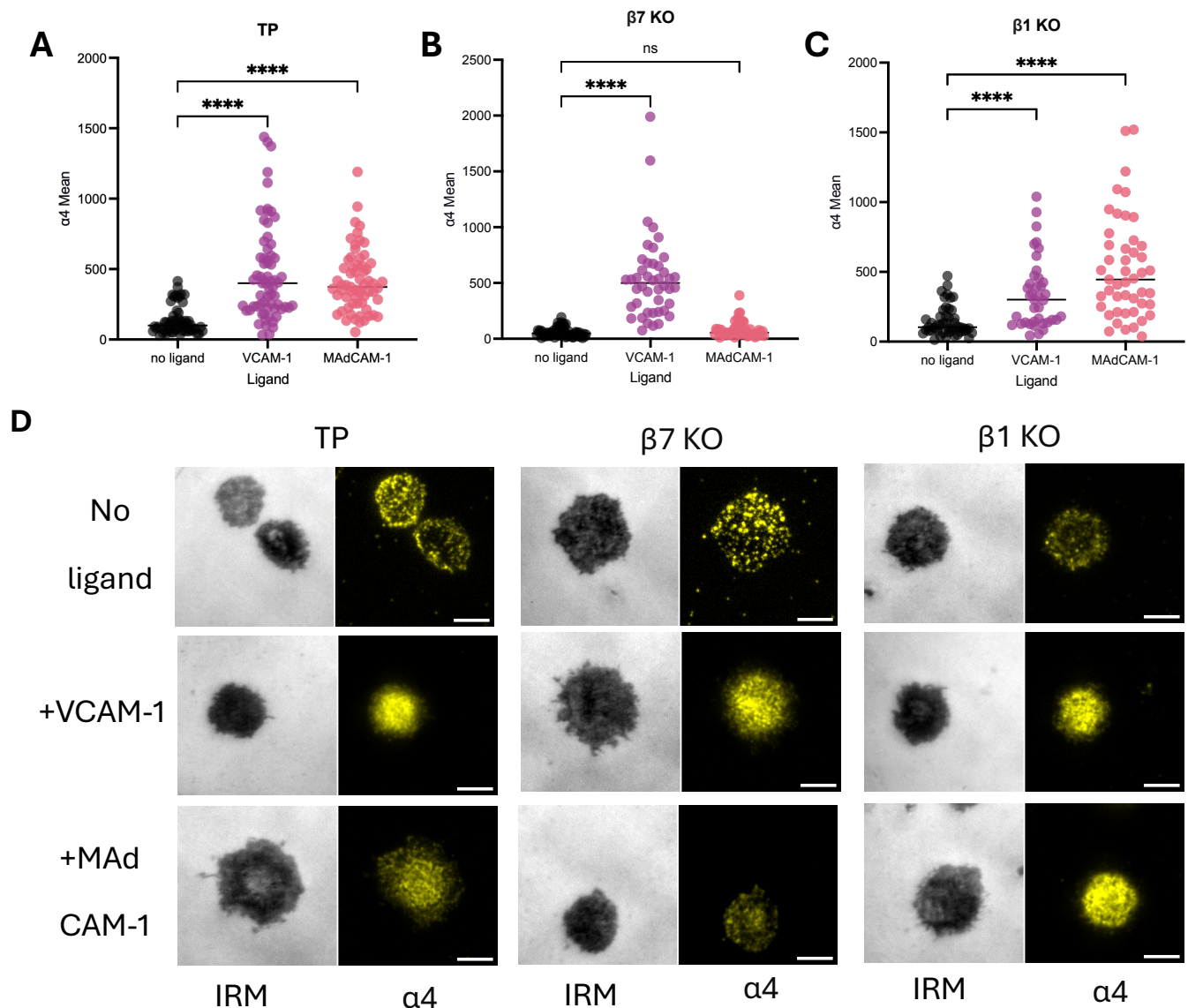


Figure 5.17: Localisation of integrin $\alpha 4$ in synapse in $\beta 1$ and $\beta 7$ knockout T cells. TP, $\beta 1$ KO and $\beta 7$ KO T cell clones were added to supported lipid bilayers containing anti-CD3 and ICAM-1 with VCAM-1 or MAdCAM-1 added in some conditions. Cells were fixed and stained with anti- $\alpha 4$ -AF647. Immune synapses were imaged using TIRFM. **(A, B, C)** Mean fluorescence intensity of $\alpha 4$ in each synapse for TP **(A)**, $\beta 7$ KO **(B)** and $\beta 1$ KO **(C)** T cell clones. Each dot represents an individually imaged synapse. Black line indicates the median. Data shown are from one representative experiment, experiment was repeated 2 times. Kruskal-Wallis with Dunn's multiple comparisons test was used to determine significance. **(D)** Representative images of synapses with no ligand, VCAM-1 and MAdCAM-1 for TP, $\beta 7$ KO, and $\beta 1$ KO T cell clones. ****, $p < 0.0001$; ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$; ns, not significant. Abbreviations: SLB, supported lipid bilayer; vascular cell adhesion molecule 1; MAdCAM-1, mucosal addressin cell adhesion molecule 1; IRM, interference reflection microscopy; TIRFM, total internal reflection fluorescence microscopy; TP, triple positive; TN, triple negative; KO, knockout.

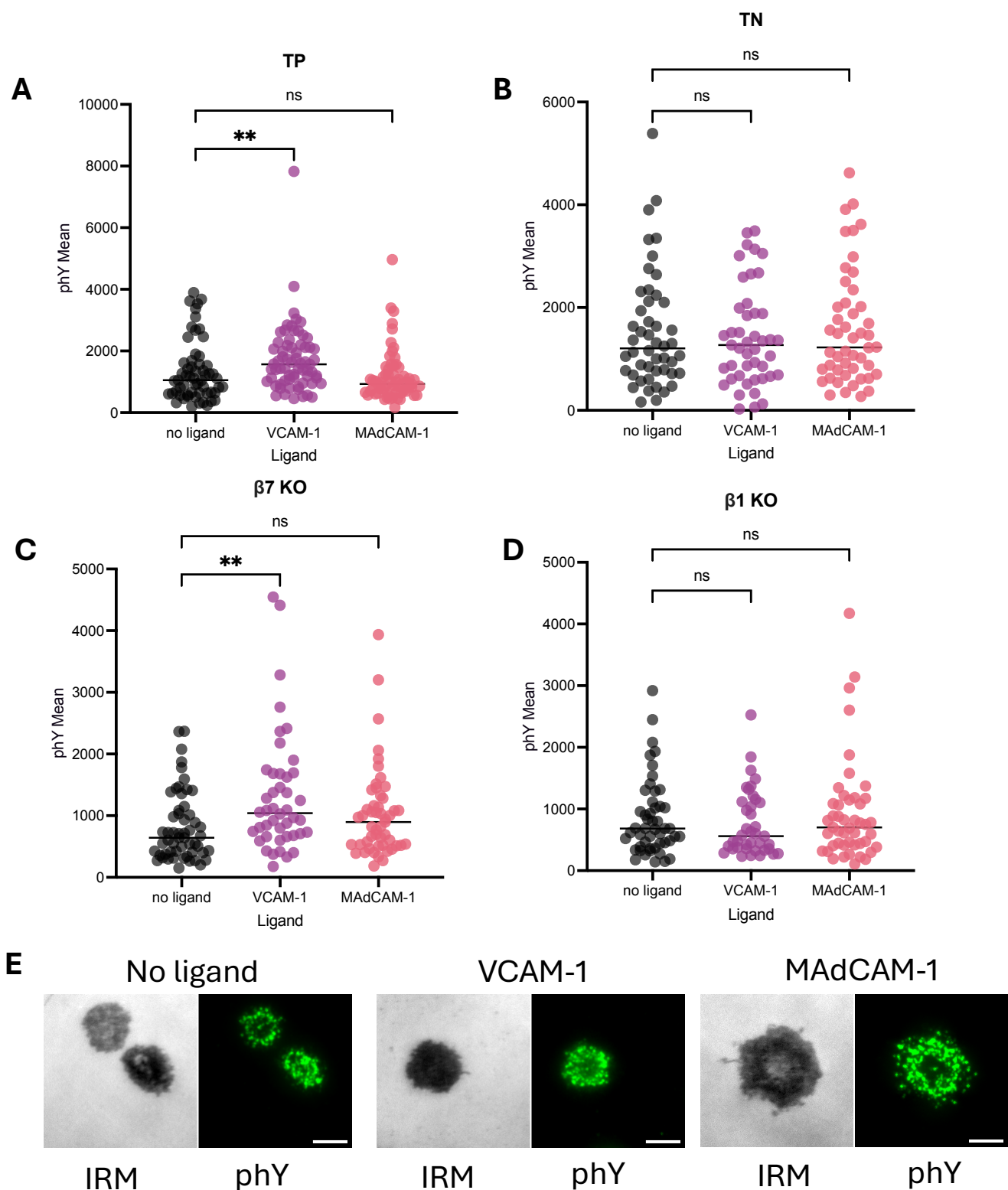


Figure 5.18: Increased phosphotyrosine in $\beta 1$ + T cells in presence of VCAM-1. TP, TN, $\beta 1$ KO and $\beta 7$ KO T cell clones were added to supported lipid bilayers containing anti-CD3 and ICAM-1 with VCAM-1 or MAdCAM-1 added in some conditions. Cells were fixed and stained with anti-phY-AF488. Immune synapses were imaged using TIRFM. **(A, B, C, D)** Mean fluorescence intensity of phY in each synapse for TP **(A)**, TN **(B)**, $\beta 7$ KO **(C)** and $\beta 1$ KO **(D)** T cell clones. Each dot represents an individually imaged synapse. Black line indicates the median. Data shown are from one representative experiment, experiment was repeated 2 times. Kruskal-Wallis with Dunn's multiple comparisons test was used to determine significance. **(E)** Representative images of synapses with no ligand, VCAM-1 and MAdCAM-1 for TP T cell clones. Scale bar, 5 μ M. ****, $p < 0.0001$; ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$; ns, not significant. Abbreviations: SLB, supported lipid bilayer; vascular cell adhesion molecule 1; MAdCAM-1, mucosal addressin cell adhesion molecule 1; IRM, interference reflection microscopy; TIRFM, total internal reflection fluorescence microscopy; TP, triple positive; TN, triple negative; KO, knockout; phY, phosphotyrosine.

Together these data shed light on integrin localisation in the immune synapse and how this could affect T cell functionality. Previously integrins were thought to be located in the pSMAC, with enrichment of LFA-1 in this region. However, the data presented here show that $\alpha 4$ integrins are distributed across the entire synapse, with enrichment in the cSMAC and pSMAC when their ligands are present in the bilayer. This suggests that in the presence of their ligands the integrins $\alpha 4\beta 7$ and $\alpha 4\beta 1$ may enhance T cell functionality by stabilising the immune synapse, thereby facilitating a longer interaction between the cells, allowing for greater effector function to be executed by the T cell. Additionally, $\alpha 4\beta 1$ appears to enhance levels of pY, suggesting that downstream signalling from this integrin heterodimer could also enhance CD8⁺ T cell function.

5.2.5 Clinical associations of $\alpha 4$ integrins

5.2.5.1 Integrin expression in SARS-CoV-2-specific T cells with clinical associations

Having established that the $\alpha 4$ integrins influence the functionality of T cells we next investigated whether this enhanced functionality is reflected in disease outcome across different clinical settings. In the context of chronic inflammatory conditions and extreme inflammation in viral infections, heightened expression of $\alpha 4$ integrins could drive T cells toward excessive effector activity, potentially contributing to tissue damage and immunopathology.

Previous data generated in our lab demonstrated that the SARS-CoV-2 NP₁₀₅₋₁₁₃-specific T cell response is associated with mild disease¹⁴² and thus these T cells may represent an appropriate or beneficial T cell response. While, as discussed in Chapter 4, the ORF3a₂₀₇₋₂₁₅-specific T cell response is associated with severe disease and thus these T cells may represent excessively activated T cells that could contribute to immunopathology. To explore whether integrin expression patterns differ between these clinically distinct T cell responses, we analysed single-cell RNA-sequencing data previously generated by other members of the Dong lab, focusing on integrin expression in T cells specific for these two epitopes. We observed significant differences in the expression of several integrin molecules between NP₁₀₅₋₁₁₃ and ORF3a₂₀₇₋₂₁₅ specific CD8⁺

T cells. Of our integrins of interest, we observed higher expression of *ITGB7* in ORF3a₂₀₇₋₂₁₅-specific compared to NP₁₀₅₋₁₁₃-specific T cells (Fig 5.19E), which supports our hypothesis that high expression of these integrins could enhance the functionality of these T cells to the point of causing pathology. However, we did not observe any significant differences in *ITGB1* or *ITGA4* between the T cells specific for these epitopes. Significant differences in other integrin subunits (*ITGAL*, *ITGA5* and *ITGB2*) were observed between the T cell populations, suggesting that other integrin molecules could also play a role in mediating T cell functionality and association with disease outcomes (Fig 5.19).

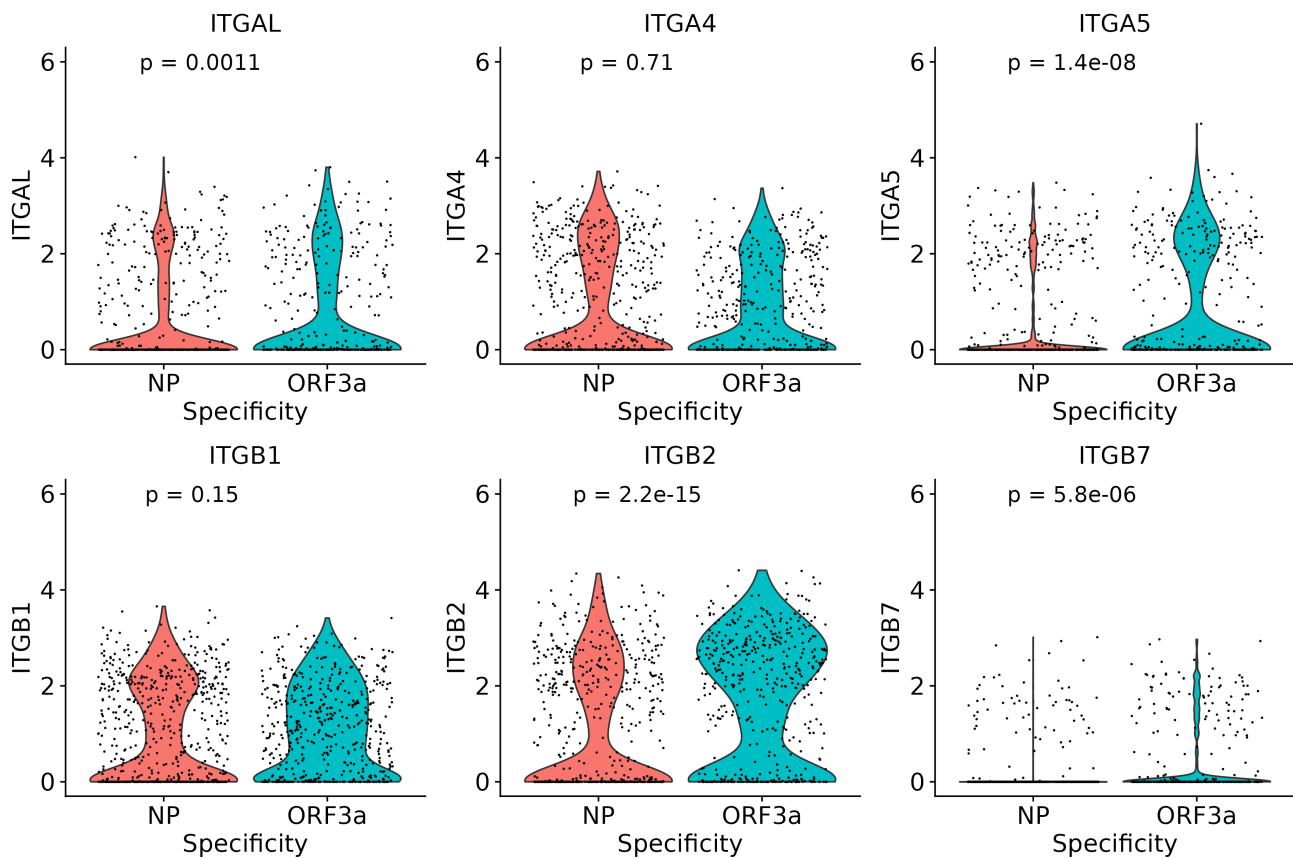


Figure 5.19: Integrin expression in SARS-CoV2-specific T cells with different specificity. SARS-CoV-2 NP₁₀₅₋₁₁₃-specific CD8⁺ T cells and ORF3a₂₀₇₋₂₁₅-specific CD8⁺ T cells were sorted *ex vivo* from PBMCs from acute and convalescent samples of COVID-19 patients and processing for single cell RNA sequencing. Expression of integrin genes in NP₁₀₅₋₁₁₃-specific CD8⁺ T cells and ORF3a₂₀₇₋₂₁₅-specific CD8⁺ T cells are shown. *P* values were calculated using Wilcoxon test.

5.2.5.2 Integrin expression correlates with survival in cancer

To further interrogate clinical associations with integrin expression we turned to the cancer genome atlas (TCGA) database as this contains large patient cohorts, survival information and bulk transcriptomic data. Previous studies have demonstrated a survival benefit of having high expression of *ITGA4* and *ITGB7* or high levels of $\alpha 4\beta 7$ expression on CD8⁺ T cells in colorectal cancer^{365, 366}. We therefore sought to examine whether this association is observed in cohorts from other cancer types. We divided lung adenocarcinoma (LUAD) patients into high and low expression of *ITGA4* and *ITGB7* and investigated whether there were survival differences between these groups. For both integrins we observed a statistically significant survival benefit for patients with high expression of *ITGA4* (Fig 5.20A) and *ITGB7* (Fig 5.20B) compared to those with low expression. This analysis could not be performed for *ITGB1* as this integrin molecule is expressed not only by immune cells, but also by many other cell types, including tumour cells. Thus, untangling its effects on T cells and how this may influence survival is not possible with TCGA bulk transcriptomic data which also contains tumour cell transcripts.

Given that the integrins $\alpha 4$ and $\beta 7$ are commonly expressed by CD8⁺ T cells and CD8⁺ T cell infiltration in tumours has been associated with positive survival outcomes in several cancers^{367, 368, 369}, we wanted to confirm that the survival benefits we observed with high integrin expression were not simply a reflection of greater CD8⁺ T cell infiltration. As the TCGA database contains bulk transcriptomic data, it is challenging to accurately determine CD8⁺ T-cell infiltration. However, using the R package *quantiseq*, which deconvolutes bulk RNA-seq profiles based on cell type specific gene-expression signatures, we were able to estimate the proportion of CD8⁺ T cells within each sample. We then performed correlation analysis to assess whether there was a correlation between integrin gene expression and CD8⁺ T cell proportion (Fig 5.20C & Fig 5.20D). We observed significant positive correlations between both *ITGA4* (Fig 5.20C) and *ITGB7* (Fig 5.20D) expression and CD8⁺ T cell proportion, that higher integrin expression could simply reflect increased CD8⁺ T-cell infiltration. Thus, to account for this we reperformed survival analysis using a Cox proportional hazards model that adjusted for CD8⁺ T cell

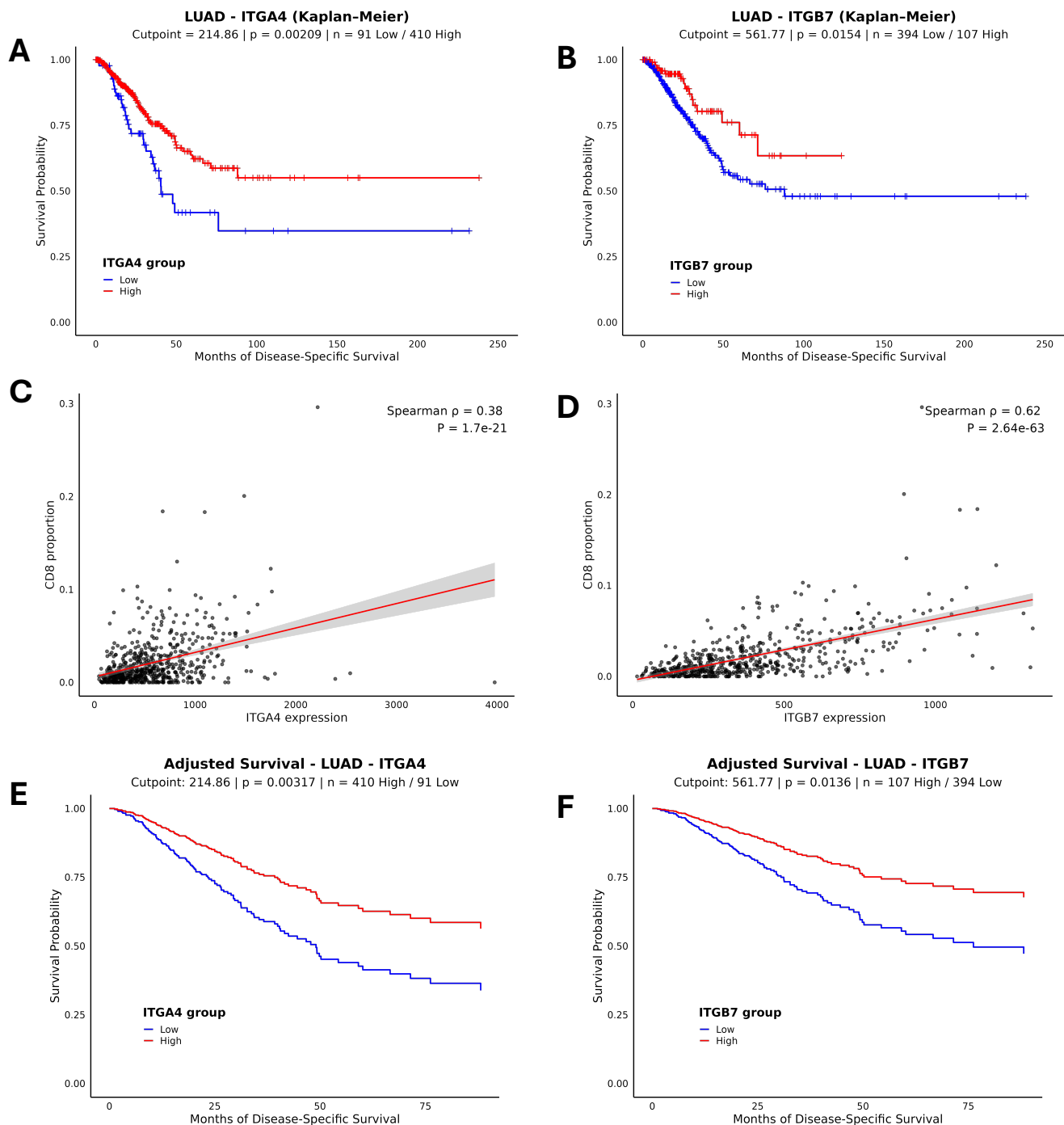


Figure 5.20: Enhanced survival in patients with higher integrin expression in lung cancer patients. Disease-specific survival data and normalised RNA-seq gene expression for LUAD were obtained from the TCGA database. For each gene (ITGA4 and ITGB7), samples were split into high- and low- expression groups based on the optimal cutpoint determined using the R package survminer. (A, B) Kaplan-Meier curves for LUAD with patients split based on (A) ITGA4 expression and (B) ITGB7 expression. Kaplan-Meier curves were fitted with the R package survival, and statistical significance was assessed using log-rank tests. (C, D) Correlation analysis between CD8+ T cell proportion and ITGA4 (C) and ITGB7 (D) expression. CD8+ T cell proportions in LUAD were estimated using the R package quantiseqr. A linear regression line with 95% confidence interval is shown. Spearman rank correlation and two-sided p-value were calculated. (E, F) Cox proportional hazards models were fitted for ITGA4 (E) and ITGB7 (F), adjusting for CD8⁺ T-cell proportion. Adjusted survival curves were plotted using the R package survival. P values correspond to the gene-group coefficient in the Cox model. Abbreviations: LUAD, lung adenocarcinoma; TCGA, the cancer genome atlas.

proportions. Even after adjustment, patients with high *ITGA4* (Fig. 5.20E) and *ITGB7* (Fig. 5.20F) expression still exhibited significant survival advantages. We repeated this analysis across additional cancer types and observed similar trends in several of them, including skin cutaneous melanoma (Fig. 5.21). In this cohort, the survival advantage associated with high *ITGB7* expression showed the same direction of effect but did not reach statistical significance (Fig. 5.21B). Together these data suggest that $\alpha 4\beta 7^+$ CD8⁺ T cells may provide a survival advantage in cancer by boosting T cell effector capacity and thereby enabling better tumour control.

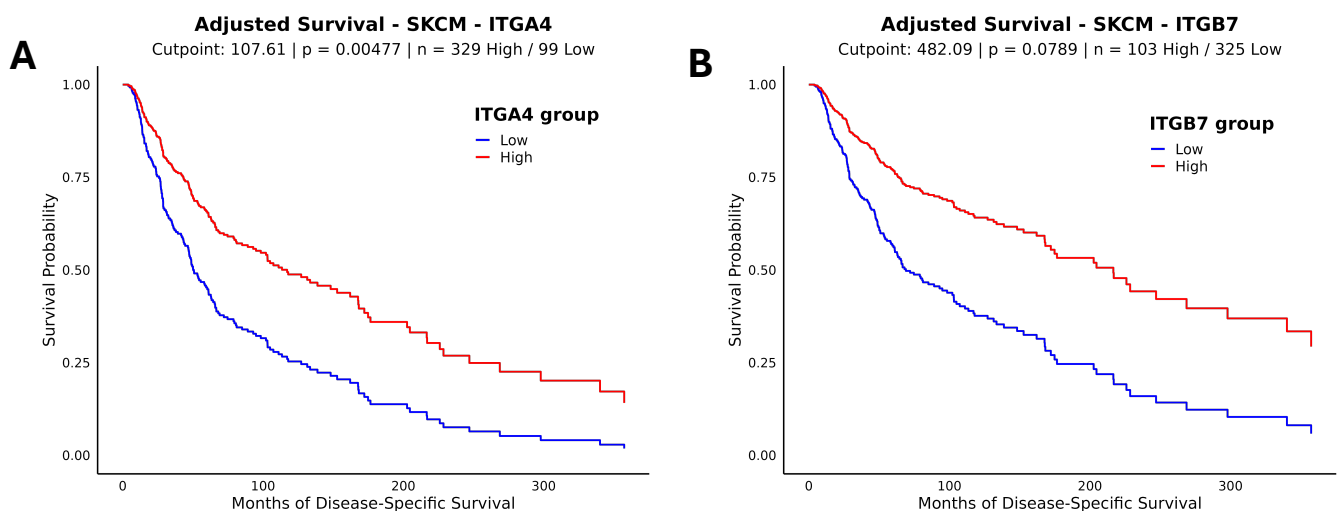


Figure 5.21: Enhanced survival in Melanoma patients with higher integrin expression. (A, B) Cox proportional hazards models were fitted for *ITGA4* (A) and *ITGB7* (B), adjusting for CD8⁺ T-cell proportion. Disease-specific survival data and normalised RNA-seq gene expression for TCGA skin SKCM were obtained from the TCGA Pan-Cancer Atlas clinical dataset. For each gene (*ITGA4* and *ITGB7*), samples were split into high- and low-expression groups based on the optimal cutpoint determined using the R package *survminer*. Adjusted survival curves were plotted using the R package *survival*. *P* values correspond to the gene-group coefficient in the Cox model. Abbreviations: SKCM, skin cutaneous melanoma; TCGA, the cancer genome atlas.

5.3 Discussion

Using functionally distinct ORF3a₂₀₇₋₂₁₅-specific CD8⁺ T cell clones we identified differences in the expression of several integrin subunits. In both TCR-matched clone pairs, the high functional avidity clone consistently expressed higher levels of the $\alpha 4$ and $\beta 7$ integrin subunits. These observations prompted us to investigate whether $\alpha 4$ -containing integrins could directly contribute to the enhanced functionality of these T cell

clones. As $\alpha 4$ can pair with either $\beta 7$ or $\beta 1$, and both $\beta 7$ and $\beta 1$ were expressed by the ORF3a₂₀₇₋₂₁₅-specific T cell clones, we explored the functional roles of both $\alpha 4\beta 7$ and $\alpha 4\beta 1$ in modulating CD8⁺ T cell responses.

Using *in vitro* functional assays, we found that through interaction with their ligands, VCAM-1 and MAdCAM-1, $\alpha 4\beta 7$ and $\alpha 4\beta 1$ could enhance the production of the effector cytokines TNF and IFN γ and increase the level of the degranulation. Preliminary data also suggested that blockade of these integrins reduces the killing capacity of CD8⁺ T cells when cocultured with a VCAM-1⁺ target cell. Immune synapse imaging revealed localisation of $\alpha 4$, $\beta 1$ and $\beta 7$ in the synapse, which was increased in the presence of their ligands, suggesting a role in immune synapse stabilisation. Localisation of the integrins in the cSMAC suggested that they may modulate TCR signalling, and we observed that increased phosphotyrosine in $\alpha 4\beta 1$ but not $\alpha 4\beta 7$ expressing cells in the presence of VCAM-1.

Previous studies have hinted at functional roles for $\alpha 4$ integrins in T cells. Mittelbrun and colleagues reported that stimulation of $\alpha 4$, in combination with anti-CD3, enhanced IFN γ production by CD4⁺ T cells and promoted a Th1 phenotype³⁷⁰, while Saukkonen and colleagues demonstrated that fibronectin costimulation, through $\alpha 4\beta 1$, increased IFN γ production in a subset of CD8⁺ T cells³⁷¹. Our findings are consistent with these previous observations and extend them by demonstrating that $\alpha 4$ integrins influence not only cytokine production but also degranulation and cytotoxic activity of CD8⁺ T cells. As integrins function as adhesion molecules there was debate over whether their enhancement of T cell function was solely due to their adhesive effects however the current consensus is that LFA-1 signalling is not only mechanosensitive but can also transduce forces and thereby activate cytoskeletal adaptors, leading to downstream signalling^{47, 53}. Therefore, we believe this is likely also the case with the integrins we have focussed on in this study.

The functional effects of $\alpha 4\beta 7$ on T cells have historically been poorly understood, but recent studies, along with our current findings, are starting to shed light on the role of this integrin heterodimer on T cell function. We demonstrated that MAdCAM-1 costimulation can increase TNF, IFN γ and CD107a expression, which was abrogated by

pharmacological blockade or genetic ablation of $\alpha 4\beta 7$. DeBerg and colleagues found that MAdCAM-1, in combination with anti-CD3, could increase the proliferative capacity of T cells *in vitro*, which was blocked by vedolizumab treatment¹⁹⁹. The authors performed RNA sequencing, following stimulation with MAdCAM-1 and anti-CD3 and observed that in CD4⁺ T cells gene expression changes resembled those of T cells stimulated with anti-CD3 and CD28, suggesting that upon MAdCAM-1 recognition $\alpha 4\beta 7$ can act as a costimulatory molecule. MAdCAM-1 stimulation of CD8⁺ T cells has also been shown to increase expression of granzyme B and several activation markers³⁵⁴. Together, these findings show that the interaction between MAdCAM-1 and $\alpha 4\beta 7$ extends beyond its well-established role in migration and adhesion and can exert substantial functional effects on $\alpha 4\beta 7$ -expressing T cells. Previous studies have suggested that although $\alpha 4\beta 7$ can bind to VCAM-1, any costimulatory effect of VCAM-1 is mediated through $\alpha 4\beta 1$, not $\alpha 4\beta 7$ ^{199, 372}. However, our current findings disagree with this as we observed increased production of TNF, IFN γ and CD107a in the presence of VCAM-1 in $\beta 1$ KO cells, suggesting that this increase is due to stimulation via $\alpha 4\beta 7$. Further experimentation is needed to reconcile the differences between these studies.

Our immune synapse analysis revealed localisation of $\alpha 4$, $\beta 7$ and $\beta 1$ in the immune synapse and that their levels were increased in the presence of their ligands VCAM-1 and MAdCAM-1. Of particular interest we observed increased localisation of these integrins in the cSMAC in the presence of VCAM-1 and MAdCAM-1, while integrins, especially LFA-1, were previously thought to be enriched in the pSMAC⁴⁹. In a 2004 study, Mittelbrun and colleagues studied $\alpha 4$ localisation in the synapse and found it to be colocalised with LFA-1 in the pSMAC, raising an apparent discrepancy with our findings. Several methodological differences in experimental set up may account for these differences. We performed immune synapse imaging using primary human CD8⁺ T cell clones and supported lipid bilayers visualised by TIRF microscopy, whereas Mittelbrun and colleagues used a CD4⁺ Jurkat cell line interacting with Raji B cells and imaged synapses using confocal microscopy. Differences in cell type, activation state and imaging could therefore contribute to the differences in our findings. Nonetheless, while TIRF microscopy provides higher-resolution imaging of the immune synapse, we should validate our observations using more physiologically relevant target cells. Our data

suggest that while interacting with their ligands, $\alpha 4\beta 7$ and $\alpha 4\beta 1$ may stabilise the immune synapse, thereby increasing the contact between T cells and their target cells, allowing for increased T cell effector function.

In $\alpha 4\beta 1^+$ CD8⁺ T cells, we also found that the presence of VCAM-1 in the bilayer increased phosphotyrosine signalling at the immune synapse, suggesting that $\alpha 4\beta 1$ -mediated enhancement of T cell functionality may go beyond immune synapse stabilisation. However, this observation disagrees with a 2018 study that showed that VCAM-1 stimulation in combination with anti-CD3, decreased phosphotyrosine levels³⁷³. The differences in length of stimulation may account for these differences; in the previous study the T cells were fixed after 5 minutes of stimulation, while we fixed T cells after 15 minutes of stimulation. Additionally, these differences could reflect differences between CD4⁺ and CD8⁺ T cells as the previous study used Jurkat cells and primary human CD4⁺ T cells. In corroboration of our findings, Nguyen and colleagues observed slowing of actin flow when T cells were stimulated with anti-CD3 and VCAM-1, together with increased phosphotyrosine, ZAP70 and NFAT activity³⁷⁴. Together, these findings suggest that $\alpha 4\beta 1$ may act as a costimulatory molecule and enhance TCR signalling. The absence of increased phosphotyrosine signalling in $\alpha 4\beta 7^+$ cells stimulated with VCAM-1 and MAdCAM-1 suggests that this integrin heterodimer may have an alternative mechanism of enhancing T cell functionality. However, it is possible that $\alpha 4\beta 7$ signalling operates with different temporal dynamics. Thus, it would be interesting to repeat these experiments with phosphotyrosine staining at multiple early timepoints, between 2 and 15 minutes after stimulation.

Our findings, together with those of DeBerg *et al*, offer a potential alternative mechanism of vedolizumab efficacy in CD patients who respond to the drug despite showing no depletion of $\alpha 4\beta 7^+$ cells in the intestinal mucosa^{199, 353}. We propose rather than inhibiting the trafficking of these cells to the intestine, vedolizumab instead is blocking the interaction of $\alpha 4\beta 7$ with VCAM-1 and MAdCAM-1, which could decrease the stability of the immune synapse and thus reduce the effector function of these proinflammatory T cells, reducing their ability to cause tissue damage. $\alpha 4\beta 7^+$ T cells have also been implicated in other inflammatory conditions; recently $\alpha 4\beta 7^+$ T cells were implicated in

the pathogenesis of liver fibrosis in mouse model of disease. The authors noted that compared to $\alpha 4\beta 7^-$ T cells, $\alpha 4\beta 7^+$ T cells from the fibrotic liver had higher expression of activation markers, the transcription factor Tbet and the proliferation marker Ki67³⁷⁵, suggesting that interaction with VCAM-1 and MAdCAM-1 had enhanced the effector function of these T cells. Additionally, upregulation of $\alpha 4\beta 7$ on the surface of activated T cells has been correlated with development of intestinal Graft versus host disease in patients after stem cell transplantation³⁷⁶.

In the context of SARS-CoV-2 infection, we hypothesise that high expression of $\alpha 4\beta 7$ and/or $\alpha 4\beta 1$ on T cells, could, in situations of excessive inflammation, push the T cells into being overly activated and contributing to tissue damage. This idea is supported by findings that levels of integrin ligands are increased in severe COVID-19 patients. When compared to healthy controls or patients with mild COVID-19, patients with severe disease were found to have significantly higher levels of VCAM-1 in the serum^{377, 378}. Additionally, VCAM-1 was upregulated at the transcriptomic level in the lungs of patients with COVID-19³⁷⁹. Although less well studied, MAdCAM-1 was also shown to be upregulated in extracellular vesicles in the sputum of COVID-19 patients³⁸⁰. Thus, it is feasible that ORF3a₂₀₇₋₂₁₅-specific CD8⁺ T cells with high expression of $\alpha 4\beta 7$ and/or $\alpha 4\beta 1$ could be overactivated through interactions with VCAM-1 and MAdCAM-1, causing them to overexert effector function and contribute to tissue damage, contributing to their association with disease severity. To investigate this possibility, it would be interesting to perform immunofluorescence staining or spatial transcriptomics of lung tissue from severe COVID-19 patients to see whether we observe high levels of T cells expressing $\alpha 4\beta 7$ and $\alpha 4\beta 1$ in areas of immunopathology.

In the context of cancer, our analysis of the TCGA database suggests that high expression of $\alpha 4\beta 7$ on immune cell populations in the tumour could improve survival through enhanced T cell activation and tumour cell killing. However, due to the bulk nature of TCGA data, dissecting the effects of these integrins directly on T cells in the tumour is challenging. While exploring response to immune checkpoint blockade (ICB) in patients with metastatic colorectal cancer, Feliu and colleagues observed that responders to ICB had higher percentage of $\alpha 4\beta 7^+$ CD8⁺ T cells than non-responders. Additionally,

patients with a high proportion of $\alpha 4\beta 7^+$ CD8⁺ T cells had increased overall survival compared to patients with a low proportion of $\alpha 4\beta 7^+$ CD8⁺ T cells³⁶⁵. Together, these data suggest that integrin expression on tumour infiltrating T cells could enhance T cell activation and killing, although this is likely only relevant in tumours that express the integrin ligands. To confirm this, the expression of $\alpha 4\beta 7$ at the protein level on T cell populations from other cancers should be studied in association with disease outcome.

Our study into the effects of $\alpha 4$ integrins on CD8⁺ T cell functionality is limited in several ways. To investigate the mechanism of action of these integrins we utilised several reductionist approaches, including plate bound assays and generation of supported lipid bilayers which do not fully recapitulate target cell membranes. To mitigate this, we also assessed the functional consequences of integrin blocking on T cells cocultured with VCAM-1⁺ target cells. However, this simple *in vitro* system still does not fully reflect the complex *in vivo* environment in tissues. Additionally, we were unable to confirm the presence or absence of MAdCAM-1 in our coculture system due to unreliable flow cytometry antibodies and thus further experiments should include using alternative techniques such as western blot or quantitative RT-PCR, to determine whether the BCLs express MAdCAM-1. Due to technical constraints on the number of immune synapses we could practically image, we conducted all SLB experiments at a 15-minute stimulation timepoint. Given that immune synapse formation and T-cell activation occur rapidly and are very dynamic processes^{51, 364}, examining only this later timepoint may have overlooked early changes in integrin localisation or phosphotyrosine levels. Therefore, in future experiments we plan to include early stimulation timepoints, between 2 and 15 minutes, for immune synapse imaging. Additionally, as the SLB experiments used anti-CD3 for stimulation instead of pMHC, repeating them with cognate pMHC would provide a more physiologically relevant method of T cell stimulation. Our study did not touch on the generation of catch bonds between integrins and their ligands and how the centripetal actin flow generated by TCR signalling influences integrin activity but investigation of this may improve understanding of how these integrins influence T cell function.

In conclusion, our current study highlights the fact that $\alpha 4$ integrins, have underappreciated and understudied roles in CD8⁺ T cell function. Our data suggest that

these integrins work as fine tuners of T cell activity. In some situations, such as cancer and acute well-controlled viral infection, high expression of the integrins enhances T cell activity and has a beneficial effect for the host. However, in chronic inflammation, autoimmunity and persistent viral infection, high expression of $\alpha 4$ integrins could cause excessive activation of CD8⁺ T cells, allowing them to contribute to immunopathology and tissue damage.

6. Summary and concluding remarks

CD8⁺ T cells are fundamental players in the anti-viral immune response, yet many questions around factors that influence their functionality remain unanswered. Focussing on the T cell response against influenza and SARS-CoV-2, we address two key research questions in this thesis: how does antigen-specificity shape the ageing trajectory of CD8⁺ T cells and which features of CD8⁺ T cells contribute to their association with severe disease.

In Chapter 3, we use scRNAseq of samples from 3 individuals over a 19-year period to investigate age-associated changes in transcriptomic profiles and TCR gene usage in influenza M1₅₈₋₆₆ specific T cells. To determine how antigen-specificity affects the ageing trajectory of these cells, we compare M1₅₈₋₆₆-specific T cells to the broader CD8⁺ T cell population and to T cells specific for epitopes from the chronic viruses CMV and EBV. We observe a reduction in diversity of the TCR repertoire of M1₅₈₋₆₆-specific T cells as the donors age from their 30s to 50s. We identified several transcriptomic changes, including changes in expression of several metabolic genes and genes associated with cytotoxicity. Several of the transcriptomic changes we identified were unique to M1₅₈₋₆₆-specific T cells, highlighting that T cells with different specificities may undergo distinct age-related changes.

Our study, along with the recent findings of Sturmlechner and colleagues, demonstrate the importance of accounting for antigen specificity when addressing the question of T cell ageing. While profiling T cells specific for the antigens from EBV, Sturmlechner et al observed that age-associated changes were not widely shared between T cells of different specificities and that each antigen-specificity had a distinct ageing signature, despite all the antigens having derived from the same virus²⁸⁸. When comparing the ageing signature M1₅₈₋₆₆-specific T cells to that of EBV/CMV-specific T cells or the broader T cell population we observed several genes that were uniquely upregulated in aged M1₅₈₋₆₆-specific T cells, including several genes associated with TCR signalling. At the phenotypic level, distinctions between ageing M1₅₈₋₆₆-specific T cells and other T cells were also observed. The majority of the M1₅₈₋₆₆ specific cells in our dataset had a Tcm or

Tem phenotype, and less than 3% of M1₅₈₋₆₆-specific cells were in the Temra cluster, which is a phenotype generally associated with ageing CD8⁺ T cells in the blood. This finding agrees with a recent study which profiled M1₅₈₋₆₆-specific T cells in individuals up to the age of 88 and did not identify M1₅₈₋₆₆-specific T cells with a Temra phenotype, even in these elderly individuals²⁷¹. Together these findings suggest that it may be overly simplistic to treat ageing CD8⁺ T cells as a uniform group as T cells with different antigen specificities appear to undergo distinct phenotypic and transcriptomic changes with age. Several factors including age at initial exposure to antigen, frequency and length of exposure to antigen and strength of TCR-pMHC likely affect the ageing trajectory of T cells. While CMV, and EBV are chronic viral infections, influenza is an acute but recurrent infection, meaning influenza-specific memory T cells may have time to recover/reset in between periods of antigen exposure which may contribute to their distinct ageing trajectory. A recent study showed that while T cells with a Temra phenotype accumulate with age in the blood, they do not increase in proportion in tissues including lungs, spleen and jejunum²⁶³. Another recent study demonstrated that cytotoxic molecule expression in memory CD8⁺ T cells varies both by viral specificity (EBV, CMV, influenza, and SARS-CoV-2) and by tissue of origin³⁸¹. These results highlight the need to account for location of initial exposure, tissue residency and antigen specificity when investigating the ageing trajectory of T cells.

In recent years several large scale cross-sectional datasets have been published profiling age-associated changes in peripheral immune cell populations^{99, 220, 221}. Although some of these studies have performed single cell 5'-RNA-sequencing which enables the TCR to be sequenced and several computational models exist^{382, 383} to predict antigen specificity from TCR sequence, current prediction accuracy remains too low for these models to be used as a standalone method. Thus, our study highlights the value of including DNA-barcoded dextramers for diverse antigen specificities in future large scale ageing cohorts to better understand how antigen specificity influences T cell ageing.

The major limitation of our study into antigen-specific T cell ageing is the limited number of donors we were able to include. Due to logistical challenges of repeatedly sampling the same donor over an almost 20 year period we are currently unable to increase the

number of donors in this longitudinal study. However, to build on the findings from this study, future work could include a cross sectional study profiling M1₅₈₋₆₆-specific T cells from individuals in their 30s and individuals in their 50s. Due to inter-donor variation we would need to include a greater number of donors in a cross sectional study.

With elderly individuals much more susceptible to severe disease and death from respiratory infections such as influenza and SARS-CoV-2^{261, 384} and likely to generate suboptimal responses to vaccines³⁸⁵, understanding how virus-specific T cells change with age is of paramount importance to enable better protection of elderly individuals from severe disease outcomes. Further cross-sectional and longitudinal studies will be necessary to fully characterise age-related changes in virus-specific T cells. It is also important to gain further understanding on how the ageing trajectory of T cells specific for epitopes within vaccines differs from that of T cells that are only activated during a natural infection. Once we gain a more complete understanding of age-related changes, *in vitro* and *in vivo* studies may then provide insight into whether these changes can be prevented or reversed, allowing T cells from elderly individuals to better protect the host from severe outcomes.

In Chapter 4, we identify pairs of SARS-CoV-2 ORF3a₂₀₇₋₂₁₅-specific T cell clones with identical TCR sequences but differences in their functionality. We use the highly functional clones as an extreme example of the severe-disease associated ORF3a₂₀₇₋₂₁₅-specific T cells that potentially contribute to immunopathology and perform proteomic and transcriptomic analysis of these clones. Through this omic analysis we identify high baseline expression of cytotoxic effectors, prolonged cytokine production and high expression of activation markers in the highly functional ORF3a₂₀₇₋₂₁₅-specific T cells. We also identify higher expression of several integrin molecules in the highly functional clones which was suggestive of a functional role which we then explored in detail. This study demonstrates how performing comparative analysis of T cell clones with identical TCRs can reveal other determinants of T cell functionality.

Our analysis of the SARS-CoV-2 ORF3a₂₀₇₋₂₁₅-specific T cell response highlights the delicate balance between beneficial anti-viral T cell response and those that can contribute to tissue damage and immunopathology. Studies on the immune response to

SARS-CoV-2 and influenza have demonstrated that T cells can have both beneficial and detrimental effects. A mouse model elegantly demonstrated the potential damage that T cells can cause in respiratory viral infections by uncoupling virus-mediated damage and T cell mediated damage³⁸⁶. CD8⁺ T cells specific for an influenza HA antigen were transferred into mice whose alveolar epithelial cells constitutively expressed the HA antigen. Without any viral infection these CD8⁺ T cells were able to cause severe lung injury, and the mice showed weight loss, compromised lung structure and increased cytokine levels in the lung³⁸⁶. Spatial transcriptomics of lung tissue from COVID-19 patients has also highlighted the contribution of T cells to immunopathology with enriched TCR signalling and cytotoxicity in areas of pathology¹⁵⁰. The activation of T cells within the lung may be spatially removed from the virus and thus activated T cells which remain and cause damage after viral clearance may be a key contributor to immunopathology. Our study demonstrates the excessively functional SARS-CoV-2 ORF3a₂₀₇₋₂₁₅-specific T cells as having a highly differentiated effector phenotype with high production of cytokines and cytotoxic molecules. Although the ORF3a₂₀₇₋₂₁₅-specific T cell response is associated with severe disease, in other situations, these factors would be thought to be protective against severe disease in viral infection. Thus, greater understanding of what differs between a controlled anti-viral T cell response and an overactivated tissue damaging T cell response is needed so that we can better understand how to prevent T cell induced immunopathology.

In Chapter 5, we utilise *in vitro* functional assays and immune synapse imaging to investigate how the integrins $\alpha 4\beta 1$ and $\alpha 4\beta 7$ influence T cell functionality and whether the expression of these integrins could be related to the association of ORF3a₂₀₇₋₂₁₅-specific T cells with disease severity. Pharmacological blockade and genetic KO of these integrins revealed that they can enhance cytokine production and killing capacity of CD8⁺ T cells. Our immune synapse analysis revealed localisation of $\alpha 4$, $\beta 7$ and $\beta 1$ in the immune synapse and that their levels were increased in the presence of their ligands, indicating that these integrins may enhance T cell functionality through stabilisation of the immune synapse. Our work in this chapter lead to the generation of our working hypothesis that integrins work as fine tuners of T cell activity. In some situations, including cancer and acute well-controlled viral infection high expression of the integrins

may enhance T cell activity and have a beneficial effect for the host. However, in chronic inflammation, autoimmunity and persistent viral infection high expression of $\alpha 4$ integrins could cause excessive activation of T cells, allowing them to contribute to immunopathology and tissue damage.

To further interrogate the molecular interactions at the immune synapse and how these may enhance T cell functionality, we plan to perform experiments to dissect temporal changes in integrin localisation. By including several stimulation timepoints we hope to elucidate the earliest changes in integrin localisation. Combining this with staining for phosphotyrosine and molecules downstream of the TCR (e.g. phospho-LAT and phospho-PLC γ) will allow us to better understand how $\alpha 4\beta 1$ signalling enhances TCR signalling and whether $\alpha 4\beta 7$ also does this. To gain further insight into the clinical effect of integrin expression on T cells we plan to perform immunofluorescence staining of lung tissue from severe COVID-19 patients to see whether we observe high levels of T cells expressing $\alpha 4\beta 7$ and $\alpha 4\beta 1$ in areas of immunopathology. If we do observe this it would support our hypothesis that high expression of $\alpha 4\beta 7$ and/or $\alpha 4\beta 1$ on T cells, could push the T cells into being overly activated and contributing to tissue damage in situation of excessive inflammation.

The remarkable clinical success of PD-1 and CTLA-4 blockade^{158, 159} illustrates the potential health impact of comprehensively understanding how specific molecules modulate T cell function. Despite the clinical use of integrin-targeting therapies for CD, substantial gaps remain in our understanding of how integrins influence T cell function. Our study, along with the recent findings of DeBerg and colleagues provides some new insight into the potential mechanism of action of Vedolizumab¹⁹⁹. We propose rather than inhibiting the migration of $\alpha 4\beta 7^+$ T cells to the intestine, vedolizumab may instead block the interaction of $\alpha 4\beta 7$ with VCAM-1 and MAdCAM-1 at the immune synapse, which could decrease the stability of the synapse and thus reduce the effector function of proinflammatory T cells, in turn reducing their ability to cause tissue damage. Further clarifying this potential mechanism of action may enable the use of integrin-blocking antibodies in other inflammatory conditions. While in this thesis we focussed solely on the integrins $\alpha 4\beta 7$ and $\alpha 4\beta 1$, recent studies have shown other integrins such as $\beta 3$ can

affect T cell function and enhance TCR signalling³⁰¹. Therefore, extending our understanding of how all integrins influence T cell biology, beyond their effects on migration and adhesion may further our ability to treat human diseases.

In inflammatory conditions pharmacological intervention is designed to dampen excessive immune activity, whereas in cancer, where tumour cells suppress immune cells, the objective is instead to enhance or reinvigorate immune cell function. To explore the clinical relevance of the $\alpha 4$ and $\beta 7$ integrins in the context of cancer, we analysed the TCGA database and observed a survival advantage of high *ITGA4* and *ITGB7* expression in several cancers, suggesting that these integrins could enhance T cell functionality in the TME which could improve survival of patients. Given the bulk nature of TCGA data this is not the ideal approach for addressing this question, but studies examining $\alpha 4\beta 7$ or $\alpha 4\beta 1$ expression directly on tumour-infiltrating T cells are limited. However, a recent study on metastatic colorectal found that patients with a high proportion of $\alpha 4\beta 7^+$ CD8⁺ T cells had increased overall survival than patients with a low proportion of $\alpha 4\beta 7^+$ CD8⁺ T cells³⁶⁵. Directly investigating $\alpha 4\beta 7$ expression on T cells in other cancers may further reveal how integrins affect T cell function and provide insight into whether introducing expression of integrins in cellular therapies could improve their performance.

To conclude, we found that antigen specificity influences the ageing trajectory of CD8⁺ T cells, as evidenced by the unique age-related transcriptomic changes observed in influenza M1₅₈₋₆₆-specific cells. In exploring which features of CD8⁺ T cells contribute to their association with severe disease, we profiled highly functional SARS-CoV-2 ORF3a₂₀₇₋₂₁₅-specific T cells which had been associated with severe disease. These cells displayed elevated expression of $\alpha 4\beta 7$ and $\alpha 4\beta 1$, which may enhance their functionality and underlie their association with severe COVID-19. Overall, this thesis addresses some key questions around virus-specific CD8⁺ T cell function and demonstrates how distinct features shape CD8⁺ T cell function across ageing and disease.

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