MONASH University

Homeostasis and Function of Virtual Memory CD8 T Cells During Ageing and Infection

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MPhil, Virology and Immunology

A thesis submitted for the degree of Doctor of Philosophy at

Monash University

April 2022

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Abstract

Classically defined CD8 T cell subsets are comprised of naïve or antigen-experienced cells (effector and memory). Virtual memory CD8 T (T_{VM}) cells are a unique population that sits in between these seemingly concrete definitions, as they are antigen-inexperienced, yet primed and express markers of activation, such as CD44, CD122 (IL-2/15Rβ) and natural killer cell receptors (NKRs). Recent evidence indicates that T_{VM} cells are not merely an activation state, but rather represent a distinct lineage of CD8 T cells, developing from $Eomes^{hi}$ precursors in the thymus with a distinct TCR repertoire and high affinity for self-peptide/MHCI. Their differentiation is completed in the periphery upon exposure to IL-15. T_{VM} cells require IL-15 to survive, as well as other factors that contribute to IL-15 signaling such as type I IFNs, Eomes, CD122 and CD8α+ dendritic cells (DCs). T_{VM} cells produce IFN-γ after innate-like stimulation with IL-12 and IL-18, and they can provide bystander protection during infections. In young mice, T_{VM} cells exhibit superior responses to TCR mediated stimuli, with rapid proliferation, cytokine production and cytotoxic capacity, compared to naïve T (T_N) cells. With increasing age, T_{VM} cells accumulate relative to T_N cells and can comprise up to 50% of antigenically naïve CD8 T cells in aged mice. T_{VM} cells also acquire a severe TCR-associated proliferative defect with increased expression of senescence markers with age, possibly a consequence of chronic cytokine stimulation. While cytokine sensitivity is a hallmark of T_{VM} cells, the impact of individual or multiple infections on T_{VM} cell prevalence and function is not well-described. Moreover, it is not known if infection or inflammation contribute to age-related T_{VM} cell dysfunction.

In this thesis, I have performed a comprehensive analysis of the impact of different infections and inflammatory cues on the generation, homeostasis, and function of T_{VM} cells in both young and aged mice. I found that infections and cytokine exposure cannot generate significant conversion of T_N cells to the T_{VM} cell phenotype, implying that, consistent with its unique developmental origin, T_{VM} cells are maintained as a distinct subset in the periphery. I also describe that despite an increasing cytokine sensitivity with age and development of senescence, innate-like T_{VM} cell function was largely maintained but not enhanced with age. I observed that T_{VM} cell population was influenced by infections, both in terms of phenotype and function, in a pathogen-dependent manner, with strikingly different impacts of helminth, LCMV, and influenza virus infections on T_{VM} cells. Furthermore, despite their cytokine sensitivity, I found that T_{VM} cell numbers remained relatively stable with several infections, and even where infection-related changes were observed, they were, with one notable exception in case of LCMV infection, not maintained long term. These data signify that the age-related changes in T_{VM} cell frequency and function, may be largely impervious to exogenous inflammatory stimuli, but rather reflect an inherent survival advantage and functional programming. These results improve our understanding of innate-like CD8 T cell function, and how it is impacted by ageing and infections, and provide clues as to how T_{VM} cell number and function might be maintained over the lifespan, which may be useful in protecting against chronic infections and cancer.

Declaration

This thesis is an original work of my research and contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

Signature:

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Date: 30 Apr. 22.

Publications during enrolment

(* indicates equal contribution/ # senior author)

Research Articles

1. Di Pietro A, Polmear J, Cooper L, Damelang T, Hussain T, Hailes L, et al. Targeting BMI-1 in B cells restores effective humoral immune responses and controls chronic viral infection. Nat Immunol. 2021 Nov 29;1–13.
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Published Conference Presentations

1. Hussain T, Nguyen A, Thiele D, Pang E.S, O'Keefe M, Good-Jacobson K, Quinn K.M, La Gruta N.L. Depletion of T_{VM} cells with LCMV infection leads to selective retention of more functional, less senescent cells. Immunology Group of Victoria 2021 Annual Scientific Meeting.
2. Hussain T, Nguyen A, Thiele D, Pang E.S, O'Keefe M, Good-Jacobson K, Quinn K.M, La Gruta N.L. Spilling the T cell; How Immune Gossip Tunes Immune Cell Function. Australia and Newzealand Society of Immunology (ASI) Annual Conference 2021.
3. Hussain T, Nguyen A, Thiele D, Pang E.S, O'Keefe M, Good-Jacobson K, Quinn K.M, La Gruta N.L LCMV infection results in a T cell subset with improved functionality. 2021 Biochemistry and Molecular Biology PhD Student Symposium.
4. Hussain T, Nguyen A, Thiele D, Pang E.S, O'Keefe M, Good-Jacobson K, Quinn K.M, La Gruta N.L. Depletion of T_{VM} cells with LCMV infection leads to selective retention of more functional, less senescent cells. Immunology at Monash (ImmuMon) 2021.
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7. Hussain T, Quinn K.M, La Gruta N.L. The Role of IL-15 in Maintenance of T_{VM} cells. Immunology Group of Victoria 2019 Annual Scientific Meeting.
8. Hussain T, Quinn K.M, La Gruta N.L. Development and maintenance of T_{VM} cells in ageing and infection. Immunology Group of Victoria 2018 Annual Scientific Meeting.

Thesis including published works declaration

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes 1 original paper published in peer reviewed journals and 1 submitted publications. The core theme of the thesis is to *understand generation, homeostasis and functions of T_{VM} cells during different infections and in the context of ageing*. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the student, working within the department of Biochemistry and Molecular Biology under the supervision of *Nicole L. La Gruta, Kylie M. Quinn and Anna Hearps*.

(The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.)

In the case of Chapter 1 and Chapter 4, my contribution to the work involved the following:

Thesis Ch	Publication Title	Status	Nature and % of student contribution	Co-author name(s) Nature and % of Co-author's contribution*	Co-author(s), Monash student Y/N*
I	Similar but different: virtual memory CD8 T cells as a memory-like cell population	Published	51%- contributed to planning the structure of article, drafted multiple sections, co-edited the manuscript and figures.	**Kylie M. Quinn** 49%- Supervised the writing process, outlined the structure of article, wrote multiple sections	N
IV	Helminth infection-induced increase in virtual memory CD8 T cells is transient, driven by IL-15, and absent in aged mice	Submitted	65%-Performed majority of the experiments, analysed results, wrote and revised multiple drafts	**Nicole La Gruta** 15% - Designed study, supervised research, co-wrote manuscript. **Kylie Quinn** 10% - Designed study, supervised research **Angela Nguyen** 2%-Performed key aged experiments, **Carmel Daunt** 1% - Maintained *Hp* lifecycle, **Daniel Thiele** 1%-Helped with *Hp* experiments, **Ee Shan Pang** 1%-Reagents and technical help for DC work, **Jasmine Li** 1% - Help with adoptive transfers, **Aidil Zaini** 1% -Set up *Tm* infections, **Meredith O'Keeffe** 1%-DC reagents and conceptual feedback**, Colby Zaph** 1% - Provided *Tm* parasites and conceptual feedback , **Nicola L. Harris** 1%- Provided *Hp* parasites and conceptual feedback	Y

*If no co-authors, leave fields blank

I have renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

Student name: Tabinda Hussain

Student signature: Date: 30 Apr. 22

I hereby certify that the above declaration correctly reflects the nature and extent of the student's and co-authors' contributions to this work. In instances where I am not the responsible author I have consulted with the responsible author to agree on the respective contributions of the authors.

Main Supervisor name: Nicole L. La Gruta

Main Supervisor signature: **Date:** 30/4/22

Acknowledgements

I have been lucky to be supervised by amazing scientists who have supported me throughout my PhD. My main supervisor, Nicole La Gruta, has played a pivotal role in improving my learning, technical expertise and critical thinking abilities that will be of great benefit in my future career. I really appreciate your patience and support through the bumpy times in my PhD, and your encouragement that pushed me to take initiatives that advanced not just my research project but also my professional development. Your drive, perseverance and intellect are inspiring, and I hope that I can emulate some of these qualities in future.

As a new and very untrained PhD student, Kylie Quinn, my co-supervisor, taught me the various lab techniques, and that was a definite highlight of my PhD journey. One cannot get a more compassionate, easy-going and smart teacher. The fact that I knew I can always ask you for help (which I did at quite odd times) has made this whole journey so much easier. You have been pivotal in shaping my approach to the kind of scientist I want to be and your mentorship and support has improved me as a person.

A big thankyou to Dr. Anna Hearps, my co-supervisor at Burnet Institute. We were very lost with HIV work before Anna came on board. Anna's advice and facilitation of HIV experiments was critical to the completion of this project. The input from my PhD committee, A/Prof Meredith O'Keeffe, A/Prof Mireille Lahoud and Dr Clovis Palmer, really advanced the direction of my project and I want to thank them for their guidance and advice through these four years.

I landed in La Gruta lab with the help of a very dear mentor, Clovis Palmer. Clovis helped me in finding a PhD position and has been very supportive throughout. People as kind and considerate as Clovis are rare and I feel very lucky to have known him.

I would like to thank Dan, for being a regular annoyance that I could always count on for distraction and bouncing off ideas. I don't think I will be able to get a better desk mate in future. I look forward to reading my name, written in golden letters, in your acknowledgements section very soon. Huge thankyou to Ange for being such a reliable lab partner, this work would have not been possible without your help and I cannot thank you enough. Your

preparedness for our experiments and our chats about random things made my long days much smoother. I will miss you guys very much.

I would also like to thank other members of La Gruta/Turner labs who are the most wonderful people to work with, especially Claerwen- for your help with everything through these 4 years and for your compassion through my rather intensive thesis writing process, Pirooz- for your expert advice and also your ability to put Dan in his place, Taylah and Vibha for being great lab mates, Brendan- for the much-needed humour, Jessie- for taking care of all the last minute orders.

This work would not have been possible without our collaborators, the dendritic cell experiments relied heavily on expertise of A/Prof Meredith O'Keefe and Dr. Ee Shan Pang. The helminth project had critical and continued support from Prof. Colby Zaph and Prof. Nicola Harris and their lab members, Aidil Zaini and Carmel Daunt. I would also like to thank A/Prof Kim Jacobson for providing LCMV virus and Prof. Mariapia Degli-Esposti and members of her lab, Iona and Xav, for reagents and valuable advice.

I would like to thank A/Prof. Frank Alderuccio and Dr. Kim Murphy for giving me the opportunity to tutor immunology. I have really benefited from the experience as it helped me not only financially but also refreshed my knowledge.

Many thanks to members of FlowCore staff (Andy, Kat, Adam and Keith) for their patience with my sorts and the MARP staff, especially Olivia Cameron, Claire Klijnsma, Mark Sheehan and Zac Teale.

I would also like to thank BDI/Biochemistry department staff for their prompt help at various times during my PhD, especially Amelia Morrison, Ico Ma and A/Prof Priscilla Johanesen.

I would like to acknowledge funding support from Monash University International Research Scholarship (MIPRS), Monash University Graduate Stipend (MGS) and Graduate Research Completion Award (GRCA) during my candidature.

I want to mention my little bundles of craziness, squeals and above all, complete joy, Amanah and Navaal. My children have endured my extremely long hours and have still loved me and given me hugs that took away all stress, you are the best thing that has ever happened to me. My dearest mother, thankyou for the confidence and independence you have given me and

for your continued prayers, they are a treasure. My dearest father, thankyou for all the love and pride you have showered on my achievements in the past. I hope you are in a much better place. I miss you every day. Thankyou to my best friend, sister and my safe place, Annie, who has always been there for me.

Finally and most importantly, my PhD would not have been possible without the unwavering support of my amazing husband, Saaim. Your hands-on parenting of the kids and continued encouragement for me has pushed me to the finish line. I owe you all of this and much more.

"Indeed, with every hardship, there is relief" (Quran 94:5)

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Abbreviations

AEC	Animal ethics committee
AMPK	AMP-activated protein kinase
AP-1	Activator protein-1
APC	Allophycocyanin
APC	Antigen presenting cell
ARL	Animal research laboratory
ART	Antiretroviral therapy
AT	Adipose tissue
BL	Baseline
CAR	Chimeric antigen receptor
cfu	colony forming units
cIL-15	IL-15/IL-15Rα:Fc
CIS	Cytokine-inducible SH2-containing protein
CML	Chronic myeloid leukemia
CMV	Cytomegalovirus
cRPMI	Complete Roswell Park Memorial Institute media
CTL	Cytotoxic T lymphocyte
CTV	Cell Trace Violet
d	day
DC	Dendritic cell
DMSO	Dimethyl sulfoxide
dPBS	Dulbecco's phosphate-buffered saline
EBV	Epstein-Barr virus
EDTA	Ethylenediamine tetraacetic acid
ERK	Extracellular signal -regulated kinase
FACS	Flow cytometry staining buffer

FAO	Fatty acid oxidation
FBS	Fetal bovine serum
GF	Germ free
GrB	Granzyme B
h	hours
HBSS	Hanks balanced salt solution
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
Hp	*H. polygyrus/ Heligmosomoides polygyrus*
HTS	High throughput sampler
IAV	Influenza A virus
IL-15c	IL-15/IL-15Rα:Fc
JCU	James Cook University
KIR	Killer cell immunoglobulin-like receptor
LAT	Linker for activation of T cells
LCMV	Lymphocytic choriomeningitis virus
LIP	Lymphopenia induced proliferation
MACS	Magnetic-activated cell sorting
MAPK	Mitogen-activated protein kinase
MARP	Monash animal research platform
medLN	mediastinal lymph node
mesLN	mesenteric lymph nodes
MHC	Major histocompatibility complex
MHCI	MHC Class I
MHCII	MHC Class II
mo	month old
MuHV	Murine gammaherpesvirus
NFAT	Nuclear factor of activated cells

NF-κB	Nuclear factor κB
NK	Natural killer
NKR	Natural killer receptor
NKT	Natural Killer T cell
OCR	Oxygen consumption rate
OXPHOS	Oxidative phosphorylation
p	phosphorylated
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PE	Phycoerythrin
pfu	plaque forming units
PI3K	Phosphotidylinostitol 3-kinase
PLC	Phospholipase
PLZF	Promyelocytic leukemia zinc finger
PMA	Phorbol 12-myristate 13-acetate
pMHC	peptide-MHC
PRR	Pattern recognition receptor
PTK	Protein tyrosine kinase
PWH	People with HIV
RBC	Red blood cell
rh	Recombinant human
RT	Room temperature
RTE	Recent thymic emigrants
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
S.t	*S. typhi/Salmonella typhimurium*
S1P	Sphingosine-1-phosphate
SEM	Standard error of mean
SLE	Systemic lupus erythematosus

SOCS	Suppressor of cytokine signaling
SPF	Specific pathogen free
SRC	Spare respiratory capacity
STAT	Signal transducer and activator of transcription
T_{CM}	Central memory T (cell)
TCR	T cell receptor
T_{EFF}	Effector T (cell)
T_{EM}	effector memory T (cell)
T_{EMRA}	Terminally differentiated effector memory T (cell)
T_{FH}	Follicular helper T (cell)
Th	T helper cell
TIL	Tumor infiltrating lymphocytes
T_{IM}	Innate memory T (cell)
Tm	*T. muris/ Trichuris muris*
T_{MP}	Memory phenotype T (cell)
T_{N}	Naïve T (cell)
T_{REG}	Regulatory T (cell)
T_{RM}	Resident memory T(cell)
T_{VM}	Virtual memory T (cell)
WEHI	Walter and Eliza Hall Institute
Zap-70	Zeta-chain associated protein kinase
γc	common gamma chain

Chapter I

1. Review of Literature

1.1. The innate and adaptive arms of our immune system

The innate and adaptive arms of our immune system have evolved to control immune challenges using distinct but complementary strategies. The innate immune system responds to challenges rapidly and provides broad but non-specific protective mechanisms through the recognition of wide classes of pathogen or cell stress associated ligands. Some key cells of the innate immune system include macrophages, granulocytes (eosinophils, basophils, neutrophils) and natural killer (NK) cells [1]. The innate immune system mediates protection primarily via phagocytosis of target cells and release of cytokines and chemokines that activate or recruit other cells of the innate as well as adaptive immune system to the site of infection [2]. Pattern recognition receptors (PRRs) are expressed on the surface of phagocytes, such as macrophages and neutrophils, and recognize microbial structures that are essential for the pathogen's survival [3–5]. Innate immune cells also have receptors that recognize cell stress-associated ligands that may be upregulated during inflammation, infection or malignancy. NK cells recognize specialized molecules expressed on the surface of stressed and infected cells, and induce lysis of target cells by releasing cytotoxic granules [6].

The adaptive immune system responds more slowly but mounts a highly specific response against pathogens that then results in long-lived memory. The two major classes of cells that mediate adaptive immunity are B cells, which produce antibodies and are therefore

responsible for humoral immunity, and T cells, which are the cell mediated arm of the adaptive immune system. Upon re-infection with the same pathogen, memory B and T cells are able to generate a response of larger magnitude that results in effective and rapid neutralization of pathogens[7] [8].

Conventional T cells can be divided into CD4 T cells, generally called helper T (Th) cells, and CD8 T cells, commonly called cytotoxic T lymphocytes (CTLs). CD4 T cells are essential for maturation of memory B cells that produce highly-specific antibodies, expansion of CTLs during primary infection and generation of memory CTL response [9], among other functions. CD8 T cells are critical for the effective control of intracellular viral and bacterial infections or cancers due to their ability to directly kill infected or malignant targets and their production of pro-inflammatory cytokines [10]. A highly variable, cell surface expressed T cell receptor (TCR) is a defining marker of all T cells. The conventional TCR is a transmemberane α/β heterodimer expressed on all T cells. Each TCR can recognize a short protein sequence (peptide), often derived from intracellular pathogens, presented in the context of the Major Histocompatibility Complex (MHC) antigen presenting molecule [11]. CD4 T cells recognize peptides presented by MHC Class II whereas CD8 T cells engage with peptide + MHC Class I (pMHCI) epitopes presented by dendritic cells (DCs) in secondary lymphoid tissues. This leads to T cell activation and rapid proliferation that results in a large population of CTLs that migrate to sites of infection and mediate protection via cytotoxicity and cytokine production [10].

Critically, the generation, homeostasis and activation mechanisms engaged by innate and adaptive immune cells are fundamental processes critical for effective triggering of immune responses and maintaining health across the lifespan.

1.2. CD8 T Cell Generation

Generation of mature CD8 T cells occurs in the thymus. T cell progenitors are generated in the bone marrow and travel to the thymus to undergo a series of maturation and antigenic education steps. These progenitors first undergo differentiation and somatic recombination, where gene segments in the TCR α and β gene loci are randomly rearranged to generate productive TCR α and β genes with wholly unique DNA sequences for each individual T cell [12]. The newly formed TCR is expressed on the cell surface of developing thymocytes and the TCR is then tested in two key processes called positive and negative selection [10].

For both positive and negative selection, the strength of the TCR/self-peptide/MHC interaction in the thymus is critical. Positive selection ensures that the TCR recognizes an individual's MHC alleles [13]. MHC molecules are glycoproteins that present peptides to T cells. MHCI is expressed on all nucleated cells and MHCII is expressed only on antigen presenting cells (APC). There are multiple genes that encode MHCI or MHCII, which mean each cell (and individual) expresses a combination of MHC molecules on their surface, thereby increasing the likelihood of expressing MHC molecules that are capable of binding to foreign peptides [14,15]. MHC genes are also exceptionally polymorphic, meaning there are many different allelic variants for each MHC gene, with over 10,000 alleles for MHCI and 3000 MHCII alleles currently identified [16]. Given that the MHC consists of multiple genes that are highly polymorphic, it is highly unlikely for two individuals to possess the exact same set of MHC molecules in an outbred population. The incredible diversity of MHC genes expressed by individuals is also a key signal for immune cells to differentiate self from non-self tissue[15].

Specific domains within the TCR recognize and bind to MHC I and II, allowing the TCR to recognize peptides held in MHC groove [17]. In the thymus, T cell precursors with TCRs of low to moderate affinity for self-peptide/MHC I or MHC II are positively selected (receive a survival signal) to become a CD8 or CD4 T cell, respectively, and undergo further development while T cell precursors that either fail to express a TCR on the cell surface or express a TCR that fails to bind to self-peptide/MHC die by neglect [18–20]. On the other hand, negative selection ensures that the TCR does not recognize self-antigen with too high an affinity. T cell precursors with TCRs of high affinity for self-peptide/MHC will be negatively selected through triggering of apoptosis [18–20]. It is therefore essential that TCRs expressed on developing CD8 T cells are able to bind to an individual's MHCI alleles but not bind too strongly to self-antigen-derived peptides [13]. After selection, mature naïve CD8 T cells that are yet to encounter their cognate antigen, exit the thymus as T_N cells.

Of note, the level of expression of CD5, a negative regulator of TCR signaling, on mature CD8 T cells after they exit the thymus is considered to denote the strength of the TCR-self-peptide/MHCI interaction, with $CD5^{hi}$ cells exhibiting modestly higher self-reactivity as compared to $CD5^{lo}$ T cells [21].

1.3. CD8 T Cell Survival and Homeostasis

After egress from the thymus, T_N cells need to survive in the periphery until they encounter their cognate antigen. T_N cells can circulate through peripheral and secondary lymphoid tissues but they primarily reside in secondary lymphoid tissues. Critically, several molecules can provide survival signals in different niches within the body. The key survival requirements

for CD8 T_N cells are; homeostatic exposure to IL-7 and IL-15 [22,23], transient self-peptide/MHCI interactions, called tonic TCR stimulus [24,25] and sphingosine-1-phosphate (S1P) signaling [26].

The initial pool of recent thymic emigrants (RTE) has a low level of IL-7Rα (CD127) expression. Upon exposure to homeostatic IL-7, the RTEs proliferate to give rise to a consolidated pool of T_N cells that have higher CD127 expression [27] and afterwards adopt a quiescent state where their survival is mainly supported by IL-7. IL-7 was found to support optimal metabolic rate and prevented T cell atrophy [23] and T_N cells are completely lost within a month of being transferred to IL-7-/- mice [22], suggesting a critical role of IL-7 in supporting T_N cell survival. Both IL-7 and IL-15 can induce expression of the anti-apoptotic protein Bcl2 which likely supports survival of CD8 T cells in the steady state [23,28–31]. However, T_N cells are not completely dependent on IL-15 for survival, although T_N cell numbers are reduced by half in mice that are IL-15 deficient [29,32,33]. In contrast, survival of memory T (T_{MEM}) cells is thought to be optimal with IL-15 and, indeed, the $CD122^{hi}$ subset of T_{MEM} cells is completely lost in the absence of IL-15 [34,35].

In their quiescent state, T_N cells also receive survival signals via homeostatic self-peptide/MHCI interactions that are repeated but transient, known as tonic signaling. The MHC molecule itself is essential for this as the T_N cell subset is gradually lost in MHCI-/- mice [36,37]. In case of T_{MEM} cells, the $CD122^{hi}$ subset's survival is independent of MHCI whereas some $CD122^{lo}$ T_{MEM} cells depend on MHCI for maintenance in the steady state [37].

S1P is a chemoattractant and is conventionally known as a guiding gradient for T cell localization during an immune response [38]. Recent evidence suggests that S1P can also serve as a survival signal for T_N cells when they are outside the secondary lymphoid tissues in

circulation. S1P is secreted by lymphatic endothelial cells is essential for maintaining mitochondrial function of T_N cells [26]

Under homeostatic conditions, T_N cells have a low rate of turnover and stay in interphase [39], whereas T_{MEM} cells have a much higher rate of background proliferation, dividing once every 7-21 days [40,41]. This is possibly due to increased sensitivity of T_{MEM} cells to IL-15 [29,32] which results in higher rates of background proliferation [42]. The average lifespan of murine T_N cells is 31-72 days [43], whereas human T_N cells can have a half-life of up to 6.5 years [44]. In contrast, different subsets of human T_{MEM} cells have wide variation in their reported longevity, where some have reported a half-life of 8-15 years for vaccinia and yellow-fever virus specific T_{MEM} cells [45,46] and others reported lifespans of less than a year for T_{EM} cells [47,48]. This highlights that naïve and memory cells, and subsets within these broad populations can have different sensitivities to survival signals, leading to their very different lifespans.

1.4. CD8 T Cell Activation

T_N cells express L-selectin (CD62L) and the chemokine receptor CCR7, both of which allow localization of T_N cells in the secondary lymphoid organs, but they lack expression of activation markers like CD44, CD49d and CD122 [10,49,50]. When a T_N cell recognizes, via the TCR, its cognate antigen presented by MHCI on the surface of a professional APC, along with additional signals (discussed below), it will become activated. The CD8 co-receptor is recruited to stabilize the TCR/pMHCI interaction. The α/β chains of the TCR have short intracellular domains which are not sufficient to transduce TCR activation signals. Within the TCR signaling complex, CD3γ/δ/ε/ζ subunits associate with the TCR and transmit the signals through phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAM) in the intracellular domain of CD3

molecules. Lck is a Src family tyrosine kinase that is typically found associated with the intracellular domain of CD8 in the steady state. Upon activation, Lck is recruited proximal to the TCR signaling complex, and phosphorylates the CD3 subunits [51] as depicted in Figure 1.1. The phosphorylated (p)-CD3ζ subunit allows binding of zeta-chain associated protein kinase (Zap-70), a Syk family protein tyrosine kinase (PTK), which is further phosphorylated by Lck. Phosphorylated (p)Zap-70 then activates linker for activation of T cells (LAT) via tyrosine phosphorylation. LAT then provides a binding site for Grb2, phospholipase C-γ1 (PLC-γ), the p85 subunit of phosphotidylinostitol 3-kinase (PI3K) and other critical signaling molecules, thus resulting in signaling cascades that can activate the Ras, mitogen-activated protein kinase (MAPK) and calcium signaling pathways [52]. These signaling cascades result in activation of various transcription factors, including nuclear factor of activated cells (NFAT), nuclear factor kB (NF-kB), and activator protein 1 (AP-1), which lead to transcription of genes that regulate CD8 T cell effector functions and proliferation [51,53] (Figure 1.1).

TCR-mediated signals alone are not sufficient to trigger activation of naïve T cells. Costimulatory receptors, like CD28 on CD8 T cells, recognize upregulation of CD80 and CD86 on activated DCs leading to maximal activation of CD8 T cells [54,55]. Furthermore, cytokine signals such as IL-2 secreted by CD4 T cells and other cells, and IL-12 produced by DCs is required for optimal T cell activation and clonal expansion [56–60] (Figure 1.1).

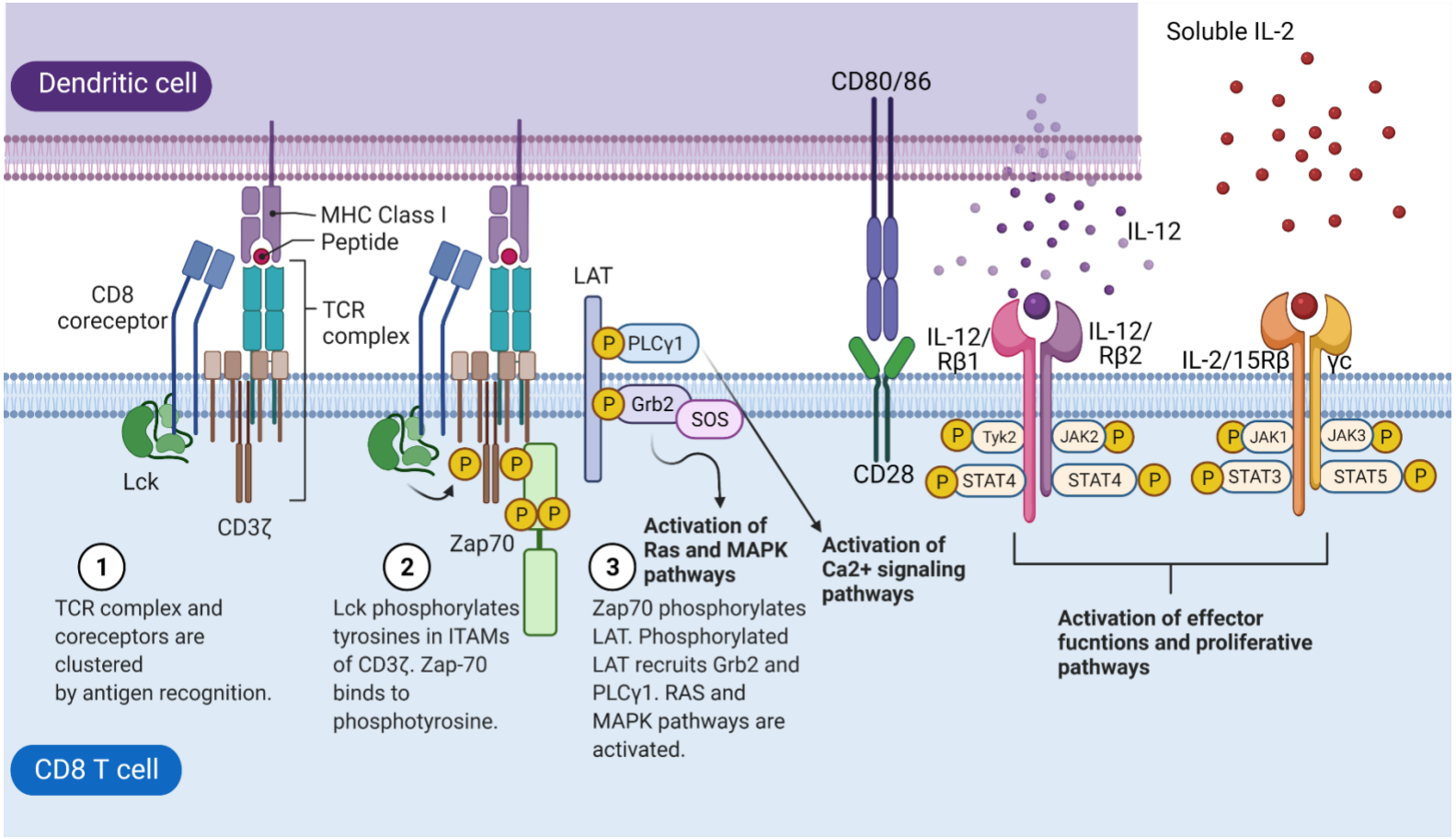

Figure 1.1. TCR signaling pathway after TCR recognition of peptide-MHCI (p-MHCI) presented on DCs. TCR and p-MHCI engagement results in phosphorylation (p) of ITAMs on CD3 by Lck. p-CD3ζ provides a binding site for p-Zap-70 leading to phosphorylation of LAT, to further recruit factors that activate Ras, MAPK and calcium signaling pathways. Additional signals from binding of CD80/86 on DCs to CD28 on CD8 T cells induces maximal activation. IL-2 and IL-12 further support activation and clonal expansion of CTLs (*created with BioRender.com*).

1.5. Effector and Memory Fates of CD8 T cells

Once a CD8 T cell is fully activated, it is called an effector CD8 T (T_{EFF}) cell. Aided by IL-2, activated CD8 T cells begin to rapidly proliferate, to establish a large pool of antigen experienced T_{EFF} cells. In mice, T_{EFF} cells lose expression of CD62L, but acquire expression of CD44 and CD49d which are markers of activation [49,61,62]. In humans, T_{EFF} cells lose expression of CD45RA, CCR7 and CD27 but gain expression of CD45RO and CD25 [10,50].

T_{EFF} cells produce key cytokines including IFN-γ, TNF and low levels of IL-2. IFN-γ directly promotes CD8 T cell cytotoxic activity [63], as well as promoting Th-1 differentiation of CD4 T cells and macrophage activation to augment anti-microbial and tumoricidal activity [64]. IL-2 can signal in an autocrine fashion to support proliferation during clonal expansion of T_{EFF} cells [65]. TNF can drive apoptosis in cells expressing TNF receptors and induce broader inflammation [66].

T_{EFF} cells can mediate cytotoxicity of target cells using extrinsic and intrinsic pathways. The key mechanism for lysis is the intrinsic pathway, which utilizes perforin and GrB that is stored in cytoplasmic granules in activated CD8 T cells. Upon TCR/pMHCI engagement, perforin is released which induces pore formation on the membrane of the infected or stressed cell through which GrB can enter and trigger apoptosis and DNA fragmentation [67]. The extrinsic pathway is initiated by engagement of death receptors, where CD95L (FasL) binds to CD95 (Fas) on target cells and triggers apoptosis of target cells by activation of caspase cascade [68,69]. While most of these pathways are targeted to infected and stressed cells recognized via TCR/pMHCI, bystander activation of CD8 T cells can also result in IFN-γ production as well as killing of infected cells; these are discussed in Section 1.8.

Once activated, CD8 T cells can differentiate into short-lived T_{EFF} cells or long-lived T_{MEM} cells. T_{MEM} cells are primed for more rapid effector function due to their prior encounter with cognate antigen, but they exist in a quiescent state in different locations of the body and mediate lasting protection from subsequent challenge with the same pathogen. T_{MEM} cells can be subdivided into distinct subsets based on their localization in the body. T_{MEM} cells that circulate through the blood and tissue and exhibit rapid effector functions are called effector memory CD8 (T_{EM}) T cells. T_{EM} cells exhibit a similar phenotype to T_{EFF} cells, with low expression of CD62L and CCR7 and play a key role in immunosurveillance of peripheral tissues. A subset of T_{MEM} cells, termed central memory CD8 T (T_{CM}) cells, have high expression of CD62L and CCR7 allowing them to home to lymph nodes [70]. T_{CM} cells have lower expression of GrB and are therefore less cytotoxic [71] but have more proliferative potential compared to T_{EM} cells [72]. Thus, T_{CM} cells provide systemic protection and can rapidly expand and provide backup at inflamed tissue sites. T_{MEM} cells that permanently reside in tissues are called resident memory CD8 T (T_{RM}) cells. T_{RM} cells are marked by their high expression of CD69 and/or CD103 (reviewed in [10,49,50]), and represent a first line of defence in tissues upon pathogen reencounter.

T_{MEM} cells that are antigen experienced but are the least differentiated population and have phenotypic and functional characteristics of T_N cells are called stem cell-like memory CD8 T (T_{SCM}) cells. These cells possess enhanced self-renewal potential and are multipotent allowing differentiation into further memory populations. T_{SCM} cells were first identified in mice as a subset with low expression of CD44, but high expression of CD62L, CD122, Sca-1 and Bcl2 [73,74]. In humans, T_{SCM} cells express CD45RA, CCR7, CD27 and CD28 consistent with T_N cells, but they also express CD122, CD95, LFA-1 and CXCR3 which reflect an antigen-experienced phenotype [75]. T_{SCM} cells are a key target cells for many immune-based interventions such as vaccines and

cellular therapies as they may have increased therapeutic potential compared to T_{CM} and T_{EM} cells.

Once the infection is cleared, most of the T_{EFF} cells die off and the long-lived T_{MEM} cells often comprise less than 5-10% of the expanded T_{EFF} population [76]. The fate decision of an activated CD8 T cell to become T_{EFF} or T_{MEM} cell is a topic of debate, where older models have proposed a branching model where these fates arise simultaneously after activation, or a linear model where a certain subset of T_{EFF} cells goes on to become T_{MEM} cells depending on the affinity of TCR/pMHCI interaction [8,77]. Recent studies that profiled the epigenetic landscape of different CD8 T cell subsets have suggested that each cell population is reflective of a further differentiation state. Methylation analysis of chromatin accessibility reveals that T_N cells are least differentiated, followed by T_{SCM} cells, T_{CM} cells and T_{EM} cells [78,79].

1.6. Classification of Memory Phenotype CD8 T cells

The aforementioned conventional classification of CD8 T cells has been complicated by the discovery of cell subsets that are antigen-naïve but express markers that have been traditionally used to indicate antigen experience [80,81]. A 'true-naïve' T_N cell has never encountered antigen and does not express markers of memory. In mice a T_N cell is $CD44^{lo}$ $CD62L^{hi}$ and in humans is characterized by co-expression of CD45RA, CD27 and/or CD28 and CCR7.

CD44 is upregulated very early after antigen encounter on mouse T cells [82,83], however more recently it has become apparent that it is not exclusively triggered by sustained stimulation with foreign cognate antigen. High expression of CD44 has been observed on T cells specific for antigen they have not yet encountered [84–86]. Prior to this observation, all CD8 T cells

expressing CD44 were regarded as T_{MEM} cells but more recently CD44 has become used to denote "memory-phenotype" cells. Memory phenotype cells (T_{MP}) cells encompass traditional antigen-experienced T cells, such as T_{EM}, T_{CM}, and T_{RM} cells, and semi-differentiated antigen-naïve CD8 T cells. These antigen-naïve T_{MP} cells include lymphopenia-induced proliferative (T_{LIP}) cells, innate memory CD8 T (T_{IM}) cells and virtual memory CD8 T (T_{VM}) cells (Figure 1.2).

T_{LIP} cells arise as a consequence of homeostatic proliferation under lymphopenic conditions. Lymphopenia can occur naturally during neonatal development or during other physiological challenges, and can drive enhanced proliferation of T_N cells, due to a relative abundance of IL-7 [87,88]. IL-7-induced homeostatic proliferation of T_N cells in neonatal mice, results in generation of T_{LIP} cells, which express CD44 and CD122, without exposure to cognate antigen [87,88]. It is important to note that LIP is IL-7 dependent and not IL-15 dependent [88,89]. There is also evidence that T_{LIP} cells are a transient T_{MP} population and revert to their naïve phenotype after LIP restores peripheral lymphoid environment to normal cell numbers [90].

T_{IM} cells arise in response to IL-4 production in the thymus. In BALB/c mice, an IL-4 dependent T_{MP} subset, called innate memory (T_{IM}) cells arise during thymic development. T_{IM} cells can be observed in the thymus of BALB/c, but not C57BL/6, mice due to the presence of IL-4 producing promyelocytic leukemia zinc finger (PLZF)+ NKT and γδ NKT cells in BALB/c mice [91,92]. T_{IM} cells are characterized by $CD44^{hi}CD122^{hi}$ expression, as well as high CXCR3 and CD62L expression [91,92] and critically depend on IL-4 for survival in the periphery but also appear to have partial dependence on IL-15 and IL-7 [93].

T_{VM} cells also arise in the thymus and their generation, homeostasis and activation will be examined as the focus of this thesis.

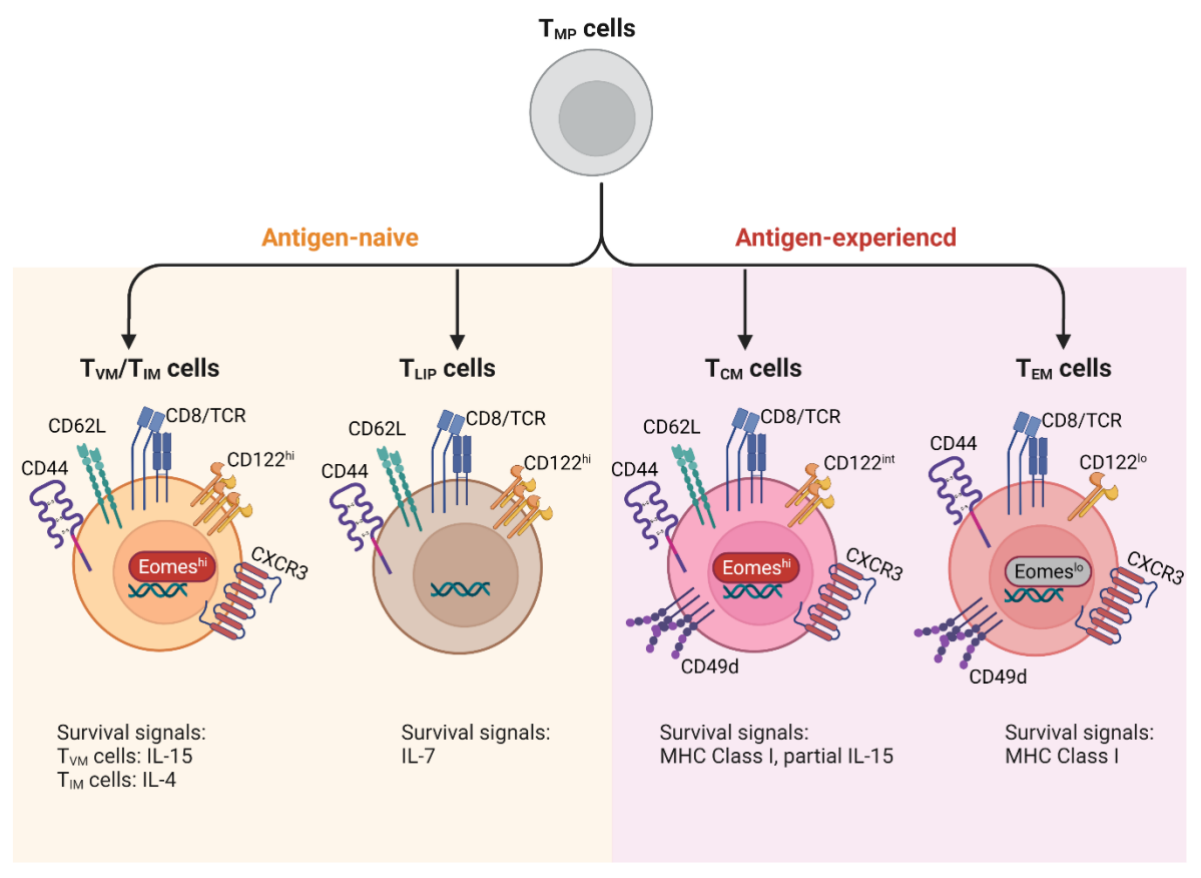

Figure 1.2. Classes of memory phenotype CD8 T (TMP) cells and their distinctive and overlapping expression profiles. (*created with BioRender.com*).

1.7. T_{VM} Cells

T_{VM} cells are antigen-naïve CD8 T cells that express markers typically associated with memory in mice, such as CD44 and CD122 [94] (Figure 1.2). NKG2A and KIRs in humans [95–97]. T_{VM} cells represent a substantial proportion of the total CD8 T cell population in adult naïve C57BL/6 mice (~10%) and humans (~5%) [86,95–98]. T_{VM} cells critically depend on IL-15 for their maintenance in the periphery in C57BL/6 mice [34,35,97], however they can proliferate in response to IL-4 as well [84,99]. Due to their semi-differentiated state, T_{VM} cells respond rapidly to TCR-mediated stimulation with proliferation [95] and contribute the first wave of T_{EFF} cells during an TCR-dependent, antigen-specific response [85]. They are also responsive to innate-like

stimulation with IL-12 and IL-18 and express receptors that are usually associated with NK cells, such as NKG2D, allowing them to mediate TCR-independent, bystander protection [94,96,97,100].

T_{IM} cells have been defined separately from T_{VM} cells because T_{IM} cells adopt the memory phenotype with $CD44^{hi}CD122^{hi}$ expression in thymus of BALB/c mice where IL-4 is available [91–93], whereas C57BL/6 mice lack IL-4 producing cells in the thymus, therefore the T_{VM} cell phenotype is only adopted after IL-15 signaling in the periphery [35,101]. The developmental origins and survival requirements of T_{VM} cells, discussed further in Section 1.10, are distinct from T_{IM} cells, the phenotype of T_{VM} and T_{IM} cells in the periphery is largely the same [86,91,94]. There are some differences between T_{VM} cells and T_{IM} cells, the foremost being that T_{IM} cells express lower levels of CD122 compared to T_{VM} cells [80,102], which explains the modest dependence of T_{IM} cells on IL-15. Additionally T_{IM} cells do not express NKG2D [102,103], an NKR that is increasingly expressed on T_{VM} cells with age (discussed in Section 1.9, 1.10) and also have reduced expression of IL-18R [102]. The lack of NKG2D expression on T_{IM} cells implies that they do not have similar bystander cytotoxic capacity as T_{VM} cells [80] however this has not been demonstrated so far. The lower level of IL-18R expression on T_{IM} cells is thought to contribute to lower IFN-γ expression by T_{IM} cells compared to T_{VM} cells after IL-12/IL-18 stimulation [102]. These data point to functional differences between these two T_{MP} populations, with T_{IM} cells having reduced innate-like functionality compared to T_{VM} cells.

1.8. TCR-Independent Functions of CD8 T cells

Classically, CD8 T cells become activated via their TCR and then mediate effector function as described above. However, T_{VM} cells and other T_{MP} cells can engage in TCR-independent, or

"bystander", functions in certain scenarios by leveraging cytokine-mediated activation or mechanisms that are classically associated with NK cells. The ability of CD8 T cells to produce IFN-γ without TCR engagement was first described during bacterial infections [104,105] and this was shown to play a critical role in controlling early bacterial replication of *Listeria monocytogenes* [106]. The key cytokines that mediate cytokine-driven activation of CD8 T cells are IL-12, IL-18 and type 1 IFNs. IL-12 and IL-18 are primarily produced by activated macrophages and DCs [107,108], while activated plasmacytoid DCs are the main producers of type I IFNs [109,110].

While it is known that type I IFNs can augment antigen-specific CD8 T cell responses [111,112], the role of type I IFNs as a direct modulator of bystander function in CD8 T cells is less well described. Type I IFNs can indirectly increase innate-like responses of CD8 T cells by upregulating DC production of IL-15 [113], which in turn can promote non-specific GrB production in CD8 T cells and initiate lysis of target cells [114]. Type I IFNs, along with IL-12, can also increase expression of IL-18 receptor on CD8 T cells [115], therefore increasing their sensitivity to innate-like stimulus and enhancing bystander cytokine production as described above in CD8 T cells. Of note, some functions of T_{VM} cells share parallels with NK cell function.

1.9. NK-like function of CD8 T cells

NK cells are key cytotoxic cells in the innate immune system and their development and function depends on integration of signals from a number of inhibitory and activating NK cell receptors (NKRs).

Inhibitory NKRs signal via cytosolic immunoreceptor tyrosine-based inhibitory motifs (ITIM) that function by tyrosine dephosphorylation [116]. They include some killer cell immunoglobulin-

like receptors (KIRs) in humans, Ly49A/G receptors in mice and NKG2A in mice and humans, which recognize various classes of MHC molecules [117,118]. Upon recognition of MHC molecules on the surface of other cells, the inhibitory receptors prevent NK cell activation and cytotoxicity [118].

Activating NKRs have intracellular domains that recruit adaptor molecules, DAP12 and DAP10 [117], that permit signal via their cytosolic immunoreceptor tyrosine-based activating motifs (ITAMs) [116]. They include NKG2C/D/E, KIR2DS, KIR3DS, Ly49H, which also recognize classical and non-classical MHC molecules [118,119]. Upon binding with activating NKR ligands, the ITAM undergoes tyrosine phosphorylation, which initiates signaling via Src family protein tyrosine kinases (PTKs), that later recruit Zap-70 and Syk tyrosine kinases, thus using signaling pathways similar to TCR activation [116]. Among activating NKRs, NKG2D is the only one known to associate with DAP10 in humans but it can recruit both DAP10 and DAP12 in mice. DAP10 has phosphorylation motif similar to those present in intracellular domain of CD28 on T cells [120]. Upon binding with NKG2D ligands, MICA, MICB and ULBP1-6 in humans and Rae-1 in mice, DAP10 is phosphorylated and signals via PI3K pathway [120,121]. Some studies have reported a direct role of IL-15 in potentiating the cytotoxic programs downstream of NKG2D activation. DAP10 has also been shown to associate with both CD122 and IL-15 signaling results in activation of Jak3 which can phosphorylate DAP10 and therefore activate signaling pathways downstream of NKG2D [122]. Activation of these signaling cascades leads to activation of cytotoxic programs and cytokine production in NK cells [116].

The activation of an NK cell is a complex decision, where the response is controlled by balancing the signals received from both activating and inhibitory receptors. If inhibitory NKR

engagement is lost and ligands expressed on stressed and infected cells are engaged by for activating NKRs, NK cells can be activated to mediate cytotoxic functions.

Adaptive CD8 T cells share many parallels with innate NK cells in their function and development. CD8 T cells and NK cells both arise from the common lymphocyte precursor in the bone marrow and undergo education during development to ensure they are not promiscuously activated in the periphery [123–125]. CD8 T cells and NK cells both have cytotoxic capacity, which can directly lyse infected and stressed cells by releasing GrB and perforin [118]. Optimal CD8 and NK cell survival and function also depend on similar cytokines, such as IL-2, IL-12, IL-15, and type I IFNs [123,126,127].

Of note, activated CD8 T cells, including T_{MP} and T_{MEM} cells, can start to express a variety of inhibitory and activating NKRs [95,97,128,129]. NKR expression on CD8 T cells can be induced by antigen-specific activation [130,131] and cytokine exposure [132,133] and increases with age [134].

Inhibitory NKR expression on CD8 T cells, such as some KIRs, Ly49a and NKG2A, is associated with suppression of TCR-induced activation in some studies [135–138]. Engagement of inhibitory NKRs with MHCI results in increased accumulation of $CD122^{hi}$ T_{MP} cells and suppression of TCR-mediated activation induced cell death (AICD) [139–141]. AICD prevention during an antigen-specific response has been associated with preferential survival of T_{MEM} cells over T_{EFF} cell [131]. NKG2A, belongs to the CD94/NKG2 class of inhibitory receptors, and recognizes non-classical MHC molecules, human leukocyte antigen (HLA)-E in humans and Qa-1b in mice, along with the leader sequence of classical MHCI molecules. Expression of NKG2A has been shown to increase on CD8 T cells *in vitro* with IL-15 stimulation [142], as well as during antiviral response in mice [129,143].

Activating NKRs, like NKG2C/D/E, are expressed on CD8 T cells in both humans and mice under various activation conditions. Bystander T_{VM}-like NKG2C+ cells accumulate during cytomegalovirus (CMV) infection [143,144], and activation of NKG2C results in IL-2 secretion, proliferation and cytotoxicity in CD8 T cells that is independent of TCR activation [145,146].

The function of NKG2D on CD8 T cells has been explored more extensively compared to other activating receptors. Multiple studies have reported that NKG2D+ T_{VM}-like cells can mediate bystander cytotoxicity during experimental influenza infections resulting in reduced viral loads [100,147]. Similar cytotoxic programs against tumor cells can be induced by cytokine stimulation [148,149]. Strikingly, in addition to its clear role in regulating innate-like functionality in CD8 T cells, various studies have reported that NKG2D can also augment TCR-dependent functions. In this regard, NKG2D can act as a costimulatory molecule and is able to augment TCR-mediated activation by enhancing cytokine production and proliferation [150–153]. Some studies have prescribed this costimulatory role of NKG2D only to the human memory CD8 T cells that lack CD28 expression [150,154]. In contrast to this costimulatory function, NKG2D has also been reported as a marker of senescence that suppresses TCR function in CD8 T cells but promotes innate-like cytotoxic programs in CD8 T cells [155].

As a deeper exploration of the current literature around T_{VM} cells and their role as a memory-like population, I include the following review article completed during my candidature:

- My review article in Immunology and Cell Biology summarizes most of the current literature on T_{VM} cells and explains the various aspects of their generation, homeostasis, and activation during an immune response.

1.10. ICB Review

Immunology & Cell Biology 2019; **97**: 675–684
www.wileyonlinelibrary.com/journal/icb

SPECIAL FEATURE REVIEW

Similar but different: virtual memory CD8 T cells as a memory-like cell population

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Keywords
CD8 T cell, Eomes, IL-15, memory, virtual memory

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Received 28 January 2019;
Revised 26 March and 26 May 2019;
Accepted 26 May 2019

doi: 10.1111/imcb.12277

Immunology & Cell Biology 2019; **97**: 675–684

Abstract

Immunological memory is a phenomenon where the immune system can respond more rapidly to pathogens and immunological challenges that it has previously encountered. It is defined by several key hallmarks. After an initial encounter, immune cells (1) expand and (2) differentiate to form memory cell populations. Memory cells are (3) long-lived and (4) facilitate more rapid immune responses to subsequent infection because of (i) an increase in cell number, (ii) a decrease in the signaling threshold required for entry into cell cycle or effector function and (iii) localization of cells to tissue sites for surveillance. Classically, immunological memory has been antigen specific but it is becoming apparent that mechanisms of immunological memory can be co-opted by innate or antigen-inexperienced immune cells to generate heterogeneity in immune responses. One such cell is the virtual memory CD8 T (T_{VM}) cell, which is a semi-differentiated but antigen-naïve CD8 T-cell population. This review will summarize current knowledge of how T_{VM} cells are generated, their memory-like hallmarks, how they are maintained during steady state, infection and aging, and propose a model to integrate key signaling pathways during their generation.

INTRODUCTION

Virtual memory CD8 T (T_{VM}) cells comprise a substantial proportion of circulating peripheral CD8 T cells, about 5–10% and 10-20% of the CD8 T cells in the blood and spleen, respectively. In mice, T_{VM} cells are phenotypically defined as $CD8^+$ T cells that are $CD44^{hi}$ and $CD49d^{lo}$ (Table 1; Figure 1a).[1-4] These two markers reflect the semi-differentiated state of T_{VM} cells, as CD44 is rapidly upregulated on T-cell receptor (TCR)- and cytokine-mediated stimulation, while CD49d upregulation requires more sustained, robust TCR stimulation from cognate antigen and it forms part of the very late antigen-4 (VLA-4) integrin complex. There is also evidence that the CD4 T-cell compartment contains antigen-naïve but memory-phenotype cells, with recent discussion as to whether they are directly analogous to CD8 virtual memory T cells,[5-8] but this review will focus on CD8 T cells.

In many previous studies, T_{VM} cells have been included in the broader category of "memory-phenotype" CD8 T (T_{MP}) cells, which are $CD44^{hi}$, sometimes $CD122^{hi}$, CD8 T cells (Table 1),[9,10] but the majority of T_{MP} cells in naïve C57BL/6 mice are T_{VM} cells.[11] Additionally, T_{VM} cells are $CD62L^{hi}$ and this has often led to misclassification of T_{VM} cells as central memory CD8 T (T_{CM}) cells, if CD49d is not included in gating strategies (Table 1).[11] As a result, many of the classical features of "memory cells," T_{MP} or even T_{CM} cells, are canonical T_{VM}-cell phenotypes, such as interleukin (IL)-15 dependence and responsiveness.[12,13]

IL-15- AND TCR-DEPENDENT GENERATION OF T_{VM} CELLS

T_{VM} cells are generated in the periphery from conventional naïve cells that emerge from the thymus and their generation is absolutely dependent on IL-15

Table 1. Comparison of markers across T_{MP}-cell types.

	Antigen naïve	Antigen experienced						
		Memory phenotype CD8 T (T_{MP}) cells						
Cell types	True naïve (T_N) mouse	Virtual memory (T_{VM}) mouse	Innate memory (T_{IM}) mouse	Lymphopenia-induced proliferation (T_{LIP}) mouse	Virtual memory (T_{VM}) human	Central memory (T_{CM}) mouse	Effector memory (T_{EM}) mouse	Effector memory Re-expressing CD45RA (T_{EMRA}) human
Key markers	$CD3^+$, $CD8^+$, $CD44^{lo}$, $CD49d^{lo}$	$CD3^+$, $CD8^+$, $CD44^{hi}$, $CD49d^{lo}$	$CD3^+$, $CD8^+$, $CD44^{hi}$, $CD49d^{lo}$	$CD3^+$, $CD8^+$, $CD44^{hi}$, $CD49d^{lo}$	$CD3^+$, $CD8^+$, $CD45RA^+$, Pan-KIRhi and/or $NKG2A^{hi}$	$CD3^+$, $CD8^+$, $CD44^{hi}$, $CD49d^{hi}$	$CD3^+$, $CD8^+$, $CD44^{hi}$, $CD49d^{hi}$	$CD3^+$, $CD8^+$, $CD45RA^+$, $CD27^-$ or $CCR7^-$
Additional markers	$CD62L^{hi}$, $CCR7^{hi}$, $Eomes^{lo}$, $CD122^{lo}$, $CD127^{hi}$, $CXCR3^{lo}$, $CX3CR1^{lo}$	$CD62L^{hi}$, $CCR7^{lo}$, $Eomes^{hi}$, $CD122^{hi}$, $CD127^{hi}$, $CXCR3^{hi}$, $CX3CR1^{lo}$	$CD62L^{hi}$, $CD122^{hi}$, $Eomes^{hi}$, $CXCR3^{hi}$	$CD62L^{hi}$, $CD122^{hi}$	$CCR7^{lo}$, $Eomes^{hi}$, $CD27^{lo}$	$CD62L^{hi}$, $CCR7^{hi}$, $Eomes^{hi}$, $CD122^{int}$, $CD127^{hi}$, $CXCR3^{hi}$, $CX3CR1^{lo}$	$CD62L^{lo}$, $CCR7^{lo}$, $Eomes^{lo}$, $CD122^{lo}$, $CD127^{lo}$, $CXCR3^{hi}$, $CX3CR1^{hi}$	

signaling. This IL-15 is transpresented on IL-15 receptor α chain (IL-15Rα) expressed by $CD8\alpha^+$ dendritic cells and developing T_{VM} cells must express the IL-15Rβ (also designated CD122 or IL-2Rβ) and eomesodermin (Eomes), a T-box transcription factor that promotes expression of IL-15Rβ.[12,14,15] Given the pivotal role of IL-15 in T_{VM}-cell generation, high expression of IL-15Rβ and Eomes can be used as additional phenotypic markers of T_{VM} cells.[12] While nonredundant roles in T_{VM}-cell generation are not observed for other common-gamma chain (γc) cytokines, IL-4 signaling can expand T_{VM}-cell populations[1,12,16,17] and T_{VM} cells express and maintain high levels of the IL-7α chain (CD127),[13] which is consistent with a role in their survival.

It is thought that naïve CD8 T cells with modestly higher basal TCR signaling have a higher propensity to develop into T_{VM} cells. In support of this, recent work has demonstrated that T_{VM} cells are preferentially derived from naïve CD8 T cells with lymphocyte-specific protein tyrosine kinase (Lck) precoupled on their CD8 co-receptor to potentiate TCR signaling[18] and from naïve CD8 T cells that express markers of heightened TCR-mediated self-reactivity, such as CD5.[13,19-21] Although T_{VM} cells may possess a TCR repertoire with increased self-reactivity, it was recently shown that they do not mediate autoimmune pathology.[18] In addition, while self-reactivity promotes T_{VM} generation, no exogenous antigen is needed for their generation; T_{VM} cells have been reported in antigen-specific but naïve populations after tetramer-based enrichment in naïve mice and in germ-free mice.[1,3,13,19,21]

Given the combined impact of cytokine and TCR signaling, we can position T_{VM} cells and other T_{MP} cells on a continuum defined by their experience with cytokine and TCR signaling (Figure 1). Using this approach, we infer that T_{VM} cells experience a combined self-reactive TCR/cytokine signal that mediates differentiation into T_{VM} cells, with this threshold being higher than tonic signaling with non-self-reactive TCRs to mediate naïve T-cell survival but not as high as *bona fide* activation with TCRs bound to their cognate antigen.

MEMORY-LIKE AND INNATE-LIKE FUNCTIONS IN T_{VM} CELLS

Consistent with their memory-like phenotype, T_{VM} cells exhibit memory-like functionality. If T_{VM} cells are stimulated through their TCR, they respond more rapidly than true naïve CD8 T (T_N) cells; they proliferate, engage cytokine production and elicit cytotoxicity earlier than T_N cells and often with similar kinetics and efficiencies to conventional antigen-experienced memory CD8 T (T_{MEM}) cells.[2,13,20] Their rapid kinetics reflect the fact

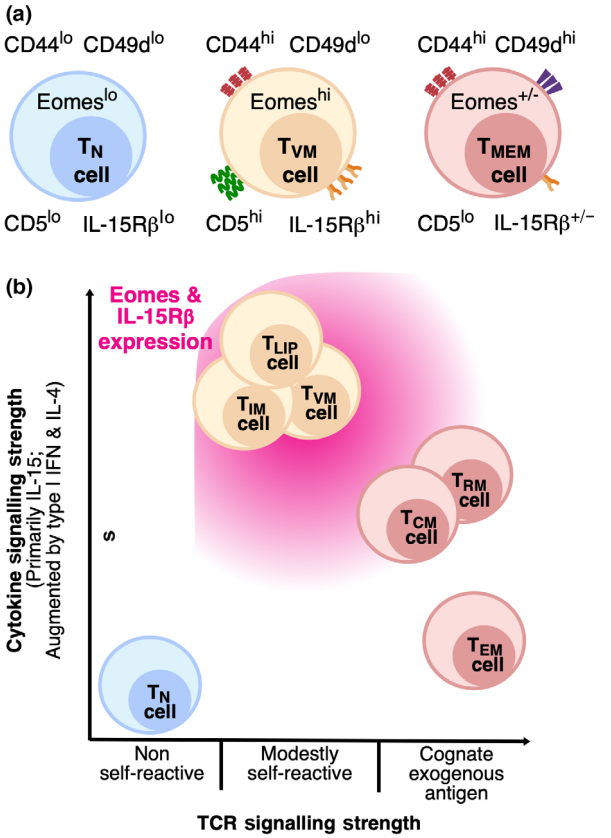

Figure 1. Defining T_{VM} cells and the proposed impact of cytokine and T-cell receptor (TCR) signaling strength. **(a)** T_{VM} cells in the periphery exhibit partial differentiation, with acquisition of traditional memory markers, such as CD44 but not CD49d, to distinguish them from T_N and T_{MEM} cells. T_{VM} cells also retain high expression of Eomes and IL-15Rβ, to reflect their IL-15 responsiveness, and are $CD5^{hi}$, to reflect their modestly self-reactive TCRs. **(b)** In this model, the combined effects of cytokine and TCR signaling strength lead to three main outcomes. (1) If cytokine signaling is low and CD8 T cells have non-self-reactive TCRs, they remain as T_N cells. (2) If cytokine signaling is high and CD8 T cells have modestly self-reactive TCRs, Eomes and IL-15Rβ expression are upregulated. Increased IL-15Rβ would enable CD8 T cells to become receptive to IL-15 and permit their cytokine-driven differentiation. While cytokines such as IL-15, type I IFN and IL-4 all augment Eomes expression to potentiate IL-15 signaling and differentiation, each cytokine may engage additional pathways to result in subtly distinct phenotypic profiles of T_{VM} (IL-15, type I IFN), T_{IM} (IL-4) and T_{LIP} (IL-7) cells. (3) If CD8 T cells encounter their cognate antigen along with a degree of cytokine signaling, they can differentiate into a variety of conventional memory subsets. [The color version of this figure can be viewed at www.wileyonlinelibrary.com/journal/icb]

that, similar to antigen-experienced T_{MEM} cells, T_{VM} cells exhibit a "poised" state for entry into cell cycle[2] and poised epigenetic profiles at the transcriptional start sites for many effector genes.[22]

T_{VM} cells are, however, more limited than T_N cells with regard to the diversity of cytokines that they produce. Following TCR stimulation, effector CD8 T (T_{EFF}) cells derived from T_{VM} cells are predominantly monofunctional, expressing interferon (IFN)γ alone with limited tumor necrosis factor and virtually no IL-2 production, while T_N-derived T_{EFF} cells are more multifunctional.[13,23] T_{VM}-derived T_{EFF}-cell populations also tend to exhibit a short-lived effector cell (SLECs; $KLRG1^+CD127^-$) profile[2,22,24] and contract significantly upon resolution of the infection.[2] In contrast, T_N-derived T_{EFF} cells tend to be memory precursor effector cells (MPECs; $KLRG1^-CD127^+$), which are more likely to develop into stable T_{MEM} populations.[2,22,24] The monofunctional, SLEC-like profile that develops in T_{VM}-derived T_{EFF} cells likely reflects the fact that T_{VM} cells are semi-differentiated prior to stimulation.

T_{VM} cells also exhibit heightened responsiveness to cytokines, particularly to IL-12, IL-18 and γc cytokines. As examples, IL-15 can drive proliferation *in vitro* in T_{VM} and T_{MEM} cells but not in T_N cells,[13] and IL-12 and IL-18 can elicit cytokine production and even bystander killing from T_{VM} cells in an antigen-independent manner.[1,2,21] This bystander killing is likely mediated by the ability of T_{VM} cells to produce IFNγ, granzyme B, and the expression of NKG2D by a subpopulation of T_{VM} cells.[21] In any case, the cytokine responsiveness of T_{VM} cells along with the expression of many markers traditionally associated with natural killer (NK) cells, such as NKG2D, strengthens the characterization of T_{VM} cells as an adaptive cell with innate-like capacity.

T_{VM} CELL LOCATION DICTATES FUNCTION

In steady state, T_{VM} cells localize to specific tissues and express a unique signature of chemokine receptors and adhesion molecules that collectively define their tissue access. T_{VM} cells express some markers associated with antigen naivety, including CD62L, which would permit circulation of T_{VM} cells to the lymph node, but they downregulate transcription of C-C chemokine receptor type (CCR)7,[21] which may limit residency and retention in the lymph node. While a subset of conventional T_{MEM} cells express C-X-3C chemokine receptor (CX3CR)1, T_{VM} cells do not express this receptor,[18] which may limit the ability of T_{VM} cells to respond to fractalkine and survey peripheral tissues. However, both T_{VM} cells and T_{MEM} cells express a high level of C-X-C chemokine receptor (CXCR)3 in the steady state,[21] which may enable circulating T_{VM} cells to migrate toward type I IFN-induced C-X-C chemokine ligand (CXCL)9/10, facilitating rapid access to sites of inflammation. While T_{VM} cells are defined by a lack of CD49d (*Itga4*) expression, they also express lower levels of *Itgb7* and

CD103 (*Itgae*).[21] CD49d and Itgb7 would normally pair together to form α4β7, while CD103 and Itgb7 form αEβ7, both of which can meditate gut-specific homing. T_{VM} cells also lack expression of CCR9, which senses C-C chemokine ligand (CCL)25 expressed by the epithelia of the small intestine to augment integrin-mediated migration. Consistent with collective loss of these molecules, T_{VM} cells lack access to the small intestine in naïve mice.[21]

While T_{VM} cells lack access to the gut, they are enriched in the liver in steady state in both mice and humans.[21] T_{VM} cells preferentially migrate to the liver across the lifespan[13,21] and localization to this site is thought to promote access to IL-15, as the liver is a rich source of this cytokine.[25] NK, Natural Killer T and γδ cells are also enriched in the liver,[26] so this reflects an additional innate-like quality of T_{VM} cells. Factors that control T_{VM} cell location are yet to be defined, but CD44 expression promotes localization of T_{MP} cells to the liver.[27] Importantly, the positioning of T_{VM} cells in the liver but not the gut during steady state may position these cells for optimal innate-like activity. It would avoid inappropriate T_{VM} activation in the inflammatory environment of the gut but facilitate rapid activation of T_{VM} cells if gut-derived pathogens gained access to the blood.

After activation, T_{VM}-derived T_{EFF} cells have a distinct migration pattern. During influenza infection, T_{VM}-derived T_{EFF} cells have been found in two studies to traffic less efficiently into the lung.[28,29] Similarly, after listerial infection, T_{VM}-derived T_{MEM} cells are found in secondary lymphoid tissues at the same frequency but in peripheral tissues at lower frequencies compared to T_N-derived T_{MEM} cells.[2] T_{VM}-derived T_{MEM} cells also tended to exhibit a T_{CM}-like profile, with high CD62L expression,[2] which may account for their preferential access to lymphoid rather than peripheral tissues.

A MEMORY-LIKE TRANSCRIPTIONAL PROFILE

Consistent with their unique phenotypic and functional profile, T_{VM} cells are transcriptionally distinct from T_N and T_{MEM} cells, while exhibiting significant overlap with both subsets.[13,18] A number of studies have assessed the transcriptional profile and chromatin accessibility for T_{VM} cells at different stages during the lifespan[13,18,21,22] and key signatures for T_{VM} cells as compared to the T_N cells are now well-defined (Figure 2). These include high expression of transcripts related to NK cells (*Klra-i* genes, *S1pr5*), granzymes and cell killing (*Grza*, *Grzb*, *Grzc*, *Grzk*, *Grzm*, *Prf1*, *Klrk1*, *Fasl*), inflammatory cytokines (*Ifng*, *Tnf, Ccl3, Ccl4, Ccl5*) and cytokine sensing (*Il2rb*, *Il4ra*, *Il18r1, Il18rap, Il12rb1, Il12rb2*), as well as differential expression of chemokine and cell adhesion markers, as described previously. A number of transcription factors are also differentially, if not exclusively, expressed in T_{VM} cells, with high expression of *Eomes*, *Runx2* and *Bhlhe40* and decreased expression of

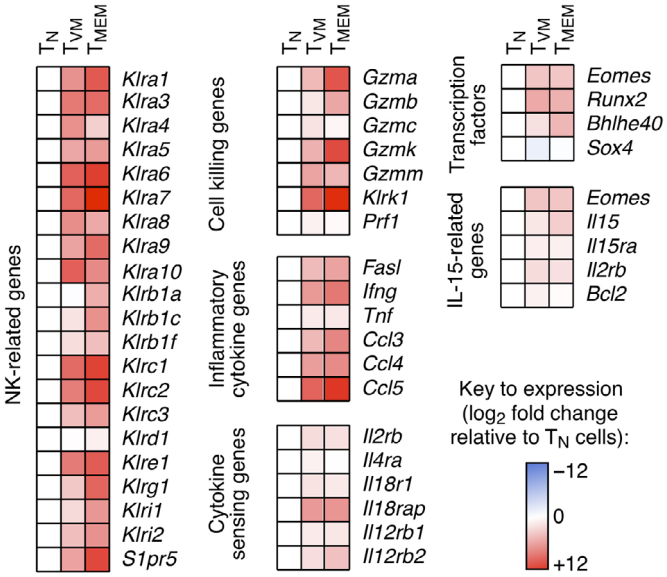

Figure 2. Transcriptional signature of T_{VM} cells. Heat map for $\log_2$ fold change in expression as compared to T_N cells for T_{VM} and T_{MEM} cells for selected genes, including NK-related, cell killing, inflammatory cytokine, cytokine sensing, transcription factors and IL-15-related genes. Data reproduced from GEO: GSE112304 using the Morpheus online tool (Broad Institute). [The color version of this figure can be viewed at www.wileyonlinelibrary.com/journal/icb]

Sox4,[13,14,21,22] which have been shown to comprise a key transcription factor hub for memory cell differentiation.[30] Finally, T_{VM} cells exhibit a clear transcriptional signal for IL-15 signaling mediators and regulators, with increased expression of *Eomes*, *Il15, Il2rb*, *Il15ra* and *Bcl2*.[13,21,22]

REFINING THE MODEL OF T_{VM}-CELL GENERATION

The T_{VM}-cell population first emerges in the neonatal mouse, initially comprising up to 60% of peripheral CD8 T cells before the T_{VM}-cell pool stabilizes at about 10–20% in young adult mice.[1] The neonate represents an intensely lymphopenic environment, which has been demonstrated to drive lymphopenia-induced proliferation and acquisition of memory markers by mature CD4 and CD8 T cells after adoptive transfer through γc cytokine signaling.[31] As a result, the current model of T_{VM}-cell generation is that IL-15 signaling in the neonatal periphery drives lymphopenia-induced proliferation and semi-differentiation in modestly self-reactive naïve CD8 T cells, to elicit a cytokine-driven, partial memory phenotype.[1,3,21] It has been proposed that T_{VM} cells are essentially equivalent to lymphopenia-induced proliferation CD8 T (T_{LIP}) cells, which are semi-differentiated CD8 T cells generated after transfer of naïve CD8 T cells into lymphopenic mice (Table 1),[32] although this is yet to be experimentally demonstrated.

T_{VM} cells confer a significant immunological advantage, particularly in the young. Neonates must first survive a period of lymphopenia with few adaptive immune cells and then survive without the protection of established antigen-specific memory populations. T_{VM} cells bridge the gap between innate and adaptive immunity, providing both innate-like and memory-like functionality during this period of immunological vulnerability. Indeed, T_{VM} cells are crucial for generation of rapid protective immunity *in vivo* in a number of bacterial and viral models.[2,16,17,29] T_{VM} cells are therefore proposed to provide an additional layer of heterogeneity within naïve CD8 T-cell populations, ensuring that antigen-naïve CD8 T cells are intrinsically diverse with regard to both (1) antigen specificity based on TCR expression and (2) preprogrammed response kinetics and functionality based on their IL-15-driven differentiation state.[22]

However, recent work highlights the need to revise the current model. Using a timestamping approach to permanently label CD8 T cells transiting through the thymus, Smith *et al.* reinforced the observation that cells emerging from the thymus early in life have a higher propensity to develop into T_{VM} cells than cells that emerge in adulthood.[22] T_{VM} cells generated early in life also exhibited an exaggerated T_{VM}-cell transcriptional profile and increased chromatin accessibility at a number of key functional genes,[22] suggesting that T_{VM} cells generated in early life are poised for more rapid effector function compared to those generated in adulthood. This highlights that there may be layers within the T_{VM}-cell compartment, with phenotypically and functionally distinct waves of neonatally generated and adult-generated T_{VM} cells.

Most surprisingly, the study by Smith *et al.* demonstrated that the neonatal thymus, rather than the peripheral lymphopenic environment, was responsible for programming the preferential development of T cells into T_{VM} cells. The authors transplanted a timestamped neonatal thymus into a timestamped adult mouse and T cells derived from the neonatal thymus contained a comparable frequency of T_{VM} cells to an intact neonate, despite having emerged into a lymphoreplete environment.[22] Additionally, after intrathymic transfer into adults, double-negative (DN) thymocytes sourced from mouse fetuses preferentially develop into T_{MP}, likely T_{VM}, cells as compared to DN thymocytes from adult mice.[33] The expression of an RNA-binding protein called Lin28b, which is known to control pluripotency, appears to contribute to preferential T_{VM}-cell development. These pivotal experiments clearly illustrate that a form of programming occurs during the development to dictate the fate of T cells in the periphery. However, it remains to be defined whether that programming is derived from a thymus-derived signal, such as IL-15 or another soluble or cell-bound mediator, or the intrinsic state of progenitor thymocytes, based on the expression of markers, such as Lin28b.

INTEGRATION OF IL-15, IFN, IL-4 AND TCR SIGNALING PATHWAYS IN T_{VM} CELLS

IL-15 and TCR signaling in the CD8 T cells themselves are clearly critical for T_{VM}-cell generation but the integration of these pathways with each other is not well understood and the potential contribution of additional signaling pathways is only beginning to be explored.

When IL-15 engages its receptor, composed of γc and IL-15Rβ, it triggers the activation of distinct pathways dependent on the strength of signal. Low-dose, steady-state IL-15 activates Janus kinase (JAK)–signal transducer and activator of transcription (STAT) 3 and 5 signaling,[34] and the STAT5 pathway is known to mediate increased Bcl-2 expression and facilitate cell survival. Higher dose IL-15 also triggers mitogen-activated protein kinase and Akt/S6 pathways that can drive proliferation.[34] This dose-dependent profile would account for the survival of T_{VM} cells in steady state and substantial expansion of T_{VM}

cells that occurs with administration of exogenous IL-15.[12,21] Signaling can also trigger a feed-forward loop, where IL-15 exposure leads to further upregulation of IL-15Rβ on CD8 T cells, making cells progressively more sensitive to IL-15.[35] IL-15 signaling also has potent mechanisms for negative regulation. The STAT5 pathway is negatively regulated by the suppressor of cytokine signaling proteins, suppressor of cytokine signaling 1 and CIS, in CD8 T cells and NK cells, respectively.[36-38] Antigen-naïve CD8 T cells lacking suppressor of cytokine signaling 1 exhibit protracted STAT5 phosphorylation and higher frequencies of T_{MP} cells,[36,37] which are presumably T_{VM}, cells. This illustrates that negative regulation of IL-15 signaling can limit T_{VM}-cell generation.

Type I IFN signaling, in contrast, augments T_{VM}-cell generation and can positively regulate IL-15 signaling through two mechanisms. First, type I IFNs induced by infection or an inflammatory signal can act directly on antigen-presenting cells to increase IL-15 production,[39] which would increase the amount of IL-15 available. Second, type I IFN signaling to the CD8 T cell itself increases sensitivity to IL-15, by increasing expression of Eomes via a IFN-stimulated gene factor 3 (ISGF3; complexed STAT1/3/IFN regulatory factor (IRF)9)-dependent pathway, which in turn increases expression of IL-15Rβ in T_{VM} cells.[40] As a result, mice that lack expression of the IFN-α receptor 1 subunit (IFNAR1) or IRF9 have significantly lower frequencies of T_{VM} cells, and this difference is exacerbated with age.[40]

IL-4 signaling also augments T_{VM}-cell generation and may positively regulate IL-15 signaling. Ablation of IL-4 or its receptor leads to a modest decrease in the frequency of T_{VM} cells,[1,12] while IL-4 production during helminth infection increases the frequency of T_{VM} cells.[16,17] In addition, the development of a population of T_{MP} cells, known as innate memory CD8 T (T_{IM}) cells is dependent on IL-4 but not IL-15 (Table 1) (reviewed in[10,41]). T_{IM} cells adopt a $CD44^{hi}$ memory phenotype in the thymus rather than in the periphery but share many phenotypic and functional characteristics with T_{VM} cells. T_{IM} cells are observed in Balb/c mice and in some genetically deficient mouse strains with heightened IL-4 signaling, including IL-2-inducible T-cell kinase, inhibitor of DNA-binding 3 (ID3), Krüppel-like factor 2 (KLF2) and CREB-binding protein knockouts. This highlights the importance of mouse strain selection when studying specific T_{MP}-cell subsets. Finally, IL-4 can also promote the expression of Eomes through a STAT6-dependent pathway[42]; so, type I IFN and IL-4 may induce similar upregulation of Eomes and IL-15Rβ to augment IL-15 signaling in CD8 T cells and promote differentiation. This suggests that, while T_{IM} and T_{VM} cells are generated by exposure to distinct cytokines, they may share developmental mechanisms.

While IL-15, type I IFN and IL-4 signaling pathways all contribute to T_{VM}-cell generation, these pathways must somehow integrate with TCR signaling. A recently defined transcription factor may provide the point of integration. *Runx2* is highly upregulated in T_{VM} cells, particularly in neonatally derived T_{VM} cells,[22] it is expressed downstream of type I IFN, IL-12 and IL-15 signaling and its expression promotes Eomes expression in CD8 T cells.[43] Importantly, Runx2 is augmented when cytokine signals are combined with TCR signals, but this requires modest as opposed to strong TCR signaling.[43] Modestly self-reactive TCRs could therefore deliver modest TCR signals alongside cytokine stimulation in the thymus to promote Runx2-mediated Eomes expression. This would lead to selective IL-15Rβ expression in precursor T_{VM} cells, making them exquisitely sensitive to the IL-15 signaling that then drives their semi-differentiation in the periphery. Accordingly, we propose this as a model of integrated IL-15, IFN, IL-4 and TCR signaling for optimal T_{VM}-cell generation (Figure 3). Of note, additional pathways, such as NFκB and inflammasome signaling, appear to impact on generation of T_{MP} cells in antigen-naïve mice.[44,45] It remains to be seen whether these pathways are impacting on T_{VM} cells specifically and, if so, how they might integrate into our proposed model.

MAINTENANCE OF T_{VM} CELLS AND THE IMPACT OF INFECTION AND AGING

Factors that control the steady-state homeostasis of T_{VM} cells in the periphery have not been well-defined, particularly whether IL-7, IL-15 or major histocompatibility complex (MHC)I are required for survival. T_{VM} cells exhibit higher rates of homeostatic proliferation than T_N cells,[1] so it is reasonable to predict that T_{VM} cells are sensitive to homeostatic cues. Dependence on MHCI or IL-15 for maintenance has not been formally assessed for T_{VM} cells specifically, but T_{MP} cells exhibit MHCI-independent but IL-15-dependent survival.[9] Given that the majority of T_{MP} cells are T_{VM} cells,[11] we predict that T_{VM} are MHCI independent and IL-15 dependent for their homeostasis.

While steady-state homeostatic cues remain to be formally defined, it is clear that increased IL-15 and type I IFN signaling can increase T_{VM}-cell population size. The administration of exogenous IL-15 causes a dramatic increase in T_{VM}-cell numbers.[12,21] Similarly, the administration of poly I:C, a toll-like receptor agonist that induces large amounts of type I IFN and IL-15, results in an IFN-dependent increase in T_{MP} cells,[46] driven by an increase in T_{VM} but not T_{MEM} cells.[40] These increases are driven by proliferation of existing T_{VM} cells,[40] but it remains to be tested whether IL-15/IFN-driven *de novo*

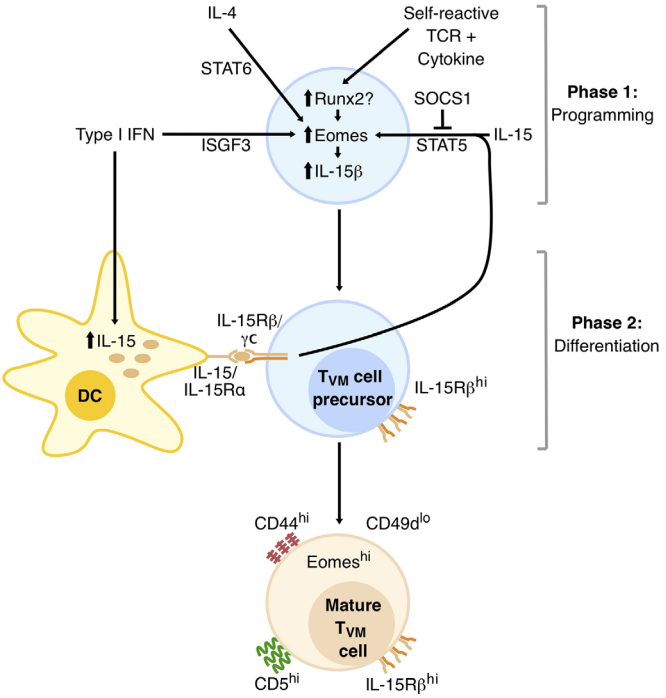

Figure 3. Two-stage model for T_{VM}-cell generation. We propose that T_{VM}-cell generation involves two phases, one of which may occur optimally in the neonatal thymus while the other may occur predominantly in the periphery. In phase 1, developing CD8 T cells experience a cytokine signal that drives increased Eomes expression. This could occur directly with type I IFN signaling via an ISGF3-dependent pathway, IL-4 signaling via a STAT6-dependent pathway or IL-15 via a STAT5-dependent pathway or indirectly with modest T-cell receptor (TCR) signals synergizing with cytokine signaling to upregulate Runx2, which would then upregulate Eomes. Increased Eomes expression would upregulate IL-15Rβ expression, resulting in an IL-15Rβ^{hi} T_{VM}-cell precursor, with heightened sensitivity to IL-15. In phase 2, DCs transpresent IL-15 on IL-15Rα to the IL-15Rβ^{hi} T_{VM}-cell precursor. Type I IFN could also act on DCs to increase IL-15 production. The IL-15 signaling to the T_{VM}-cell precursor not only would be negatively regulated by SOCS1 but would also (i) drive a feed-forward loop to further increase IL-15Rβ expression and (ii) drive partial differentiation with acquisition of markers that reflect their ontogeny: expression of CD44 but not CD49d (cytokine-driven, not TCR-driven, differentiation), high expression of Eomes and IL-15Rβ (cytokine stimulation) and high expression of CD5 (modest TCR stimulation). [The color version of this figure can be viewed at www.wileyonlinelibrary.com/journal/icb]

generation of T_{VM} cells also contributes to increased cell number. Contraction of expanded T_{VM} cells is also not well-defined and it is not known whether expanded T_{VM} cells are functionally equivalent to steady state T_{VM} cells. Given that IL-15 and type I IFN signaling impacts on T_{VM}-cell population size, conditions that induce such signaling may modulate homeostasis of T_{VM} cells in the periphery; two such conditions include infection and aging.

With regard to infection, a history of multiple infections certainly shifts the composition and tissue distribution in the CD8 T-cell compartment.[47] It can even shift the basal production of type I IFNs, as mice that were administered a series of infections to model "normal" exposures exhibited significant enrichment of the type I IFN metagene compared to naïve mice.[48] While the cumulative impact of multiple infections on T_{VM}-cell homeostasis has not been defined, it is well established that viral infections can cause expansion of T_{MP} and presumably T_{VM} cells.[46] In addition, a single infection with helminths was sufficient to increase T_{VM}-cell frequency in the periphery, in an IL-4 dependent manner, leading to more effective clearance of subsequent murid gammaherpesvirus 4 infection[16] or listerial infection.[17] This illustrates that pathogenic exposures have a significant acute impact on T_{VM}-cell homeostasis and function but the long-term impact of such exposures remains to be characterized.

With regard to aging, the relative proportion of T_{VM} cells within the CD8 T-cell compartment increases substantially with increasing age, comprising up to 40% of the peripheral CD8 T-cell pool in mice and 25% in humans.[11,13,19-21,49] While T_{VM} cells are highly functional in the young, they lose function with increasing age, exhibiting impaired TCR-mediated proliferation and increased apoptosis in response to peptide stimulation.[13,20] At the same time, they adopt a senescent rather than exhausted phenotype, with increased levels of γ-H2Ax and hyperphosphorylation of components in mitogen-activated protein kinase pathways, such as p38, extracellular signal-regulated kinase 1/2 and Jun N-terminal kinase.[13,50] Despite these TCR-associated functional defects, T_{VM} cells

proliferate robustly in response to IL-15 even at advanced ages,[13] which reflects that IL-15Rβ expression and IL-15 sensitivity is not only maintained but increased with age in T_{VM} cells, resulting in increased Bcl-2 expression and a significant survival advantage.[13,20] This preferential survival of aged T_{VM} cells likely contributes to the increased frequency of T_{VM} cells in advanced age.

The question remains, what drives the relative accumulation of T_{VM} cells with age? Over the normal course of aging, individuals will experience a series of infections, resulting in repeated exposure of the T_{VM}-cell population to IL-15, type I IFN and IL-4 signals. Older individuals also develop persistent low-grade inflammation because of the increase in senescent cells, with increased expression of IFN-related genes and IL-15 transcription.[51-54] This state of heightened inflammatory signaling is known as "inflammaging" and it is correlated with poorer CD8 T-cell responses to diseases and vaccination.[55,56] In either scenario, cumulative infection-induced or age-related inflammatory signals could mediate preferential retention of T_{VM} cells, but this sustained or repeated cytokine signaling to aged T_{VM} cells may simultaneously undermine their TCR-driven proliferative capacity. As a result, aged T_{VM} cells survive relatively well in the aged environment but are not as effective for generating primary antigen-specific immunity.[13,20]

A PUTATIVE T_{VM} CELL IN HUMANS

It should be noted that a putative T_{VM} cell has been identified in humans.[57] These cells are naïve-like, in that they express CD45RA, but they also express Eomes and NK cell receptors, including NKG2A and/or KIR3DL1 and KIR2D, and they lack expression of CCR7 and CD27 (Table 1).[21,57] These cells first emerge in neonatal humans, they are enriched in the liver and they increase in proportion substantially with age.[13,21,57] In terms of functionality, they are cytokine responsive (IL-12 and IL-18), exert rapid cytotoxicity[57] and are highly proliferative in young adults but lose proliferative capacity with age.[13] Given that putative T_{VM} cells are $CD45RA^+$ $CD27^-$ and $CCR7^-$, they may have been included in classical gating strategies for effector memory CD8 T cells that re-express CD45RA (T_{EMRA}).[58] More specific markers for human T_{VM} cells would therefore aid in differentiating these cells from antigen-experienced T_{MEM}-cell subsets. Crucial steps forward in this regard would be to comprehensively dissect memory marker expression in *bona fide* naïve CD8 T-cell populations using MHCI tetramer-based naïve enrichment[59] and to perform single-cell transcriptional profiling to identify a core signature for mouse and human T_{VM} cells across the lifespan.

SUMMARY

Antigen-naïve, semi-differentiated T_{VM} cells represent a unique subset of CD8 T cells with many memory-like characteristics. Their experience of cytokine signaling clearly leads to a poised state with regard to cell cycle, differentiation, effector function, transcriptional profile and epigenetic landscape, and many of these features overlap with conventional T_{MEM} subsets. This suggests that they employ many of the same molecular mechanisms as conventional T_{MEM} cells in their generation and function. In addition, they exhibit many hallmarks of memory: modest expansion, marked differentiation, a steady-state survival advantage, accelerated TCR-driven responses, decreased signaling thresholds and localization of T_{VM} cells to specific tissue sites. T_{VM} cells may in fact represent a valuable resource to dissect transcriptional profiles driven by cytokine as opposed to those driven by TCR and those that are driven synergistically by cytokine and TCR. This knowledge will refine our definition of what mediates and constitutes the phenomenon of immunological memory.

ACKNOWLEDGMENTS

We acknowledge support and funding for this work from the Rebecca L Cooper Foundation (KMQ) and a Monash University International Research Scholarship and Monash University Graduate Stipend (TH). We apologize to colleagues whose work we could not cite because of space limitations.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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1.11. T_{VM} cells are a distinct lineage of CD8 T cells

Studies in the past 2-3 years have advanced our understanding of T_{VM} cell generation. Early studies indicated that T_{VM} cells had features that suggested self-reactivity, such as high CD5 expression indicative of strong TCR signaling in the thymus [97,156]. Later, Stepanek and colleagues demonstrated that T_{VM} cell differentiation was linked to TCR usage. T_{VM} cells were shown to have a TCR repertoire distinct from T_N cells, where high affinity TCR/pMHCI interactions skewed towards T_{VM} cell fate in the periphery [157]. A later study by Savage and colleagues described T_{VM} cell generation as a distinct fate programmed during thymic development [101]. This study echoed the findings of Stepanek and colleagues and described the TCR repertoire of T_{VM} cells as not only distinct from T_N cells but also highly recurrent when analyzed across different mice [101,157]. T cells bearing these TCR sequences reproducibly differentiated into T_{VM} cells in the periphery, further indicating that the TCR affinity dictates the decision to develop into a T_{VM} cell [101]. As previously described (Section 1.10), IL-15 is a key cytokine for maintaining T_{VM} cells, as T_{VM} cells are completely lost from C57BL/6 mice lacking IL-15 or CD122 [34,35,97]. While T_{VM} cell precursors in the thymus develop independently of IL-15, their future sensitivity to the cytokine was programmed during thymic development. When TCR retrogenic mice were generated that expressed TCRα sequences repeatedly observed either in the T_{VM} or T_N cells, T cell precursors with T_{VM} TCRs expressed higher levels of the transcription factor, Eomes, in the later stages of maturation in the thymus [101]. These T_{VM} cell precursors were found to have an increased proportion of Ki67 expressing cells and higher CD5 expression compared to thymocytes bearing T_N cell TCRs [101]. Eomes directly upregulates

expression of CD122 [158], therefore after thymic egress T_{VM} cell precursors complete their differentiation to a $CD44^{hi}CD122^{hi}$ T_{VM} cell lineage in the periphery upon exposure to IL-15 on CD8α+ DCs [35,101]. Thus, rather than being a differentiation state of CD8 T cells that is adopted upon exposure to peripheral cytokines or a lymphopenic environment, these recent studies demonstrate that T_{VM} cells are a distinct lineage of CD8 T cells whose development is initiated in the thymus via high affinity recognition of pMHCI and completed in the periphery through exposure to homeostatic cytokines, in particular IL-15.

1.12. T_{VM} Cell Peripheral Establishment and Maintenance

In the periphery, T_{VM} cells are absolutely dependent on IL-15 [34,35,97]. While the impact of tonic TCR/MHCI signaling on T_{VM} cell survival has not been directly assessed, $CD44^{hi}CD122^{hi}$ T_{MP} cells have been shown to survive without MHCI [37], implying that unlike T_N cells [159], T_{VM} cells may not require pMHCI interactions for homeostatic maintenance.

Previous studies had suggested that T_{VM} cells were highly dependent on CD8α+ DCs for homeostatic IL-15 exposure. T_{VM} cell numbers were reduced by more than half in *Batf3-/-* mice [35], which have a selective loss of this DC subset [160–162]. This suggested that CD8α+ DCs were dominant in the provision of IL-15 to T_{VM} cells to permit their survival but other DC subsets can also produce IL-15, albeit at a lower level [35,163]. Of note, CD11b+ DCs can induce expansion of T_{MP} cells *in vitro* [164], which was suggestive of T_{VM} cell expansion. However, it remained unclear until recently as to whether other DC subsets could provide IL-15 to T_{VM} cells.

Recent work demonstrated that IL-15 levels in the periphery are tightly regulated during normal homeostasis, to limit the access of T_{VM} cells to IL-15. An elegant study by Lund and

colleagues showed that the size of the T_{VM} cell population is indirectly controlled by CD25+Foxp3+ CD4 T regulatory (T_{REG}) cells [165]. In this work, β-1 integrin+ (CD29) T_{REG} cells were found to be particularly efficient at suppressing IL-15 signaling from CD11b+ DCs, thereby limiting the amount of IL-15 available to T_{VM} cells [165]. This was evidenced by the dramatic expansion of T_{VM} cells within 4-6 days of T_{REG} depletion, due to increased transpresentation of IL-15 by CD11b+ DCs. This expanded T_{VM} cell population was maintained for at least 4 weeks after T_{REG} depletion [165]. The findings raise the possibility that conditions such as infections where T_{REG} cells are reduced or CD11b+ DCs are increased, T_{VM} cells may have the opportunity to access more IL-15 and can therefore proliferate. Infections or other immune perturbations could therefore impact on the long-term maintenance of the T_{VM} cell population.

1.13. T_{VM} Cells in Infection and Cancer

As described in previously in Section 1.10, T_{VM} cells mediate protection against infections such as *Listeria monocytogenes* and influenza A virus (IAV) in mouse models using both TCR-dependent and bystander pathways [85,166–168]. Similarly, T_{VM} cells could mediate *in vitro* control of viral replication through NKR-mediated killing [169]. In line with their cytokine-sensitive nature, T_{VM} cells were observed to be increased during CMV infection in macaques [143] and were more frequent in people with active human immunodeficiency virus (HIV) and those on anti-retroviral therapy (ART) as compared to healthy individuals [169], correlating with increased levels of IL-15. Studies of helminth infection in mice have shown that T_{VM} cells increase in an IL-4 dependent manner by more than twofold after infection, and this increase was maintained for nearly 8 weeks after infection [84,167].

In addition to the proliferation observed during these infections, several studies have also shown that increased T_{VM} cells were associated with better outcomes during subsequent exposure to another pathogen. Helminth-induced T_{VM} cells were shown to mediate better control of a subsequent infection with murine gammaherpesvirus (MuHV) [84] and *Listeria monocytogenes* infection [167]. Additionally, T_{VM}-like cells that increased in response to chronic CMV infection in macaques could inhibit Simian immunodeficiency virus (SIV) replication *ex vivo* [143].

T_{VM} cells may also be key mediators and sources of local immune control in tissues. Hou et al found that a subset of CCR2+ T_{VM} cells expressing higher levels of CXCR3 could mediate rapid infiltration into the lungs and were associated with improved viral clearance. The same study also showed that T_{VM} cells had increased potential to seed T_{RM} cell populations in lungs compared to T_N cells during IAV infection [166].

More recently, studies have demonstrated anti-tumour activity of T_{VM} cells. It was shown that T_{VM} cells had superior capacity to infiltrate solid tumors, like prostate adenocarcinoma, compared to T_N cells in mouse models [101] indicating that T_{VM} cells made a significant contribution to the anti-tumor response. In addition to this, increased frequencies of T_{VM} cells have been observed after chemotherapeutic treatment in humanized mouse models of lymphomas [170] and in human chronic myeloid lekuemia (CML) patients in remission compared to those with a chronic phase CML [171,172]. Wang et al demonstrated activation of T_{VM} cells, with increased GrB and IFN-γ production, in humanized mouse models of lymphoma and demonstrated that T_{VM} cells were able to engage MHCI-independent killing programs to exert tumor control [170].

1.14. Ageing and Dysfunction in T_{VM} Cells

Infection-driven inflammation and activation by cognate antigen can also affect T cell survival. These triggers can drive rapid expansions in the CD8 T cell compartment, leading to expanded T_{MEM} cell populations and even reduced T_N cell populations. With age, the thymic mass and cellularity reduces resulting in a gradual and irreversible loss in the output of T_N cells [173–175]. In addition to this quantitative decline in output, functional defects such as reduced proliferative capacity, defects in TCR signaling and compromised effector function have also been reported in aged CD8 T cells ([176,177].

Ageing results in increased levels of inflammation [178–180] which is associated with higher incidence of infections and cancer in the elderly [181–183]. The chronic low-grade inflammation contributes to limited CD8 T cell responses in advanced age, where T_N cells undergo severe attrition in aged mice and humans, reducing from >80% to 20-30% of total CD8 T cells [184,185] (depicted in Figure 1.3). At the same time ageing leads to a significantly increased frequency of T_{VM} cells in mice and humans [95,98,186], expanding from 5-10% in young mice to 20-40% of all CD8 T cells in aged mice [95,186]. In contrast to T_{VM} cells from young mice and humans, which proliferate more rapidly than T_N cells [85,94], T_{VM} cells from aged mice and humans have reduced proliferative capacity in response to TCR stimulation compared to T_N cells [95,168,186]. The TCR mediated dysfunction was found to be associated with multiple markers of senescence including increased expression of p-extracellular signal -regulated kinase (ERK)1/2, p-p38, γ-H2Ax and Bcl-2 in aged T_{VM} cells compared to other CD8 T cell subsets [95]. While T cell senescence has been observed in memory CD8 T cell populations in aged individuals [187,188], these observations of senescence in T_{VM} cells were unusual given that these are antigenically

naïve cells. Collectively, the increased frequency of T_{VM} cells, coupled with a marked decline in TCR-mediated functions, makes this subset of particular interest in context of age-associated immune defects (Figure 1.3).

While T_{VM} cells express several of the hallmarks of senescence in aged mice as mentioned above, their senescent phenotype is distinct from other CD8 T cells, and sources of dysfunction observed in other aged CD8 T cells are absent in T_{VM} cells. Expression of exhaustion markers, including PD-1, Lag-3 and TIGIT, is increased on both T_N and T_{MEM} cells with age, however T_{VM} cells maintain low expression of these markers across the lifespan [95]. Another example of distinct dysfunction is that T_N cells lose the ability to divide asymmetrically with age, a property considered important for generating optimal memory responses [189,190]. In contrast, T_{VM} cells undergo asymmetric divisions while young and retain this ability with age [189]. These studies imply that the factors driving senescence and dysfunction in T_{VM} cells may be unique and require dedicated investigation. Given that T_{VM} cells are poised for increased homeostatic turnover and prolonged survival due to their reactivity to IL-15, their senescent phenotype may be a consequence of their unique sensitivity to cytokine stimulation. Indeed, while T_{VM} cells lose TCR mediated proliferation with age, they appear to retain their proliferative response to IL-15 [34,95]

Recently, dysfunction in CD27-CD28- double negative, terminally differentiated effector memory T (T_{EMRA}) cells has been linked to p-p38 and p-ERK1/2 forming an inhibitory complex with stress induced proteins called sestrins. This complex disrupts TCR signaling and limits antigen specific proliferation and cytokine responses [191]. Increased expression of NKG2D has been shown to correlate with observations of TCR dysfunction in terminally differentiated CD8

T cells [155]. Sestrin 2 has been shown to directly associate with NKG2D/DAP12 which then inhibits TCR signaling and stimulates innate-like killing programs in terminally differentiated CD8 T cells in humans and in CD8 T cells from aged mice [155]. Transcriptional analysis has shown that NKR expression is increased on T_{VM} cells with age [95], however it is not known if this also translates to increase in innate-like killing in aged T_{VM} cells. If so, it would imply that T_{VM} cells are also downregulating TCR-mediated functions to favour innate-like functionality with increasing age, similar to the senescent cells described by Akbar and colleagues [155].

An additional factor that may alter T_{VM} cell frequency and function with age is the cumulative infection history over the lifespan. Studies comparing laboratory mice with wild/feral mice have found significant differences in the distribution of CD8 T cells in the wild mice compared to laboratory mice, suggesting that history of infections can alter the landscape of CD8 T cells [192,193]. However, similar observations were not made when T_{VM} cell population was assessed in laboratory mice versus feral mice [102]. Studies have observed significant increases in T_{VM} cells with helminth, CMV and HIV infections [84,143,167,169], but these analyses have not been extended out to advanced age to assess how infections impact T_{VM} cell over prolonged periods. Additionally, while a few studies have explored the near-term impact of helminth infections [84,167], there has been no investigation of the impact of a wide range of distinct infections or sequential infections on T_{VM} cells.

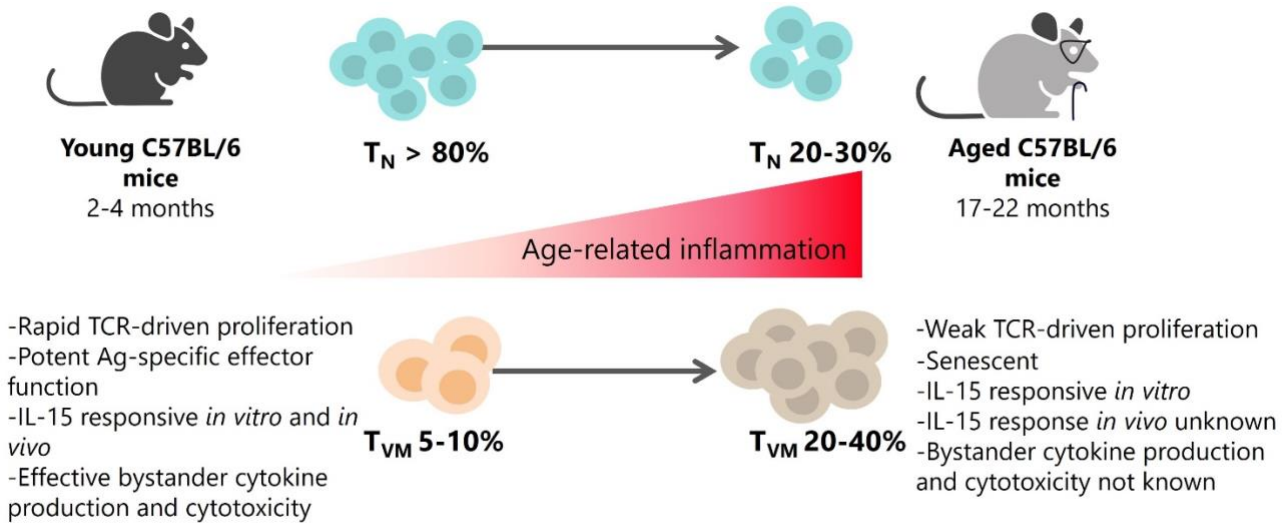

Figure 1.3. Age-related accumulation and dysfunction in T_{VM} cells and attrition of T_N cells. With increasing age, the T_{VM} subset is preferentially retained while the T_N cell subset declines [95,98,185,194]. The current model is that this shift in composition supports survival of CD8 T cells with higher affinity for self-peptide/MHCI and is associated with increasing inflammatory signals, such as IL-15 and type I IFNs during ageing [102,156]. With selective retention of T_{VM} cells, we also see age-related dysfunction with a decline in proliferation and effector functions but retention of IL-15 responsiveness [95,186].

1.15. Specific Aims

The broad aim of this thesis is to investigate the impact of different infections and inflammatory cues on the generation, homeostasis, and function of T_{VM} cells in both young and aged mice. It is clear that T_{VM} cells require IL-15 to survive during homeostasis, but I aim to define how the T_{VM} population responds, both quantitatively and qualitatively, to changes in IL-15 availability. Infection-driven inflammation increases levels of cytokines, like type I IFNs and IL-15, to which T_{VM} cells are particularly sensitive, therefore infection history is likely to modulate T_{VM} cell maintenance. Helminth infections are already known to drive T_{VM} cell

expansion but this has been attributed to IL-4, with the role of IL-15 during helminth infection not yet explored. Given that only a small range of infection types have been assessed with regard to their ability to modulate T_{VM} cell maintenance and function, I aimed to assess a broader range of acute and chronic infections inducing different inflammatory profiles, individually and sequentially, in mouse and human samples, to identify broader patterns of T_{VM} cell modulation. Finally, given the dramatic shifts already observed in T_{VM} cell frequency, phenotype and function with some infections and natural ageing, I will also track how the IL-15 and various early-life infections impact T_{VM} cell number and function over the lifespan, and determine if infection-driven changes to T_{VM} cell population exacerbate or attenuate the dysfunction acquired by the T_{VM} population with age.

The specific aims of my project are therefore:

1. To define how IL-15 impacts T_{VM} cell generation, maintenance and function in young mice and aged mice.
2. To determine whether helminth infection-induced changes in the T_{VM} cell number, phenotype and function are sustained in advanced age, how this influences T_{VM} cell senescence, and the role of IL-15 in driving these changes.
3. To determine whether distinct types of infections, either individually or combined, are able to modify T_{VM} cell generation, maintenance or function in young mice and aged mice.
4. To determine whether a chronic viral infection (HIV) associated with sustained inflammation, impacts T_{VM} cell frequency, phenotype and function in humans.

Chapter II

2. Materials and Methods

2.1. Mice

All C57BL/6 mice used in this study were either from the colony bred at Monash Animal Research Platform (MARP) or at Walter and Eliza Hall Research Institute (WEHI), Victoria, Australia, except for dirty mice which are described below. We acquired CD45.1+ mice (B6.SJL-PtprcaPep3b/BoyJArc) from the Animal Resources Centre, Perth, Western Australia. TCRα-/- mice were bred and housed in Animal Research Laboratory (ARL) at Monash University. *Cish-/-* mice were housed at WEHI and tissues were processed at Monash University. Uninfected young and aged mice were housed in a specific pathogen free (SPF) facility, whereas mice infected with pathogens were housed in the QC2 quarantine facility of ARL. Experimental procedures conducted during this study had prior approval from MARP Animal Ethics Committee (AEC). Young mice used in this study were typically between 8-12 weeks of age, whereas aged mice ranged from 17-20 months old (mo) of age. All experiments used female mice, except for *Cish-/-* mouse work which was conducted with young male mice. In experiments with aged mice, animals that exhibited splenomegaly or other significant abnormalities or malignancies were excluded from experiments.

For dirty mice experiments, young, 5 mo male and aged 20 mo male dirty mice were analyzed. These mice were acquired from James Cook University (JCU), Townsville, Queensland. Aged (20 mo) dirty mice were housed in an unsealed building. The young dirty mice received bedding from the aged dirty mice but were not co-housed. Young dirty mice were analysed

alongside SPF housed age and sex matched C57BL/6 control mice acquired from the same parental line.

2.2. Infections

2.2.1. Influenza A virus

For Influenza A virus (IAV) infections, A/Hong Kong/X31 (HKx31) H3N2 strain [195] was used. After general anaesthesia with isoflurane inhalation, mice were administered 10,000 plaque forming units (pfu) of HKx31 in a volume of 30 µL of 1X Dulbecco's phosphate buffered saline (dPBS) (Gibco) intranasally.

2.2.2. *Salmonella typhimurium*

For *Salmonella typhimurium* infections, the BRD509 strain of *S. typhimurium* was used. This strain is a ΔaroA ΔaroD mutant of the SL1344 strain of *S. typhimurium* [196]. Mice were briefly warmed in their cages before administering 200 colony forming units (cfu) of BRD509 intravenously via the tail vein in 100 µL of dPBS.

2.2.3. Lymphocytic choriomeningitis virus

For lymphocytic choriomeningitis virus (LCMV) infections, the WE strain was used, which causes an acute infection [197]. Mice were briefly warmed in their cages before administering 3000 pfu of LCMV-WE intravenously via the tail vein in 100 µL of dPBS.

2.2.4. *Trichuris muris*

Trichuris muris (*T. muris* or Tm) is a whipworm, used as mouse model for human Trichuriasis [198]. To infect mice with *Trichuris muris*, 200 *T. muris* eggs were administered in 100-200 µL of Milli-Q water orally using a 22G gavage needle.

2.2.5. *Heligmosomoides polygyrus*

Heligmosomoides polygyrus (H. polygyrus or *Hp)* is a natural roundworm infection of mice. To infect mice with *H. polygyrus*, mice, 200 stage 3 larvae (L3) were administered in 100-200 µL of Milli-Q water orally using a 22G gavage needle.

2.3: Exogenous treatments and adoptive cell transfers

2.3.1. cIL-15 treatment

For IL-15 administration and stimulation assays, a complexed form of IL-15 (cIL-15) was used. Complexed IL-15 was selected as it persists longer *in vivo* and is efficiently trans-presented to CD8 T cells via binding to Fc receptors via an Fc domain, leading to more sustained and more potent IL-15 signaling [97,199]. To generate this complex, carrier-free recombinant murine IL-15 (Peprotech, London, UK) was combined with a recombinant fusion protein, carrier-free mouse IL-15Rα:Fc (R&D systems, Minnesota, USA), incubated for 30 mins at 37 ˚C to permit binding, and administered intraperitoneally at dose of 2 µg or 4 µg per mouse. An example formulation for 5 mice plus overage is described in Table 2.1, with 200 µL administered per mouse.

Table 2.1. Formulation of cIL-15.

Reagent	Concentration	Volume
IL-15	1.8 µg/mL	10.8 µL
IL-15Rα:Fc	8.2 µg/mL	49.2 µL
dPBS	1x	1140 µL
Final Volume		1200 µL

2.3.2. Anti-IL-15 treatment

To block IL-15 signaling, an IL-15/Rα neutralising monoclonal antibody (Ab) (clone GRW15PLZ, eBiosciences, Massachusetts, USA) was used. Mice were administered 40 µg of anti-IL-15/Rα Ab intraperitoneally on day (d) 0 of *H. polygyrus* infection and then again at d4.

2.3.3. Pyrantel treatment

To cure mice of *H. polygyrus* infection, mice were administered pyrantel pamoate at 10 mg/kg body weight. The drug was made fresh in Milli-Q autoclaved water and given orally twice one day apart. Mice were drug treated 2-3 weeks post infection and then rested for 10-14 days before further infection or analysis.

2.3.4. CD8 T cell adoptive transfers

Sorted CD8 T cell subsets were pelleted (500 x*g*, 6 mins, 4˚C) and washed twice in dPBS to remove all protein. Cells were counted and administered intravenously via tail vein in 100 µL dPBS.

2.4. Tissue sampling, processing and cell counts

2.4.1. Spleen and lymph nodes

Mice were euthanised using CO_2 asphyxiation. For the isolation of splenocytes from secondary lymphoid organs, spleens were harvested separately, whereas lymph nodes (axillary, brachial, cervical, inguinal, mesenteric) were pooled in cRPMI (Appendix 9.2). For some experiments only, mesenteric lymph nodes (mesLN) and mediastinal lymph (medLN) nodes were taken separately.

Spleen and lymph nodes were mechanically disrupted using a 3 mL syringe plunger and then filtered through a 70 μm sieve (BD Falcon, BD Biosciences, Massachusetts, USA). Cells were then pelleted (~600 x*g*, 6 mins, 4 °C) and resuspended in appropriate volume of cRPMI. In case of spleens, red blood cell (RBC) lysis was performed using RBC Lysing Buffer Hybri-Max (Sigma-Aldrich, Missouri, USA) for 1 minute and 30 seconds at room temperature (RT) with intermittent manual resuspension or votexing. Hanks balanced salt solution (HBSS) (Appendix 9.2) was used to stop lysis, followed by immediate centrifugation (~600 x*g*, 6 mins, 4 °C). Splenocytes were then resuspended in appropriate volume of cRPMI to get approximately 10-15x10^6 cells/mL.

Cell counts were performed using ABX Micros 60, Hematology analyzer (HORIBA Medical, Japan). In some experiments, cell counts are based on Accuchek counting beads (Invitrogen, Thermo Fisher Scientific, USA). After thoroughly mixing counting beads with a pipette, a fixed volume (usually 20 μL) and a known concentration (200,000 beads/mL) of Accuchek beads was added to a known volume of cell suspension. During flow cytometric analysis, Accuchek bead

events were gated first on FSC-A vs SSC-A and then again on B530 vs SSC-A to get total bead events. Total lymphocyte counts were then computed based on the following equation:

$$\text{Total cells/ ml of sample} = \frac{cell\ events}{bead\ events} \times \frac{(bead\ volume)}{(bead\ volume + cell\ volume)} \times total\ beads\ per\ ml$$

2.4.2. Blood and Serum

Peripheral blood was harvested via cardiac puncture either into a syringe containing 20 µL of 0.5 mM ethylenediamine tetraacetic acid (EDTA) to prevent clotting or directly into tubes for serum analysis.

For analysis of blood lymphocyte phenotype and count, blood was briefly spun down in a minifuge and then lysed using a water lysis technique. In this technique, a fixed volume of blood was transferred into 7.5 mL of water and then quenched with 2.5ml of 3.5% w/v salt solution within 5-10 seconds to return the solution to osmotic balance. After lysis, tubes were incubated on ice until they were ready to centrifuge. Cells were then pelleted (~600 x*g*, 6 mins, 4 °C) and resuspended in appropriate volume of cRPMI (Appendix 9.2).

For analysis of serum, blood was allowed to clot at RT for 30 mins to 1 hour and the centrifuged (1000 x*g*, 10 mins, RT or 4 °C). Serum was collected into fresh Eppendorf tubes and stored at -20 °C to -80 °C.

2.4.3 Liver

Liver was perfused using 10-15 mL of PBS of containing 2 mM EDTA via portal vein and then harvested in cRPMI. Liver tissue was mechanically disrupted with a 5 mL syringe plunger and then filtered through 100 µm sieve. The cell suspension was then pelleted (400 x*g*, 10 mins, 4 °C), resuspended in 10 mL of flow cytometry staining buffer (FACS) buffer (Appendix 9.2), transferred to a 15 mL tube and centrifuged again (400 x*g*, 10 mins, 22 °C). For density gradient separation, cells were resuspended in 37.5% Percoll® (Merck, Sigma Aldrich, Missouri, USA, Missouri, USA) and centrifuged (690 x*g*, 12 mins at 22 °C) with no deceleration. The hepatocyte layer and supernatant was aspirated and the remaining cell pellet was resuspended in 1 mL of Ammonium-Chloride-Potassium (ACK) lysis buffer (Appendix 9.2) to lyse RBCs and incubated for 3 mins at RT. Lysis was quenched with FACS buffer (Appendix 9.2), cells were pelleted (400 x*g*, 7 mins, 4 °C) and then resuspended in appropriate volume of cRPMI (Appendix 9.2).

2.4.4. Lungs

Lungs were perfused via the right ventricle of the heart with 10 mL of PBS containing 2 mM EDTA. and then harvested into 1 mL of cRPMI. Lungs were finely cut into small pieces, resuspended in 5 mL of collagenase-DNAase I mix (Appendix 9.2) to digest connective tissue and incubated at 37 ˚C, 5% CO_2 for 30 mins. Following digestion, lung pieces were mechanically disrupted by drawing up and down in a 3-5 mL syringe plunger and then filtered through a 70 µm filter. Digestion was quenched with 20 mL of cRPMI (Appendix 9.2). Lungs cells were centrifuged (600 x*g*, 6 mins, 4 ˚C), resuspended in cRPMI and filtered through a 70 µm sieve. For density gradient separation, the cell suspension was underlaid with isotonic Percoll® solution (Appendix 9.2) and centrifuged (800 x*g*, 20 mins, RT) with no deceleration.

Lymphocytes separated at the interface and were harvested into 10 mL of cRPMI, pelleted (800 x*g*, 6 mins, 4 ˚C) and then resuspended in an appropriate volume of cRPMI.

2.4.5. Dendritic cell preparation

To obtain an optimal yield of dendritic cells (DCs) from spleens, a digestion and light density gradient separation method was used. Spleens were finely cut with curved scissors until no visible chunks remained in 100-200 µL of DC digestion mix (Appendix 9.2), then transferred into a 15 mL tube and resuspended in a final volume of 2 mL of DC digestion mix and incubated for 20 mins at RT. Cell were resuspended using intermittent manual resuspension. Digestion was quenched with 10 mL of cRPMI and cell suspension was then passed through a 70 µm filter into a 50 mL tube and centrifuged (700 x*g*, 7 mins, 4 ˚C). Cell were resuspended in 1.5 mL of Nycoprep (Appendix 9.2) and then overlayed onto 1.5 mL of neat Nycoprep in a 5mL tube. Another 1-2 mL of fetal bovine serum (FBS) (Sigma-Aldrich, Missouri, USA) was overlayed on top and the tube was centrifuged (1700 x*g*, 15 mins, 4 ˚C) with no deceleration. DCs were harvested from the light density fraction along with all the FBS and transferred to a 15 mL tube and washed with 10 mL of DC-magnetic activated cell sorting (MACS) buffer (Appendix 9.2).

2.4.6. Human PBMC samples

HIV infected peripheral blood mononuclear cells (PBMC) and uninfected samples were a part of Melbourne HIV Cohort, curated by ImmunoVirologyResearch Network (IVRN) at Australian Centre for HIV and Hepatitis Research (ACH2). This project was approved by IVRN steering

committee, and the work was approved by Monash University Human Research Ethics Committee (MUHREC), application no. 22344.

2.5. Cell lines

2.5.1. YAC-1

YAC-1 cells are a mouse lymphoma cell line. YAC-1 cells have constitutive expression of Rae-1, which is a target of activating NKR, NKG2D. The YAC-1 cell line was a kindly provided by Prof. Andrew Brooks (Department of Microbiology and Immunology, University of Melbourne). The cells were grown in cRPMI at 37 ˚C with 5% CO_2 and cultures were split when cell density reached approximately $8x10^5$- $1x10^6$ per mL. YAC-1 aliquots were frozen down in 10% dimethyl sulfoxide (DMSO):90% FBS and stored in liquid nitrogen.

2.5.2. 2.4G2 hybridoma cell line

To block non-specific binding of antibodies to Fc receptors found on surface of immune cells, 2.4G2 supernatant was used. 2.4G2 is a B cell hybridoma cell line that is used to raise monoclonal antibody specific for a common epitope between CD16 (FcγRIII) and CD32 (FcγRII). 2.4G2 cells were cultured in cRPMI (Appendix 9.2) at 37 ˚C with 5% CO_2 until spent. The supernatant was isolated with centrifugation (600 x*g*, 6 mins, 4 ˚C) and filtered through a 0.22 µm vacuum filter to remove impurities. The 2.4G2 supernatant was then stored in aliquots at -80 ˚C.

2.6. CD8 T cell enrichment and B cell depletion

2.6.1. B cell depletion

During splenocyte processing, splenocytes were depleted of B cells prior to RBC lysis. Briefly, spleen cell suspension was plated on 150 X 15 mm plastic dish (Falcon) coated with anti-IgG and anti-IgM (Abacus, Sydney, Australia) and incubated at 37 ˚C for 30-45mins. B cells would thereby bind firmly to the dish. Cells in suspension were recovered by removing the supernatant and washing the plate once with HBSS (Appendix 9.2) recover the non-adherent and therefore T cell-enriched fraction.

2.6.2. CD8 T cell enrichment

To enrich CD8 T cells from mouse spleens, a magnetic bead enrichment protocol using a CD8α+ T cell isolation kit (130-095-236, Miltenyi Biotech, Gladbach, Germany) was followed. Splenocytes were pelleted, resuspended in 300 µL of MACS buffer with 50 µL of Biotin-antibody cocktail and incubated at 4 ˚C in the fridge for 5 min. After incubation, 100 µL of anti-biotin microbeads and 200 µL of MACS buffer (Appendix 9.2) was added to the cell suspension and incubated for 10 mins at 4 ˚C. Cells were then added directly to LS column. The flowthrough containing enriched CD8 T cells was collected by washing the columns with 3 mL of MACS buffer twice. The enriched CD8 T cells were pelleted (600 x*g*, 6 mins, 4 °C) and resuspended in appropriate volume of cRPMI.

2.7. Polyclonal stimulation for intracellular cytokine staining

For polyclonal stimulation to assess intracellular cytokine production, 96-well U bottom plates (Nunc, Roskilde, Denmark) were coated with anti-mouse(m)CD3ε, anti-mCD8α, anti-mCD11a

in PBS overnight (Table 2.2), and washed twice with PBS the next morning to remove all unbound antibody. Sorted CD8 T cells were resuspended in recombinant human (rh) IL-2 (Roche) at 10 U/mL in cRPMI and plated at a concentration of 10,000-30,000 cells per well. Cells were incubated for 36 hours at 37 ˚C with 5% CO_2, with addition of 1 µL/mL brefeldin A (BD Biosciences, Massachusetts, USA) added to all wells during the last 4-6 hours to inhibit cytokine export. Cells were then pelleted and intracellular marker staining protocol was followed (Section 2.9.2).

Table 2.2: Concentration of antibodies for polylconal stimulation

Reagent	Concentration
anti-mCD3ε	1 or 10 µg/mL
anti-mCD8α	10 µg/mL
anti-mCD11a	5 µg/mL

2.8. Proliferation assays

2.8.1. Cell Trace Violet staining

To track T cell proliferation, either whole splenocytes, PBMCs or sorted CD8 T cell subsets were stained with Cell Trace Violet (CTV) (Invitrogen, ThermoFisher Scientific, USA) dye. PBS and cRPMI was pre-warmed at 37 ˚C. RBC lysed splenocytes were stained in a 1 µM CTV staining solution made in 1-2mL volume of warm PBS and then incubated at 37 ˚C for 20 mins. CTV staining was quenched with addition 10 mL of warm cRPMI and cells were incubated for an additional 5 mins at 37 ˚C to permit dye conversion. Cells were washed twice in cRPMI or MACS

buffer (600 x*g*, 6 mins, 4 ˚C), before proceeding to cell sorting of whole splenocytes or PBMCs. If sorted CD8 T cell subsets were stained with CTV to prepare for adoptive transfers, cells were washed in dPBS to remove any residual FBS prior to transfer..

2.8.2. Bulk proliferation assay

For mouse bulk CD8 T cell proliferation assays, 24 well flat-bottom plates (Nunc, Roskilde, Denmark) were coated overnight with similar concentration of antibody as given above (Section 2.7, Table 2.2). Of note, 10 µg/mL of anti-mCD3ε was used instead of 1 µg/mL in some proliferation assays. CTV labelled cells, as described in Section 2.8.1, were plated at a concentration of 10,000-20,000 cell per well for 60-65 hours at 37 ˚C with 5% CO_2 before staining for viability and flow cytometric analysis.

For human bulk proliferation assays, 24 well flat-bottom plates (Nunc, Roskilde, Denmark) were coated overnight with anti-human (h) CD3ε (OKT3) at 1 µg/mL and anti-hCD11a (MEM83) at 19.5 µg/mL and washed twice with PBS the next morning to remove all unbound antibody. Human CD8 T cell subsets were plated at a concentration of 3000-5000 per well for 90 hours at 37 ˚C with 5% CO_2. At the end of the stimulation period, cells were stained with a fixable viability dye and fixed with Cytofix/Cytoperm buffer (BD Biosciences, Massachusetts, USA) for 20 mins. The cells were then washed twice before proceeding to flow cytometric analysis (Section 2.11).

2.8.3. Single-cell proliferation assay

To assess capacity of single cells to proliferate in response to polyclonal stimulation, 96-well plates were coated with antibodies overnight as described in Section 2.7 and Table 2.2. Cells were sorted directly into the wells containing cRPMI with 10 U/mL rhIL-2 (Roche/Merck). Cells were then incubated at 37 ˚C with 5% CO_2 for 5 days.

2.8.4. IL-15 proliferation assay

To assess *in vitro* IL-15-driven proliferation, sorted CTV-labelled cells (Section 2.8.1) were incubated at 37 ˚C with 5% CO_2 either with 100 ng of cIL-15 (Section 2.3.1) for 5 days with murine CD8 T cells or with 200ng of cIL-15 for 7 days with human CD8 T cells.

2.9. Cell staining for Flow Cytometry

2.9.1. Tetramer staining and surface molecule staining

Monomers were made in laboratory of Prof. Andrew Brooks (Department of Microbiology and Immunology, University of Melbourne), and were tetramerised with Streptavidin-Phycoerythrin (PE) or Streptavidin-Allophycocyanin (APC) (Invitrogen, Thermo Fisher Scientific)). To identify antigen-specific CD8 T cells, approximately 1 x 10^6 splenocytes were aliquoted into a 96-well U-bottom plates (CoStar® Corning, NY, USA), pelleted and Fc receptors were blocked with 2.4G2 for 10 mins at RT. Splenocytes were then stained with fluorophore conjugated peptide-MHC Class I tetramers (0.825 ng/µL) for 30-45 mins at RT. Tetramer was washed off once, and then surface antibody cocktail was added to cell pellets in a 50 µL volume of FACS buffer and stained for 20-30 mins on ice. Cells were then washed twice before proceeding to fixation and permeabilization (Intracellular markers in Section 2.9.2;

Intranuclear markers in Section 2.9.3; Phosphorylation markers in Section 2.9.4) or directly to flow cytometric analysis (Section 2.11).

2.9.2. Intracellular marker staining

For intracellular staining of cytokines and cytosolic molecules, cells were first stained with a fixable viability dye and surface markers as described in Section 2.9.1. Cells were then pelleted (935 x*g*, 3 mins, 4 ˚C), resuspended in 100 µL of BD Cytofix/Cytoperm buffer for 30 mins at 4 ˚C and then washed twice in 1x BD Perm/Wash buffer (BD Biosciences, Massachusetts, USA). Antibodies for intracellular markers were diluted at required concentration in 1x BD Perm/Wash buffer and cells were then stained for 30 mins at 4 ˚C. Cells were washed twice with 1x Perm/Wash buffer to remove unbound antibody, with a final wash in FACS buffer (Appendix 9.2) to remove the permeabilization buffer. Cells were resuspended in 100-200 µL of FACS buffer and taken for flow cytometric analysis.

2.9.3. Intranuclear marker staining

For intranuclear markers such as transcription factors, cells were first stained with a fixable viability dye and surface markers as described in Section 2.9.1. Cells were then pelleted (935 x*g*, 3 mins, 4 ˚C), resuspended in 100 µL of 1x FoxP3 Fixation/Permeabilization solution (eBioscience, Thermo Fisher Scientific, USA), according to manufacturer's instructions, for 30-45 mins at RT, and then washed once in 1x Permeabilization buffer (eBioscience, Thermo Fisher Scientific, USA). Antibodies for intranuclear markers were diluted at required concentration in 1x Permeabilization buffer and cells were then stained for 30 mins to 1 hour at RT. Cells were

washed twice with 1x Permeabilization buffer to remove unbound antibody, with a final wash in FACS buffer (Appendix 9.2) to remove the permeabilization buffer. Cells were resuspended in 100-200 µL of FACS buffer and taken for flow cytometric analysis (Section 2.11).

2.9.4. Phosphorylation staining

For phosphorylated markers, whole splenocytes or sorted CD8 T cell subsets were used. Cells were either analyzed for basal phosphorylation of desired markers or stimulated. Cells were polyclonally stimulated with rat anti-mCD3ε/CD8α/CD28 or with phorbol 12-myristate 13-acetate (PMA) and ionomycin (Sigma Aldrich, Missouri, USA) to use as positive controls or to assess TCR-induced phosphorylation of extracellular signal-regulated protein kinase (ERK) 1/2. The concentration of stimulation antibodies and PMA/ionomycin is given in Table 2.3.

For polyclonal stimulation, cells were first aliquoted into wells and allowed to equilibrate on ice for 10 mins. Stimulation antibodies were added on ice to allow binding but slow down the TCR activation process, as recommended by manufacturer's protocol. Stimulation cocktail (rat anti-mCD3/CD8α/CD28) was added to the wells on ice and antibodies were allowed to bind to cells on ice for 15 mins. A goat anti-rat IgG (Abcam, USA) was then added while keeping the plate on ice and allowed to cross-link for another 10 minutes. During this time, 2x Lyse/Fix solution (BD Biosciences, Massachusetts, USA) was prepared and warmed to 37 ˚C. Once the cross-linking step was over, the plate was incubated at 37 ˚C for 5, 8 and 12 mins. PMA/Ionomycin stimulated cells were incubated for 10 mins at 37 ˚C. At the end of each round of stimulation, the phosphorylation was fixed by adding an equivalent volume of 2x Lyse/Fix

solution to each of the wells and incubating at 37 ˚C for 10 mins. Cells were pelleted (935 x*g*, 3 mins, 4 ˚C) and then washed twice with ice-cold PBS.

Fixed cells were resuspended in ice-cold Perm Buffer II (BD Biosciences) and permeabilized overnight at -20 ˚C. Cells were then washed twice with FACS buffer and Fc receptors were blocked with 2.4G2 solution (Section 2.5.2, Appendix 9.2). Unconjugated antibodies for p-ERK1/2 or p-p38 (Cell Signaling Technology, Massachusetts, USA) were diluted at required concentration in FACS buffer and cells were stained for 30 mins-1 hour on ice. Cells were washed twice and then stained with fluorophore-conjugated anti-rabbit secondary mAb (Cell Signaling Technology, Massachusetts, USA) for 30 mins at RT. Cells were washed twice in FACS buffer and phospho-staining was analyzed with flow cytometry (Section 2.11).

Table 2.3. Stimulation antibodies for phosphorylation assays

Reagent	Concentration
Rat anti-mCD3ε	1 µg/mL
Rat anti-mCD8α	10 µg/mL
Rat anti-mCD28	5 µg/mL
Anti-Rat IgG	2 µg/mL
PMA	0.05 µg/mL
Ionomycin	0.5 µg/mL

2.9.5. Annexin-V staining

To detect early and late apoptotic cells, Apoptosis Detection Kit (Invitrogen, Thermo Fisher Scientific, USA) was used. Briefly, cells were pelleted and stained with viability dye and surface

markers as described in Section 2.9.1 and then washed once in 1x Annexin V binding buffer (Invitrogen, Thermo Fisher Scientific, USA). Cells were incubated with fluorescently-conjugated Annexin V diluted at required concentration in 1X binding buffer for 15 mins at RT. Cells were washed once with 1x Annexin V binding buffer and, if required, incubated with Propidium Iodide Staining Solution (Invitrogen, Thermo Fisher Scientific, USA) for 10 mins at RT. Cells were washed once with FACS Buffer (Appendix 9.2), resuspended in FACS buffer and analyzed with flow cytometry.

2.10. Assays of innate-like function

For assessment of innate-like IFN-γ and GrB production, sorted murine CD8 T cells were cultured in cRPMI (Appendix 9.2) containing mIL-12 (Peprotech, London, UK), mIL-18 (Peprotech, London, UK) and cIL-15 (Section 2.3.1) at the concentrations described in Table 2.4. Cells were incubated in stimulation media for 16 hours at 37 ˚C with 5% CO_2 with addition of 1 µL/mL brefeldin A (BD Biosciences, Massachusetts, USA) added to all wells during the last 4-6 hours to inhibit cytokine export. Cells were then pelleted and stained for intracellular markers as described in Section 2.9.2.

Table 2.4. Concentration of cytokines for stimulation

Reagent	Concentration
mIL-12 p70	20 ng/mL
mIL-18	20 ng/mL
cIL-15	100 ng/mL

For assessment of innate-like cytotoxic capacity, YAC-1 target cells were prepared from cultures in the exponential phase and stained with CTV as described in Section 2.8.1. YAC-1 cells were washed twice, counted and resuspended in cRPMI (Appendix 9.2) at 37 ˚C until ready to plate out in wells. Sorted CD8 T cell subsets resuspended in cRPMI, counted and plated in wells with or without purified anti-NKG2D Ab (Biolegend, California, USA) at 10 µg/mL. Cells were incubated with anti-NKG2D Ab to permit blocking for 30-45 mins at 37 ˚C. Cytokine stimulation media was prepared as outlined in Table 2.4. YAC-1 cells were mixed with Accuchek beads (Invitrogen, Thermo Fisher Scientific, USA) in a 1:1 ratio in cRPMI then added to the pre-plated effector cells at a 10:1 ratio (effectors:targets/beads mix). Controls were also plated out as targets/bead mix only and effectors only, and all cells were incubated with cytokine stimulation media. After overnight incubation (12-16 hours), viability staining was performed as described in Section 2.9.1. The relative number of effectors, targets, and bead events were calculated through acquisition with flow cytometry (Section 2.11). Cytotoxic capacity was computed using the following equation:

$$\%\ \text{killing} = \left(1 - \left(ratio\ of\ bead\ to\ targets\ in\ control\ wells / ratio\ of\ beads\ to\ targets\ in\ coincubated\ wells\right)\right) \times 100$$

2.11. Flow cytometric acquisition and analysis

All flow cytometric analysis was performed using BD Fortessa X-20 or BD Symphony A3 systems with FACSDiva software (BD Immunocytometry Systems, California, USA). For automated acquisition of samples from 96-well plates, a high-throughput sampler (HTS; BD

Immunocytometry Systems, California, USA) was used. Briefly, samples were resuspended in 120-200 µL of FACS buffer (Appendix 9.2) and acquired at a flow rate 2 µL/s. The HTS was programmed to mix the sample prior to acquisition. To prevent contamination and clogging, the flow cell was washed with 400 µL of sheath fluid before acquisition of the next sample. Data were analysed with FlowJo version 10.8.1 (Tree Star Inc., Ashland, Oregon, USA).

2.12. Isolation of cells by fluorescence activated cell sorting (FACS)

Following CD8 T cell enrichment or B cell depletion, as described in Section 2.6.2, and antibody staining, lymphocytes were resuspended at 4 x 10^7 cells/mL in sort buffer (Appendix 9.2) and filtered through a 40 µm sieve (BD Falcon, BD Biosciences, Massachusetts, USA) into 5 mL polypropylene FACS tubes (BD Falcon). FACS was performed on BD Influx and BD FACSAria systems (BD Immunocytometry Systems, California, USA) at the Monash FlowCore facility (Monash University, Clayton campus) or at ARAFlowcore Facility (Monash University - Alfred Research Alliance), previously called AMREPFlow Facility (Burnet Institute, Melbourne). For sorting single cells, cells were sorted directly into 96-well plates. For bulk sorting, a three-way sort was performed into 1.5 ml sterile tubes (Eppendorf, Hamburg, Germany), or polypropylene FACS tubes (BD Falcon, BD Biosciences, Massachusetts, USA) with filtered FBS.

2.13. Statistical analysis

All data analysis was performed using GraphPad Prism (v9.3.1; San Diego, USA). Where data was not normally distributed, Mann-Whitney test was applied for unpaired data and Wilcoxon matched pairs test was applied for paired data. For test of normal Gaussian distribution,

Shapiro-Wilk test was applied and alpha=0.05 was considered to pass the normality test. For data that was normally distributed, non-parametric t-test was used. For all bar graphs, error bars represent standard error of mean (SEM).

Chapter III

3. Innate and antigen-specific responses of T_{VM} cells over a life course

3.1. Introduction

T_{MP} cells are known to depend on IL-15 for survival and not on MHC Class I [29,32]. There have been several observations of loss of T_{MP} cells in IL-15-/- mice and an expanded subset of $CD122^{hi}$ T_{MP} cells has been observed in mice with increased IL-15 signaling [113,200,201]. We now know that these T_{MP} cells are predominantly T_{VM} cells in C57BL/6 mice, and cytokine sensitivity is a fundamental characteristic of this subset [35,94]. Thymocytes possessing a distinct, high affinity TCR repertoire with elevated expression of transcription factor Eomes are reported as precursors of T_{VM} cells [101]. Cytokine dependence of T_{VM} cells is evident both during thymic development, where type I IFNs are required to maintain expression of Eomes [202], and in the periphery, where IL-15 is required to complete the differentiation to the T_{VM} cell lineage [35,101]. T_{VM} cells also require other factors involved in the IL-15 signaling pathway, including expression of the IL-15Rβ chain (CD122) and CD8α+ DCs, which are essential for transpresentation of IL-15. Mice lacking either of these present with significantly reduced or absent T_{VM} cells [35].

There have been multiple demonstrations of T_{VM} cell's rapid response to both TCR as well as IL-15 stimulation, but these have primarily been *in vitro* studies carried out on T_{VM} cells from young mice. For instance, IL-15 can induce proliferation of both young and aged T_{VM} cells *in vitro* [95], whereas exogenous IL-15 administration can also direct an increase in T_{VM} cells in

young mice [35,97]. There is also evidence of enhanced sensitivity to IL-15 in aged T_{VM} cells, evidenced by their increased expression of CD122 and p-Signal transducer and activator of transcription (STAT)5 after *in vitro* IL-15 treatment compared to young T_{VM} cells [34]. p-STAT5 regulates the expression of anti-apoptotic protein Bcl2, that is highly expressed in T_{VM} cells [95]. High expression of Bcl2 is likely to support preferential accumulation of T_{VM} cells with age, where they can make up to 50% of naïve CD8 T cell pool in aged mice. Given that T_{VM} cells acquire increased CD122 and p-STAT5 expression with age, one may expect that they would also exhibit increased proliferative capacity in response to IL-15 *in vivo*, however this remains unknown.

Other key innate-like functions of T_{VM} cells include their capacity to respond to IL-12 and IL-18 stimulation, which results in effector molecule production and induction of antigen-independent protective pathways mediated by IFN-γ and granzyme B (GrB) production [94,96]. While these pathways are highly active in young T_{VM} cells, it is not known if ageing is associated with a retention or increase in such innate-like functionality. Consistent with their cytokine-sensitive phenotype, T_{VM} cells also express several NKRs [97,203]. IL-15 is known to play a major role in NKR expression on NK cells [132,204], and can also upregulate expression of NKG2D on CD8 T cells *in vitro* [133]. Studies have shown that CD8 T cells can mediate cytotoxicity in a bystander manner via NKG2D [100,155], therefore NKR expression on T_{VM} cells is likely to aid their bystander cytotoxic response. The increased CD122 expression on aged T_{VM} cells may predict their enhanced NKR expression with age, thus equipping them with enhanced bystander cytotoxic responses. To date the impact of IL-15 on NKR expression, and associated innate responses, in young and aged T_{VM} cells has not been described.

Young T_{VM} cells are highly effective mediators of Ag-specific response, where they proliferate earlier than T_N cells after TCR activation and mediate cytokine production and cytotoxicity more readily than T_N cells [85,95,95] . However, with ageing the T_{VM} cell subset preferentially accumulates compares to T_N cells and acquires intrinsic TCR associated defects, reducing its ability to proliferate in response to TCR stimulus as well as reducing cytokine polyfunctionality upon TCR activation [95,168]. Alongside these defects in TCR mediated functionality, T_{VM} cells also acquire increased expression of senescent markers like, p-p38, ERK1/2 and γH2Ax [95]. With their retention of cytokine (and IL-15 in particular) responses and acquired defects in TCR responses that have been demonstrated *in vitro* , it is possible that aged T_{VM} cells adopt a more innate-like role in the periphery. In support of this, there have been recent studies that have shown that NKR expression on terminally differentiated CD8 T cells is correlated with an adaptation of innate-like immune function at the expense of TCR responses [155,191]. It is not known if a similar reciprocal functionality occurs in the case of aged T_{VM} cells as well, where their innate cytotoxic and proliferative responses are kept intact with higher NKR expression at the expense of reduced TCR signaling.

Here, we assessed whether the innate-like response of T_{VM} cells changes with age and whether this correlates with the phenotypic differences occurring over the course of ageing, such as those observed in cytokine and NKR expression. While it is clear that T_{VM} cells from young mice exhibit efficient innate and TCR-mediated functions, here we extend these analyses not only to T_{VM} cells from aged mice, but also determine TCR-mediated and IL-15 mediated responses *in vivo*, to better define intrinsic differences between young and aged T_{VM} cells. These data provide an understanding of how innate functions of T_{VM} cells are maintained with age and whether aged T_{VM} cells have adopted a more innate-like role in the periphery.

3.2. Results

3.2.1 Phenotypic changes in T_{VM} cells with ageing

We have previously shown that, in agreement with their highly cytokine sensitive profile, young and aged T_{VM} cells have increased expression of CD122 and consequently heightened expression of STAT5 and Bcl2 compared to other CD8 T cell subsets [34,95]. Our key aim was to assess if this cytokine-sensitive phenotype translated to increased innate-like functions, like improved bystander killing and IL-15 mediated proliferation in young and aged T_{VM} cells. Thus, we first analysed the broad hallmarks of innate-like immune function, including cytokine and NKR expression, to give an idea of how innate-like functionality may be modulated with age in T_{VM} cells. Young (8-12 week old) and aged (18-20 mo) mice were humanely killed and splenic T_{VM} along with T_N and T_{MEM} cells were analysed. As previously reported, we observed an increased frequency of T_{VM} cells in aged mice [98], however this did not correspond to an increased number of T_{VM} cell numbers [95] (Figure 3.1 A). As expected, the number of T_N cells was severely reduced, as reported in previous studies [185,194] whereas T_{MEM} cell number was increased in the aged mice compare to their young counterparts (Figure 3.1 A).

T_{VM} cells are better poised to undergo homeostatic proliferation due to their sensitivity to IL-15 [35,97], as well as their increased affinity for self-peptide/MHC [101,157,205]. Therefore, we hypothesized that aged T_{VM} cells would have increased basal proliferation compared to young T_{VM} cells. When Ki67 expression, which is an intranuclear marker indicating recent division, was assessed on CD8 T cell subsets, the proportion of cells showing recent division was increased

in T_{VM} cells compared to T_N cells from young mice, with T_{MEM} cells showing the highest proportion of dividing cells (Figure 3.1 B). In aged mice, the proportion of recently divided cells was most substantially and significantly increased in the T_{VM} cell subset, whereas a reduced proportion of T_{MEM} cells were dividing with age (Figure 3.1 B). These data show that ageing results in changes to homeostatic turnover such that the T_{VM} cell subset has the largest proportion of dividing cells, which may be due to their increased sensitivity to homeostatic IL-15 and/or tonic peptide-MHC stimulus with age.

To check if indeed T_{VM} cells were becoming increasingly cytokine sensitive, we assessed receptor expression of γc family of cytokines. We next assessed expression of CD122, CD127 and CD124, key components of IL-15, IL-7, and IL-4 receptors, respectively. T_{VM} cells showed increased expression of CD122 (Figure 3.1 C), as described in our previous reports [34], and CD127 with age (Figure 3.1 D) but have downregulated expression of CD124 with age (Figure 3.1 E). Notably, irrespective of age, T_{VM} cells had the highest expression of these cytokine receptors among all CD8 T cell subsets. Increased expression of CD122 and CD127 can indicate increasing sensitivity to IL-15 and IL-7 respectively, and could also indicate preferential accumulation or proliferation of T_{VM} cells with high CD122 and CD127 expression.

We next assessed expression of Eomes on T_{VM} cells. Eomes is a key transcription factor which regulates the transcription of CD122 [158] and CD124 [92,206] and its high expression is a key marker of T_{VM} cells in young mice [35,101]. Eomes expression showed no consistent change with age in T_{VM} cells (Figure 3.1 F). This was unexpected, given that T_{VM} cells have significantly increased expression of CD122 with age, indicating that there may be other factors driving the expression of CD122.

CD5 is considered an indicator of TCR signaling strength and self-reactivity during thymic development [21] and its high expression on T_{VM} cells is considered to indicate the higher affinity for self-ligands and stronger TCR signaling they have experienced during thymic development [97,101]. In line with this, young T_{VM} cells had the highest expression of CD5 across all CD8 T cell subsets, however T_{VM} cells showed a nearly 50% reduction in CD5 expression with age (Figure 3.1 G), such that the expression was largely normalized across subsets with age. It is unclear whether this shift in CD5 expression is as a result of preferential survival of an altered TCR repertoire with age as suggested by previous studies [156,185] or whether CD5 is no longer representative of TCR signaling strength in advanced age.

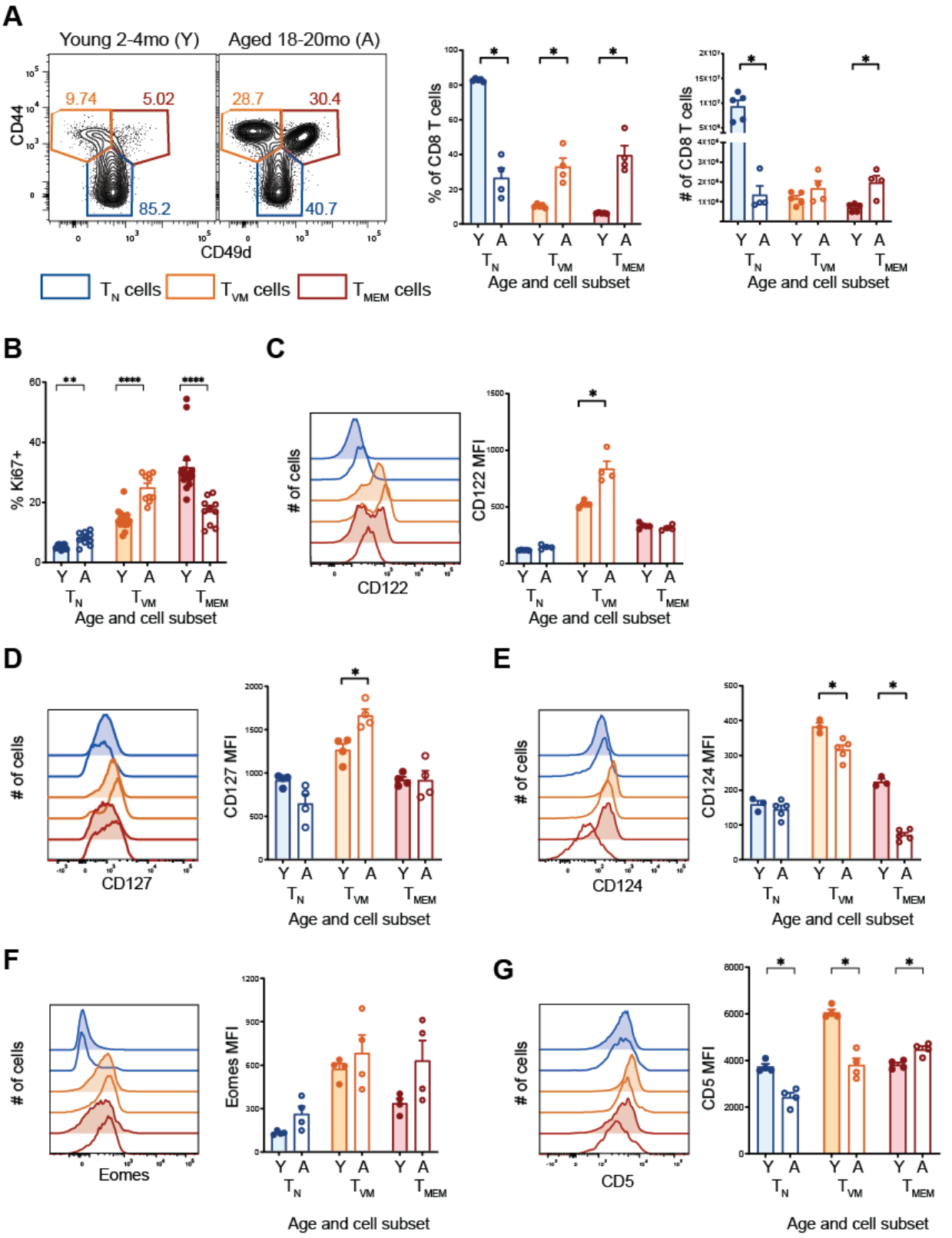

Figure 3.1. Phenotypic characterisation of young and aged T_{VM} cells. A) Representative contour plots gated on live TCR+CD8+ T cells from young (Y) and aged (A) mice depicting T_N, T_{VM} and T_{MEM} cells and bar graphs depicting frequency and number of each subset. B) Frequency of T_N, T_{VM} and T_{MEM} cells that

are Ki67+ in young and aged mice. Representative histograms and bar graphs showing the median fluorescence intensity (MFI) of C) CD122, D) CD127 E) CD124 F) Eomes, and G) CD5 on T_N, T_{VM} and T_{MEM} cells from young and aged mice. Data is representative of 2-3 different experiments with n=4-5 mice per group, except B is pooled data from 3 separate experiments. * $p<0.05$, ** $p<0.01$ using Mann Whitney test. Bar graphs represent mean ± SEM.

Previously, transcriptional analysis of young and aged CD8 T cell subsets from our group found that T_{VM} cells from young mice express a multitude of inflammatory chemokine receptors at a higher level compared to T_N and T_{MEM} cells. Chemokine receptor expression on CD8 T cells is critical for their migration to lymph nodes to undergo antigen-dependent activation, and for trafficking to sites of infection to mediate effector functions[207,208].

CCR2, a receptor for CCL2, is the master recruiter for monocytes and macrophages to sites of infections and is also expressed on activated T cells [209]. CCR2 expression on T_{VM} cells controlled recruitment to lungs during respiratory infections and also indicated ability of effector differentiation in T_{VM} cells [166]. We found that T_{VM} cells had the highest frequency of CCR2 expression (~30%) compared to T_N cells (~0%) and T_{MEM} cells (<20%), and this proportion of CCR2+ T_{VM} cells increased by 3-fold with age (Figure 3.2 A). CCR2 expression also increased on a per cell basis by more than 2-fold on T_{VM} cells with age, whereas T_{MEM} cells only had modestly increased expression compared to young T_{MEM} cells (Figure 3.2 B).

We assessed CD122 expression on CCR2+ and CCR2- T_{VM} cells, as this can be of relevance in understanding the heterogenous response of T_{VM} cells to stimuli, where T_{VM} cells with high CD122 and CCR2 expression may be more sensitive to chemokine and cytokine stimulus, and therefore better poised to traffic to sites of infection. We observed that CCR2+ T_{VM} cells had nearly 2-fold more CD122 expression compared to CCR2- T_{VM} cells, whereas the same difference was not observed for T_{MEM} cells (Figure 3.2 C).

CXCR3, which senses CXCL9/10/11 [210], is potently expressed on effector CD8 T cells[211]. In recent studies, CXCR3 was shown to regulate recruitment of bystander memory CD8 T cells and T_{VM} cells to sites of infection [166,212]. CXCR3 is expressed on the majority of T_{VM} cells in C57BL/6 and BALB/c mice [84,85]. Our data also showed that it was expressed by nearly all (>70%) T_{VM} cells (Figure 3.2 D), however the level of CXCR3 expression was modestly, but significantly downregulated in aged T_{VM} cells, whereas T_N cells acquired slightly increased expression with age (Figure 3.2 D).

CXCR5 is expressed by follicular helper T cells (T_{FH}) cells but more recently has been described in CD8 T cells as well. During viral infections, CXCR5+ CD8 T cells are able to mediate cytotoxicity of infected cells and suppress exhaustion [213,214]. CXCR5+ CD8 T cells are also considered a stem-cell like population with increased proliferative capacity [215,216]. A greater proportion of T_{MEM} cells expressed CXCR5 compared to T_{VM} cells in young mice (Figure 3.2 E). Expression of CXCR5 was not assessed at the aged timepoint.

Together these results demonstrate that T_{VM} cells are better positioned than other CD8 T cell subsets to respond to a variety of cytokine and chemokine stimuli, which results in their semi-differentiated state and explains their ability to act as first responders during primary infections in young mouse models [85,94]. The further increased expression of these cytokine and chemokine receptors in aged T_{VM} cells suggests an enhancement of their innate functionality with age.

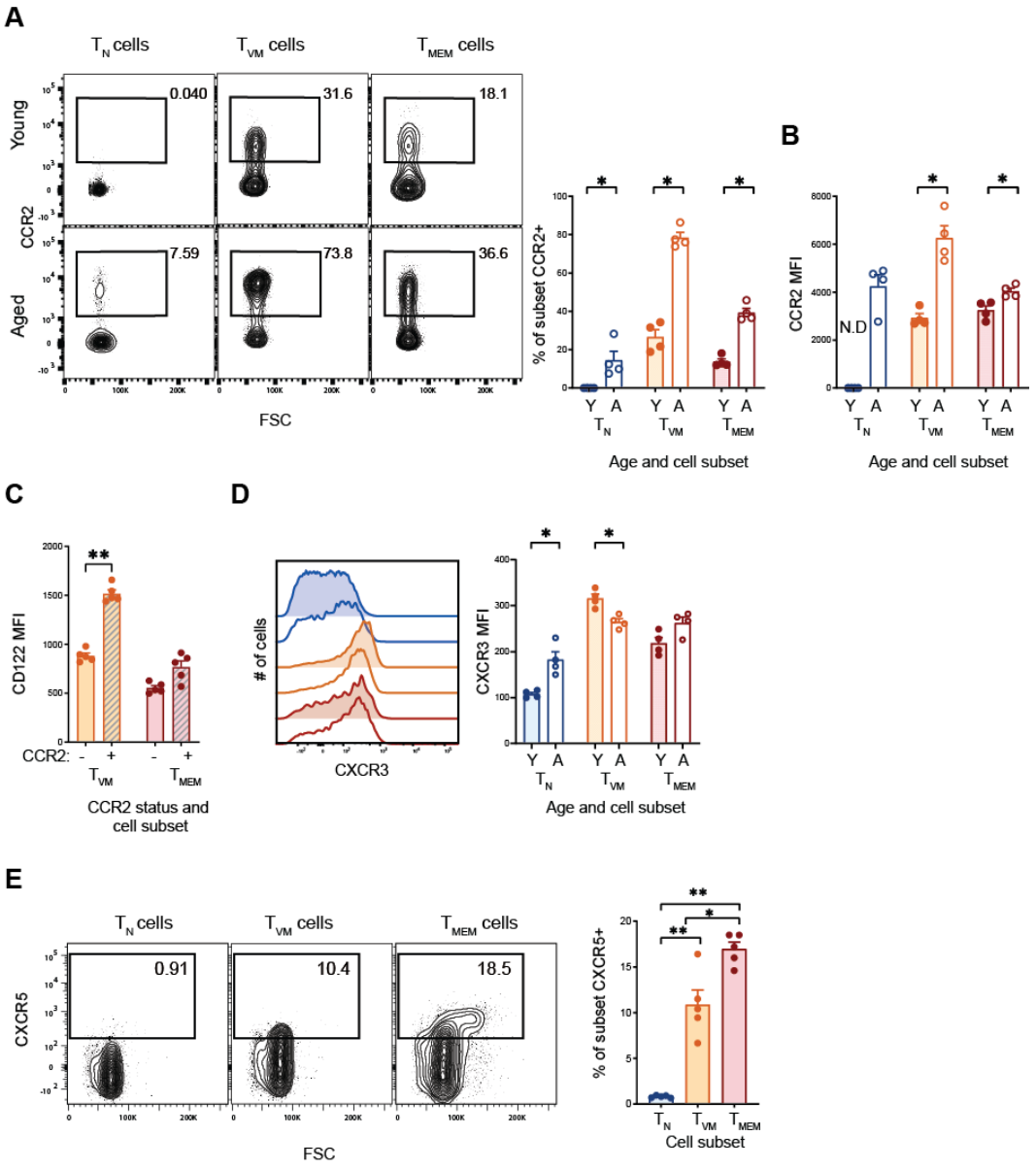

Figure 3.2. Chemokine receptor expression on young and aged T_{VM} cells. A) Representative contour plots and bar graph showing the frequency of young T_N, T_{VM} and T_{MEM} cells that express CCR2 and B) the MFI of CCR2 in young and aged T_N, T_{VM} and T_{MEM} cells. C) MFI of CD122 on CCR2- and CCR2+ T_{VM} and T_{MEM} cells in young mice. D) Representative histograms depicting expression of CXCR3 in young and aged T_N, T_{VM} and T_{MEM} cells and bar graph showing MFI of CXCR3 on each subset. E) Representative contour plots gated on T_N, T_{VM} and T_{MEM} cells and depicting frequency of expression of CXCR5 across the three subsets in young mice. Data is representative of 2 different experiments: n=4-5 mice per group, *p<0.05, **p<0.01 using Mann Whitney test. Bar graphs represent mean ± SEM. N.D: not detected.

3.2.2. Innate-like functions of young and aged T_{VM} cells

Previous work from our lab has shown that T_{VM} cells from aged mice retain responsiveness to IL-15 *in vitro* [95], supporting the notion that T_{VM} cells may switch from predominantly adaptive-like functions in young mice to a more innate-like cell in advanced age, a shift that has been described for other CD8 T cell subsets in humans [155]. We assessed whether ageing augmented innate-like functionality of T_{VM} cells with age. T_{VM} cells are known to express NKRs including NKG2D and NKG2A[97,203], which are thought to impart innate-like responses, including cytotoxicity, and NKR expression is often sensitive to IL-15 signaling [132,133,204]. In aged mice, we observed a >5-fold increase in the proportion of T_{VM} cells that expressed NKG2D as well as a significant increase on a per cell basis (Figure 3.3 A, B). Similarly, pan-NKG2A/C/E expression was also increased on aged, compared to young, T_{VM} cells (Figure 3.3 C). Notably, increases in NKRs were only observed on T_{VM}, and not T_N and T_{MEM}, cells.

Recent reports have suggested that sestrins, which are stress-induced proteins, can inhibit TCR signaling by localizing with AMPK and the MAP kinases in CD4 T cells[191]. It was later shown that similar inhibitory pathways existed in CD8 T cells as well, where NKG2D co-localization with DAP12 and sestrins dampened TCR signaling and activated innate like killing programs in terminally differentiated CD8 T cells in both humans and mice[155]. As we observed a significant increase in NKG2D expression on T_{VM} cells with age, we assessed whether it was inversely correlated with TCR function in young and aged T_{VM} cells. To investigate this, T_{VM} cells were sorted from young and aged mice based on NKG2D expression, CTV labelled, and then stimulated *in vitro* for 65 hours with anti-CD3/-8/-11a Ab (described in Section 2.8.2). When CTV dilution was assessed in young cells, NKG2D- T_{VM} cells proliferated significantly

more than NKG2D+ T_{VM} cells (Figure 3.3 D). When proliferation of NKG2D+ and NKG2D- T_{VM} cells from aged mice was assessed in a separate experiment, NKG2D- T_{VM} cells did not differ significantly from NKG2D+ T_{VM} cells in their proliferative capacity (Figure 3.3 E). Further investigation is required to determine if expression of NKG2D on T_{VM} cells accurately predicts TCR-mediated functionality in T_{VM} cells from aged mice.

We next examined whether the increased NKR expression with age resulted in enhanced innate-like killing capacity. Other studies have reported that T_{VM} cells produce IFN-γ with cytokine stimulation [94,96] and that bystander activated CD8 T cells are able to mediate killing in an NKG2D-dependent manner [100]. IL-12 and IL-18 are known to activate T_{VM} cells , and lack of IL-15 leads to reduced NKG2D expression and GrB production in T_{VM} cells [97]. As NKG2D is an activating NKR that triggers apoptosis in target cells upon engagement with the Rae-1 ligand, we focused on NKG2D-mediated killing of YAC-1 cells (which express the NKG2D ligand, Rae-1) with IL-12/15/18 stimulation in culture. Briefly, sorted young and aged T_N, T_{VM} and T_{MEM} effectors were co-incubated with CTV labelled YAC-1 cells, at a 10:1 effector to target (E:T) ratio in cytokine stimulation media and T cell cytotoxicity was assessed (as described in Section 2.10).

T_{MEM} cells had the highest killing capacity, which was nearly two-fold greater than young T_{VM} cells, but ageing had no impact on T_{MEM} cell cytotoxic capacity (Figure 3.3 F). Young T_{VM} cells appeared to mediate slightly more cytotoxicity of targets than aged T_{VM} cells, but aged T_{VM} cells had significantly poorer viability in culture (30-40%) compared to young T_{VM} cells (65%) after overnight incubation with YAC target cells (Figure 3.3 G). When killing capacity was

normalized to viability, no difference was observed between young and aged T_{VM} cells (Figure 3.3 H).

To confirm that the non-antigen specific cytotoxicity was mediated by engagement of NKG2D receptors, NKG2D was blocked by addition an anti-NKG2D antibody prior to coincubation of effector T_{VM} cells with targets. To ensure that NKG2D was fully saturated with blocking antibody, T cells were incubated with the blocking antibody and then stained with fluorescently labelled anti-NKG2D to demonstrate that no NKG2D+ signal was observed (Figure 3.3 I). Surprisingly, while NKG2D blockade resulted in a significant reduction in the killing capacity of young T_{VM} cells (Figure 3.3 J), it did not have the same effect on aged T_{VM} cells, and possibly even slightly (albeit not significantly) augmented the killing capacity of aged T_{VM} cells. These results suggest that, at least for young T_{VM} cells, antigen-independent killing is largely mediated by NKG2D.

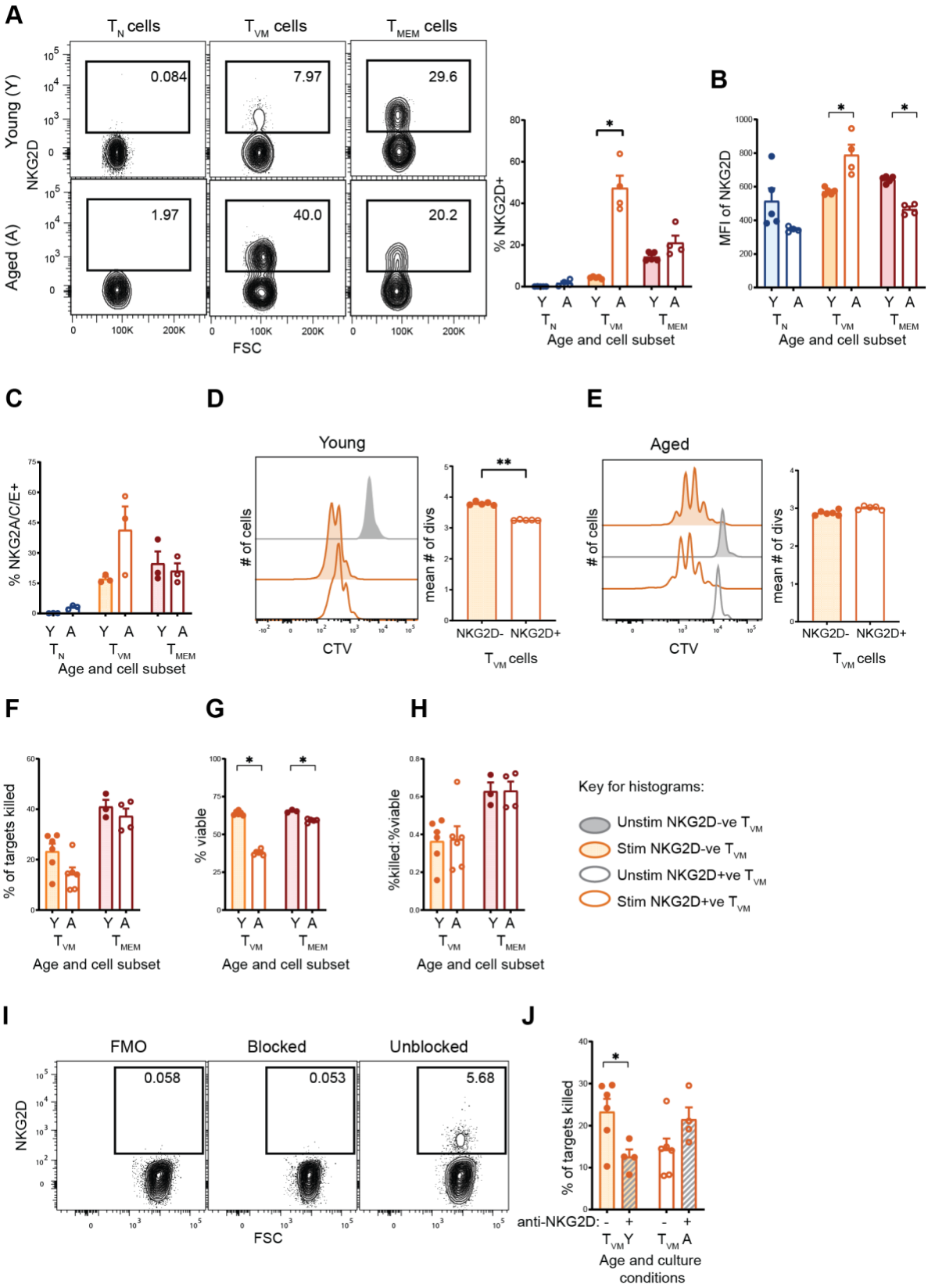

Figure 3.3. NKR expression and innate-like cytotoxicity of young and aged T_{VM} cells A) Representative contour plots showing NKG2D expression on gated live, TCR+CD8+ T_N, T_{VM} and T_{MEM} cells from young

(Y) and aged (A) mice and bar graphs depicting the frequency of NKG2D expressing cells in each subset with B) showing the MFI of NKG2D in young and aged CD8 T cell subsets. Data is representative of 3 experiments. C) Frequency of T_N, T_{VM} and T_{MEM} cells that are expressing NKG2A/C/E. D) Representative histograms showing CTV dilution and bar graphs showing the mean number of divisions in sorted NKG2D+ and NKG2D- T_{VM} cells after 65 hours of stimulation with anti-CD3/8/11a Ab from young and E) aged mice. F) Percentage of specific killing, G) frequency of viable cells and H) ratio of the percentage of specific killing to the frequency of viable cells in 16h co-cultures of young and aged T_{VM} and T_{MEM} cells with YAC-1 targets and IL-12/15/18 stimulation. I) Representative contour plots showing NKG2D expression on young T_{VM} effector cells with or without anti-NKG2D blocking antibody and J) Percentage of specific killing by young or aged T_{VM} cells with and without the addition of anti-NKG2D blocking antibody. C-J were performed once. All *in vitro* analysis is based on sorted cells pooled from 4-5 mice per group * $p<0.05$, ** $p<0.01$ using Mann Whitney test. Bar graphs represent mean ± SEM.

The ability of T_{VM} cells to produce IFN-γ in response to IL-12 and IL-18 has been demonstrated in young T_{VM} cells [94]. Additionally, IL-15 is known to enhance GrB production in CD8 T cells [114]. Therefore, to assess the IFN-γ as well as GrB, we measured IFN-γ and GrB production after IL-12 and IL-18 stimulation, with and without IL-15 in a 36-hour(h) assay. The presence of IL-15 had no impact on the production of IFN-γ by T_{VM} or T_{MEM} cells (Figure 3.4 A), however we observed a nearly 50% increase in GrB production in T_{VM} cells with IL-15 supplementation (Figure 3.4 B). IL-15 has been shown to stimulate GrB production via activation of the STAT5 pathway in both NK cells and CD8 T cells [97,114]. We also observed increased IFN-γ and GrB in T_N cells with IL-15 supplementation (Figure 3.4 A, B), which was surprising as T_N cells have very low expression of CD122 (Figure 3.1 C).

Since we observed very poor viability of CD8 T cells subsets after a 36h stimulation, we tested a shorter stimulation period and assessed the capacity of aged T_{VM} cells to produce IFN-γ and GrB like young T_{VM} cells. After 16h stimulation with IL-12/IL-18/IL-15, we observed that T_N cells were largely unresponsive to shorter periods of cytokine stimulation (Figure 3.4 C, D). T_{VM} cells maintained responsiveness to *in vitro* cytokine stimulation with age. In contrast, aged T_{MEM} cells showed a near complete loss of their ability to produce IFN-γ and GrB with age, signifying

that aged T_{MEM} cells lose response to these cytokine cues (Figure 3.3 C, D). Similar observations were made in our earlier study where T_{MEM} cells had a complete loss of IL-15 mediated proliferation with age [95].

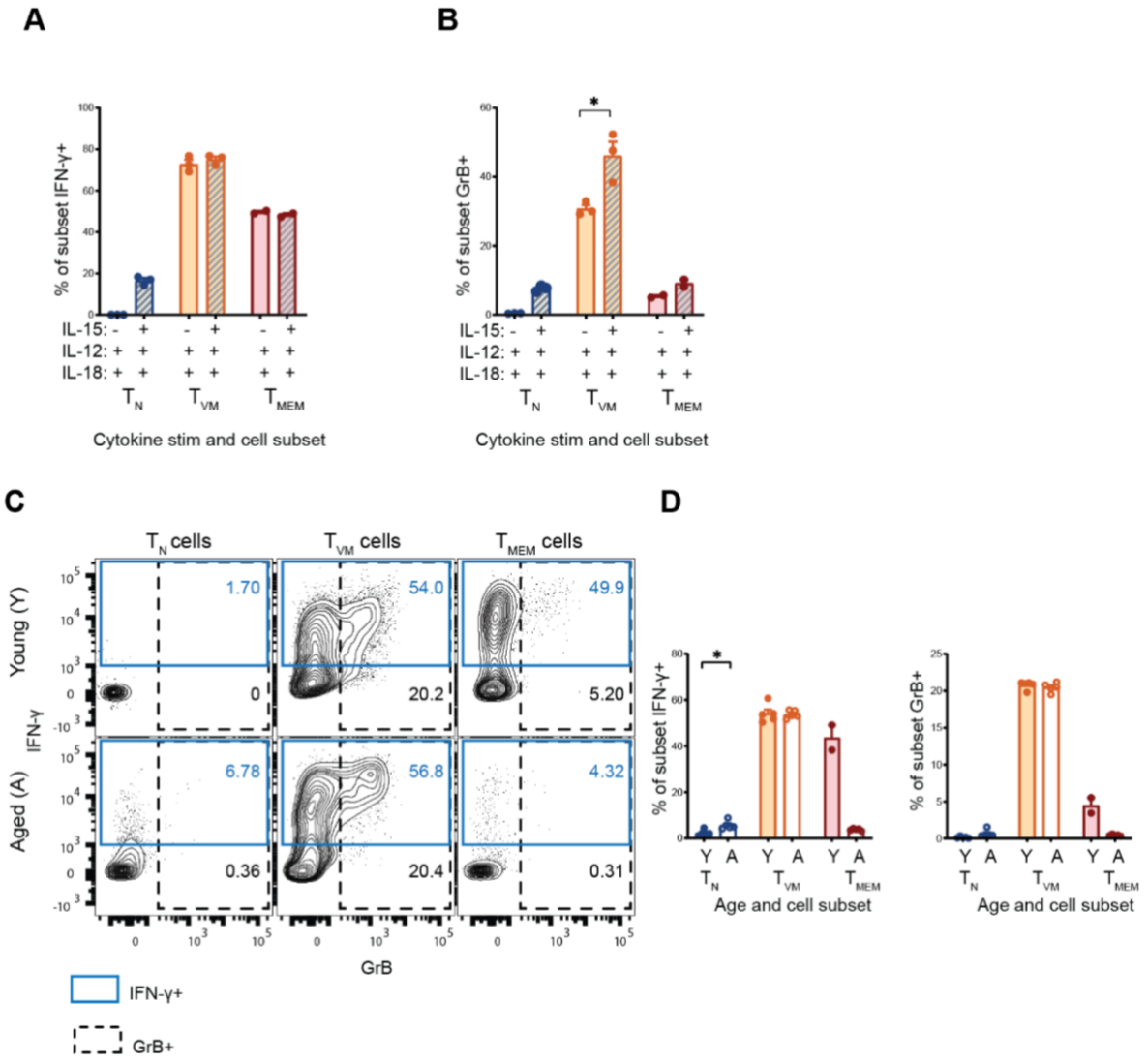

Figure 3.4. Innate-like cytokine production in young and aged T_{VM} cells A) Bar graph depicting frequency of A) IFN-γ and B) GrB production in T_N, T_{VM} and T_{MEM} cells after 36h culture with IL-12/18 or IL-12/15/18 stimulation. C) Representative contour plots depicting total IFN-γ and GrB production in young and aged T_N, T_{VM} and T_{MEM} cells and D) bar graphs showing the respective frequencies IFN-γ+ and GrB+ cells in each subset after 16h of IL-12/15/18 stimulation. For A and B, data is from 1 experiment. For C and D, data are representative of 2 experiments with cells pooled from 3-5 mice per group. *p<0.05, **p<0.01 using Mann Whitney test. Bar graphs represent mean ± SEM.

3.2.3. Modulation of IL-15 response in T_{VM} cells with age

Young T_{VM} cells proliferate in response to IL-15 stimulation *in vitro* [95] and this IL-15 sensitivity appears to be largely maintained in T_{VM} cells from aged mice [34]. We had observed significant phenotypic differences in young and aged T_{VM} cells (Fig 3.1, 3.2), therefore, we next assessed *in vivo* differences modulated by increased IL-15 signaling. To determine the extent to which T_{VM} cells from young and aged mice responded to increased IL-15 or IL-15 signaling *in vivo* we took two approaches. Firstly, we analysed T_{VM} cells in mice lacking a key suppressor of cytokine signaling (SOCS) family member, cytokine-inducible SH2-containing protein (CIS). The SOCS family of proteins has 8 members, including (CIS, gene: *Cish*) and SOCS1-7. Both CIS and SOCS1 are negative regulators of IL-15 signaling pathway and inhibit the enzymatic activity of JAK1 [200,217,218]. CIS is also reported as a negative regulator of TCR signaling [219]. Analysis of T_{VM} cell number in *Cish*-/- mice revealed, surprisingly, no difference in T_{VM} cell frequency or number compared to WT controls (Figure 3.5 A). CD122 expression was modestly reduced on T_{VM} and T_{MEM} cells from *Cish*-/- mice, down by 20% compared to WT controls (Figure 3.5 B). These results suggest that CIS might not limit IL-15 signaling in T_{VM} cells, or possibly T cells more broadly. Indeed, CIS has mainly been shown to limit IL-15 signaling in the context of NK cells [218].

Given that no numeric differences in T_{VM} cells were observed in a mouse model of enhanced IL-15 signaling, we next determined the impact of exogenous administration of IL-15. Previous studies have reported an immediate increase in T_{VM} cells with IL-15 treatment [35,97] but it is not known i) whether this increase translates to differences in functional responses, and ii) whether aged T_{VM} cells display a similar sensitivity to increased IL-15 *in vivo*.

To evaluate the impact of increased IL-15 signaling *in vivo*, we first selected an appropriate dose of IL-15 complexed with the IL-15Rα:Fc (cIL-15) (described in Section 2.3.1), which delivers a potent IL-15 signal [199,204]. Young (8-12 week old) mice were treated with two different doses of cIL-15 and CD8 T cell number and phenotype was assessed at day (d)5 post treatment. T_{VM} cells increased nearly 3-fold with 2 µg of c-IL15 and by 10-fold with 4 µg of c-IL15 (Figure 3.5 C). While T_N and T_{MEM} cell numbers were not significantly increased with low dose treatment, they increased 2-3 fold after high dose administration of cIL-15 (Figure 3.5 C). These results suggest that T_{VM} cells have enhanced *in vivo* sensitivity to IL-15 compared to other subsets and their response is proportional to level of IL-15. Given that the 2 µg dose of cIL-15 appeared to act more selectively on T_{VM} cells, this dose was selected for use in further experiments. To determine whether the IL-15-mediated increase in T_{VM} cells was transient or sustained over extended periods, young mice were treated with cIL-15 and CD8 T cell number and phenotype was assessed in the spleen at d5 and d90 post treatment. T_{VM} cell numbers were significantly increased by d5 after cIL-15 treatment but had almost returned to baseline by d90 (Figure 3.5 D). This illustrates that the increase in T_{VM} cell number with increased IL-15 signaling is transient.

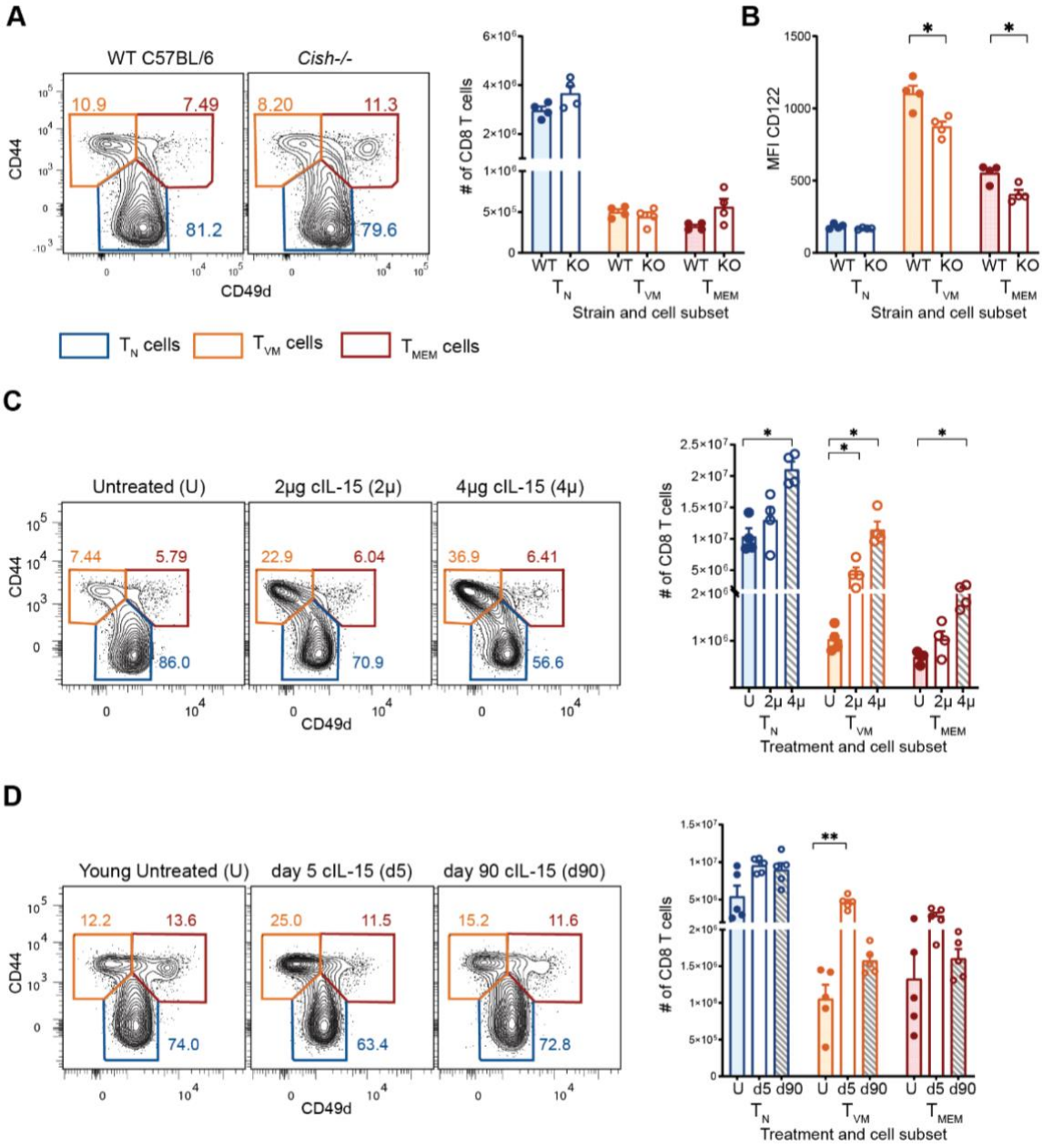

Figure 3.5. Response of young mice to exogenous IL-15 treatment. A) Representative contour plots gated on live, TCR+CD8+ T cells showing the frequency of cells that are of T_N, T_{VM} and T_{MEM} phenotype and bar graph of the number of cells in each subset in the spleen of young wildtype C57BL/6 (WT) and *Cish*$^{-/-}$ (KO) mice. B) MFI of CD122 on each subset. C) Representative contour plots depicting the frequency of cells that are T_N, T_{VM} and T_{MEM} phenotype and bar graph of the number of cells in each subset in the spleen of young WT mice that are untreated (U), treated with 2 µg (2µ) or 4 µg (4µ) of cIL-15, at d5 post treatment. D) Representative contour plots showing the frequency of cells that are T_N, T_{VM} and T_{MEM} phenotype and bar graph of the number of cells in each subset in the spleen of young WT mice untreated or at d5 or d90 after treatment with 2 µg of cIL-15. Data is representative of 2 experiments with n=4-5 mice per group, except analysis of Cish$^{-/-}$ mice and 4 µg cIL-15 treatments, which were performed once. *p<0.05, **p<0.01 using Mann Whitney test. Bar graphs represent mean ± SEM.

We previously demonstrated that aged T_{VM} cells retained the ability to proliferate in response to IL-15 *in vitro*[95]. To determine the extent to which aged T_{VM} cells retain the ability to respond to IL-15 *in vivo*, young (8-12 week old) and aged (18-20 month old) mice were treated with 2 µg of cIL-15 (section 2.3.1) and splenic CD8 T cell number was assessed 7 days later. As shown previously for 5 days post-treatment (Figure 3.5 C), young T_{VM} cells had significantly increased in 7 days after cIL-15 treatment (Figure 3.6 A, B). While aged T_{VM} cells showed a trend toward higher numbers with cIL-15 treatment, the variation in T_{VM} cell numbers in aged mice, meant that this increase was not significant (Figure 3.6 A, C). Aged mice were then treated with a higher (4 µg) dose of cIL-15 and similar results were observed as seen with high dose treatment in young mice (Figure 3.5 C), with a >10-fold increase in aged T_{VM} cells 7 days after treatment (Figure 3.6 D). Paired analysis of blood taken at d0, d7 and d14 post treatment revealed that the increase observed at d7 after cIL-15 treatment contracted slightly on d14, however T_{VM} cells were still significantly increased compared to untreated controls (Figure 3.6 E). As in young mice, administration of the high dose of cIL-15 also drove a >3-fold increase in T_N and T_{MEM} cells, with the expansion being markedly greater in aged, compared to young, T_N cells. Within the T_N cell population, aged mice contain a larger proportion of CD44intermediate ($CD44^{int}$) T_N cells due to attrition of the CD44low ($CD44^{lo}$) T_N cells over a life course [185]. It is possible that the increased effect of cIL-15 administration on aged T_N cells is due to a heightened sensitivity of $CD44^{int}$ T_N cells to IL-15 signaling. Indeed, there have been studies that showed that human and mouse T_N cells can proliferate and differentiate to a T_{CM} and T_{VM} like phenotype, respectively, after IL-15 stimulation [97,220]. We will explore the ability of mouse T_N cells to respond to IL-15 stimulation in Chapter 4.

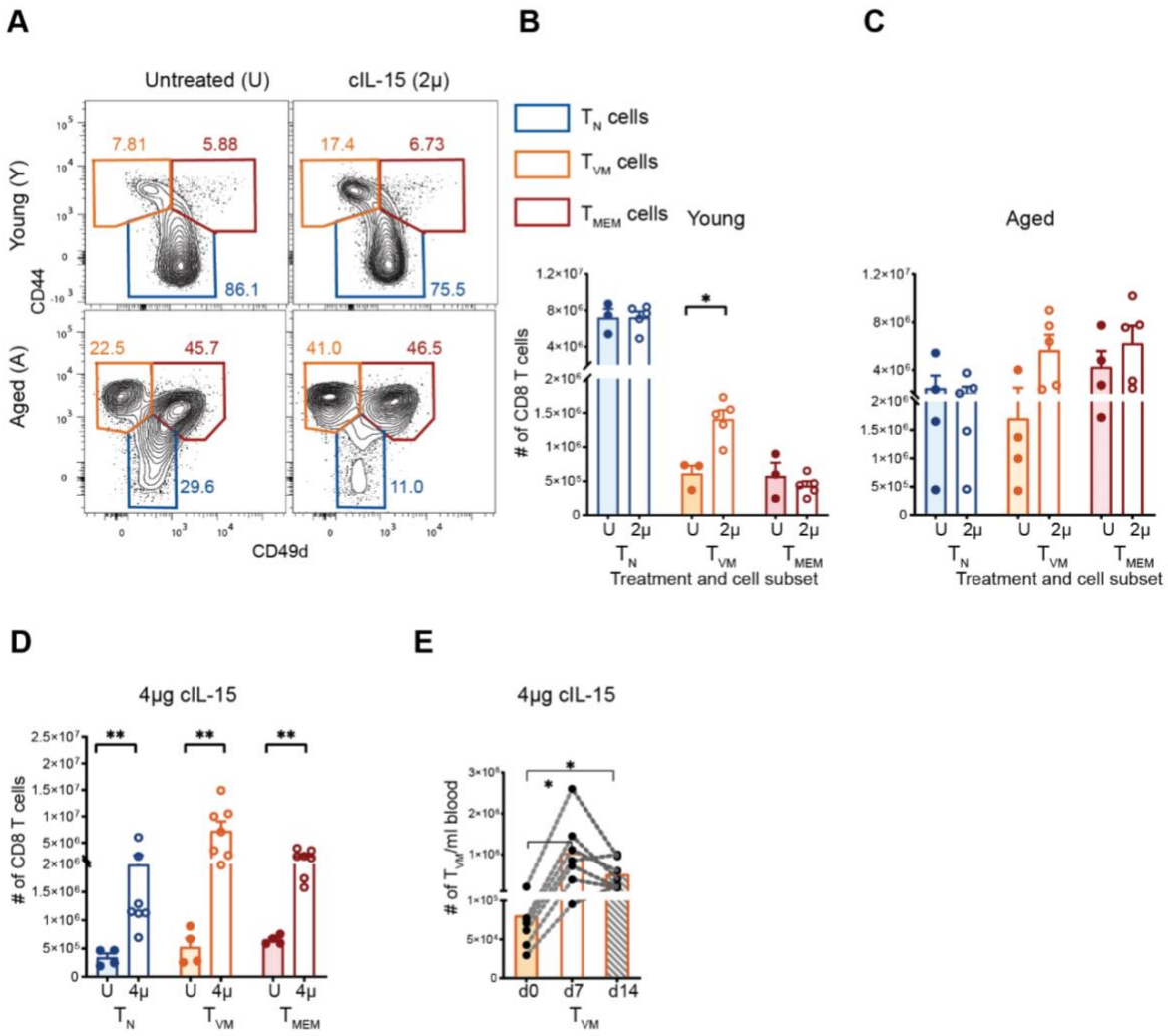

Figure 3.6. Comparison of CD8 T cell response to IL-15 treatment from young and aged mice. A) Representative contour plots gated on live, TCR+CD8+ T cells showing frequency of cells that are T_N, T_{VM} and T_{MEM} phenotype and B) bar graph of the number of cells in each subset in the spleen of young and C) aged WT untreated (U) mice or at d7 after treatment with 2 μg of cIL-15. D) Bar graph of the number of cells in each subset in the spleen of aged WT mice untreated or at d14 after treatment with 4 μg of cIL-15 and E) paired analysis of blood drawn from the same mice at d0, d7 and d14 post treatment. Data is representative of 1 experiment with n=4-6 mice per group. *p<0.05, **p<0.01 using Mann Whitney test. Bar graphs represent mean ± SEM.

3.2.4. Phenotypic and functional responses to IL-15 in young and aged T_{VM} cells

Given the marked expansion of T_{VM} cells with cIL-15 treatment, we determined whether increased IL-15 availability also caused phenotypic and functional changes in young and aged T_{VM} cells. Both CD122 and NKG2D dictate innate-like functionality in T_{VM} cells, therefore we first looked at the expression of these receptors. Expression of CD122 was increased modestly on T_{VM} and T_{MEM} cells in young cIL-15-treated mice at day 5 by nearly 20% (Figure 3.7 A), but this increase was not maintained over time on either subset (Figure 3.7 B). No difference in CD122 expression was observed on aged T_{VM} cells after treatment (Figure 3.7 C). The frequency of NKG2D+ T_{VM} and T_{MEM} cells was increased in young mice after cIL-15 treatment by nearly 40% (Figure 3.7 D), however the level of NKG2D expression on young T_{VM} cells was reduced after cIL-15 treatment (Figure 3.7 E). cIL-15 treatment of aged mice had no impact on frequency of NKG2D+ cells or level of NKG2D expression on T_{VM} cells or T_{MEM} cells (Figure 3.7 F, G). These data suggest that IL-15 treatment increased proliferation of NKG2D+ T_{VM} cells in young but not aged mice.

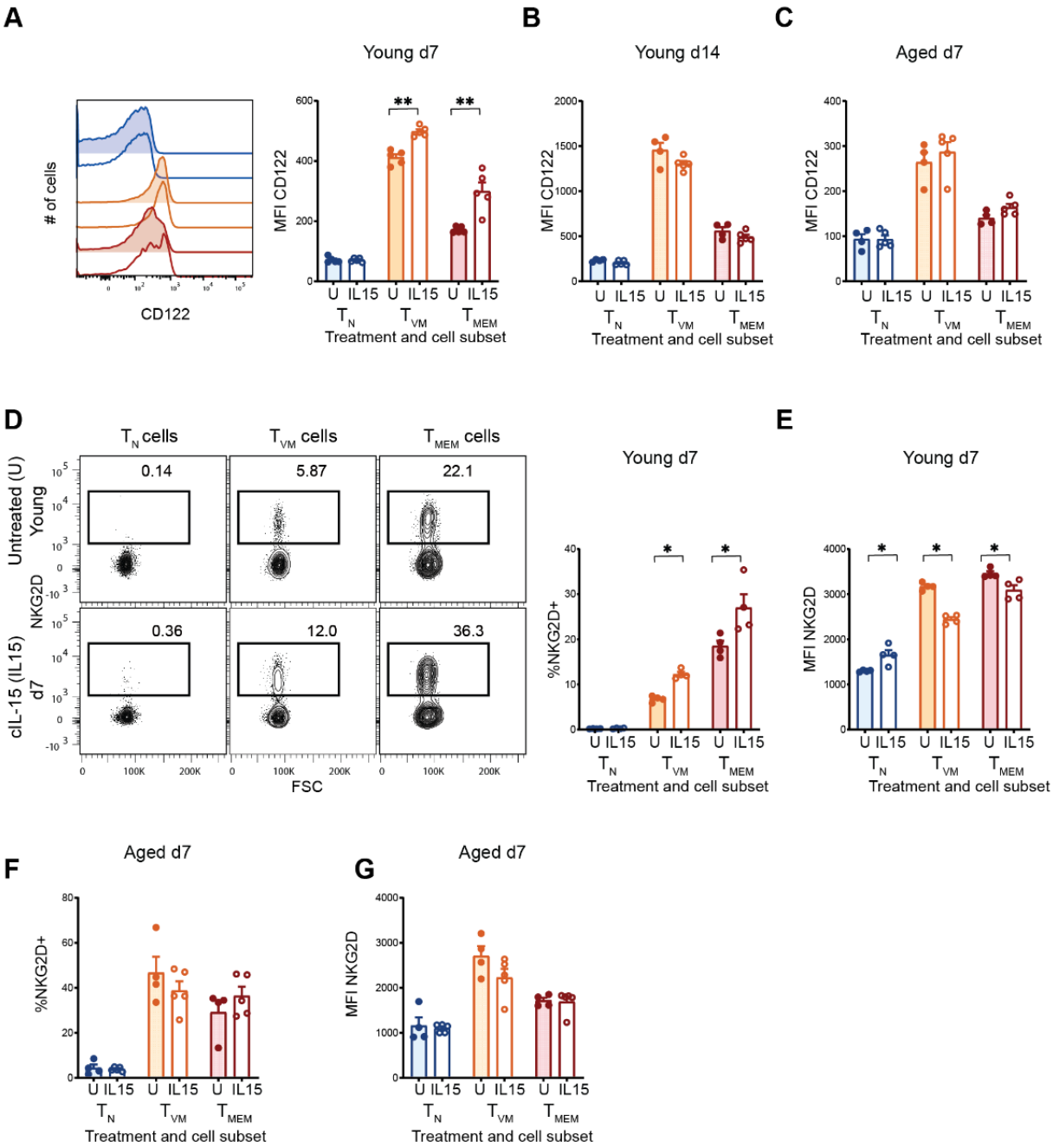

Figure 3.7. I) Impact of cIL-15 treatment on phenotype of young and aged CD8 T cells A) Representative histograms showing expression of CD122 on live/TCR+CD8+ T_N, T_{VM} and T_{MEM} cells from untreated (U) and cIL-15 treated (IL15) young mice at A) d7 and B) d14 after treatment. C) MFI of CD122 on CD8 T cell subsets in aged treated and untreated mice at d7 after treatment. D) Representative contour plots showing NKG2D expression on live, TCR+CD8+ T_N, T_{VM} and T_{MEM} cells from untreated and cIL-15 treated young mice with bar graphs of the frequency of T cell subsets that are NKG2D+ and E) MFI of NKG2D on the NKG2D+ CD8 T cell subsets. F) Bar graphs showing the frequency of T cell subsets that are NKG2D+ and G) MFI of NKG2D on the NKG2D+ CD8 T cell subsets in untreated and cIL-15 treated aged mice at d7 post treatment. All mice were treated with 2 µg of cIL-15. Young mice data is representative of 3 experiments with n=4-5 mice per group. Aged mice data is from 1 experiment with n=4-5 mice per group. *p<0.05, **p<0.01 using Mann Whitney test. Bar graphs represent mean ± SEM.

There have been some reports of *in vitro* IL-15 stimulation of human CD8 T cells led to downregulated CD5 expression [221]. We also observed that cIL-15 treatment resulted in reduced CD5 expression on young T_{VM} cells and T_{MEM} cells compared to their untreated subsets, with a more marked downregulation observed on aged T_{VM} cells after treatment (Figure 3.8 A, B).

Key transcription factors for T_{VM} cells upstream and downstream of IL-15 signaling include Eomes and Bcl-2 [31,35,158,222]. Bcl2 was downregulated by 20-30% on young and aged T_{VM} cells with cIL-15 treatment (Figure 3.8 C, D). Eomes expression also trended down on young and aged T_{VM} cells after cIL-15 treatment, however these differences were not significant (Young p=0.2 Aged: p=0.066) (Figure 3.8 D).

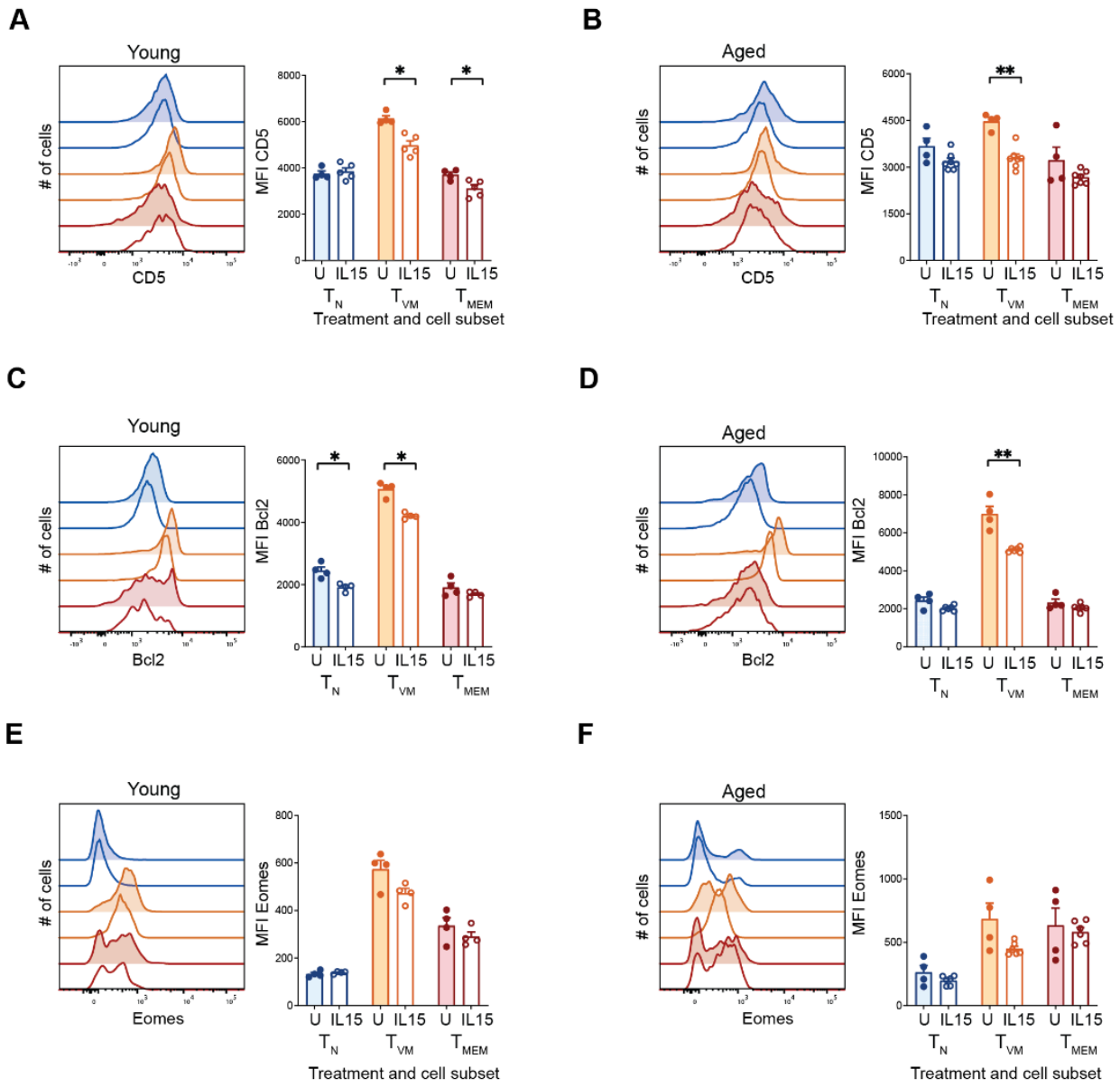

Figure 3.8. II) Impact of cIL-15 treatment on phenotype of young and aged CD8 T cells. Representative histograms and bar graphs showing expression of CD5 and MFI on CD8 T cell subsets in A) young and B) aged untreated and cIL-15 treated mice. Representative histograms and bar graphs showing expression of Bcl-2 and MFI in CD8 T cell subsets in C) young and D) aged untreated and cIL-15 treated mice. Representative histograms and bar graphs showing expression of for Eomes MFI in CD8 T cell subsets in E) young F) and aged untreated and cIL-15 treated mice. Young mice were treated with 2 µg of cIL-15 and aged mice were treated with 4 µg of cIL-15, all data is from 1 experiment analyzed at d14 after treatment with n=4-6 mice per group. *p<0.05, **p<0.01 using Mann Whitney test. Bar graphs represent mean ± SEM.

The above results indicate that young and aged T_{VM} cells are responding to IL-15 cues in a similar manner, lending support to the notion that aged T_{VM} cells remain responsive to IL-15

[34,95]. To further compare the intrinsic responsiveness of young and aged T_{VM} cells to IL-15, and to control for differences between the young and aged environments, we sorted young and aged T_{VM} cells and pooled them before transfer to congenically distinct young mice that were then treated with cIL-15 within the next 18 hours (Figure 3.9 A). A week after adoptive transfer, spleens from recipient mice were harvested and T cell proliferation was assessed by CTV dilution. The young T_{VM} cells showed robust proliferative capacity as described previously *in vitro* [95], dividing up to 4 times on average *in vivo* (Figure 3.9 B). In terms of phenotype, we found similar results as was previously seen in IL-15 treated intact young mice (Figure 3.7). The treated T_{VM} cells had significantly increased CD122 expression (Figure 3.9 C). The expression of NKG2D mirrored our previous results with an increased proportion of NKG2D+ T_{VM} cells seen after treatment of young T_{VM} cells, alongside a moderately reduced expression of NKG2D (Figure 3.7 D, E).

In case of aged T_{VM} cells adoptively transferred to young mice, there was a modest but reproducible decline in IL-15 mediated proliferation compared to young T_{VM} cells *in vivo* (Figure 3.9 B). In previous reports by our group, we observed that IL-15 mediated proliferative capacity did not differ between young and aged T_{VM} cells *in vitro*[95]. It is possible that given the high CD122 expression on aged T_{VM} cells (Figure 3.1 C), and their high basal proliferation (Figure 3.1 B), aged T_{VM} cells may reach their saturation point earlier than young T_{VM} cells, thus limiting the extent of aged T_{VM} cell proliferation. This data combined with our previous reports of aged T_{VM} cells having more CD122 expression and increased pSTAT5 after *in vitro* IL-15 treatment [95], suggest that enhanced expression of these markers of sensitivity to IL-15 do not necessarily result in a corresponding increase in IL-15 mediated proliferation of aged T_{VM} cells.

In contrast to the IL-15 treatment of intact aged mice (Figure 3.7 C), we observed a very modest, but significant increase in CD122 expression on aged T_{VM} cells after adoptive transfer and IL-15 treatment (Figure 3.9 C). However, the increase in young cIL-15 treated T_{VM} cells was three-fold that observed in aged T_{VM} cells (Figure 3.9 C). Similar to our previous results in intact aged mice, we did not observe an increase in proportion of NKG2D+ cells after IL-15 treatment in aged T_{VM} cells, but there was marked downregulation of NKG2D expression in aged treated T_{VM} cells (Figure 3.9 D, E). It is possible that the lack of increase in NKG2D+ aged T_{VM} cells and the limited change in CD122 expression after IL-15 treatment is because the aged cells, which have an increased basal expression of NKG2D (Figure 3.3 A) and CD122 (Figure 3.1 C), are already maximally expressing these receptors. Collectively, these results indicate that aged T_{VM} cells can respond to IL-15 cues *in vivo* however due to their already elevated basal proliferation and activation states, the impact of IL-15 on the proliferation and phenotype of aged T_{VM} cells is attenuated. Titrating the amount of administered IL-15 may be able to interpret the extent of the age-related defect in IL-15-induced proliferation in a better way.

T_{VM} cells exhibit efficient innate and antigen-specific functions in young individuals, where they respond more rapidly than T_N cells to TCR mediated stimuli [85,95]. However, their TCR-associated function declines in aged individuals [95,186]. Given their exquisite sensitivity to IL-15, which is not greatly impaired by the ageing process, we next investigated the integration of the innate and adaptive functions of T_{VM} cells and assessed whether their sensitivity to IL-15 can influence their responsiveness to TCR mediated stimuli. T_N, T_{VM} and T_{MEM} cells were sorted from young and aged mice that were either untreated or at d14 after cIL-15 treatment, stained with CTV and polyclonally stimulated *in vitro* (as described in Section 2.8.1). Polyclonal stimulation used two different concentrations of anti-CD3 (1 µg/mL vs 10 µg/mL) (Section 2.7). After 65 hours

of stimulation, the mean number of divisions was calculated using CTV dilution. A modest reduction in TCR-driven proliferative capacity was seen in T_{VM} cells from young, cIL-15 treated mice compared to young, untreated mice using a lower concentration of anti-CD3 (Figure 3.9 F), although this difference was not significant at the higher concentration (Figure 3.9 G). There was no difference in TCR-driven proliferation in aged T_{VM} cells with cIL-15 treatment (Figure 3.9 H), at either dose of cIL-15 (2 µg vs 4 µg) or with either dose of anti-CD3. Of note, the TCR-driven proliferative capacity of aged T_{MEM} cells increased with cIL-15 treatment using a lower dose of anti-CD3 (Figure 3.9 H) but the increase was not observed with higher dose of anti-CD3 (Figure 3.9 I).

These results indicate that short term IL-15 exposure can modestly impede TCR-mediated responses in young T_{VM} cells but does not appear to influence aged T_{VM} cells.

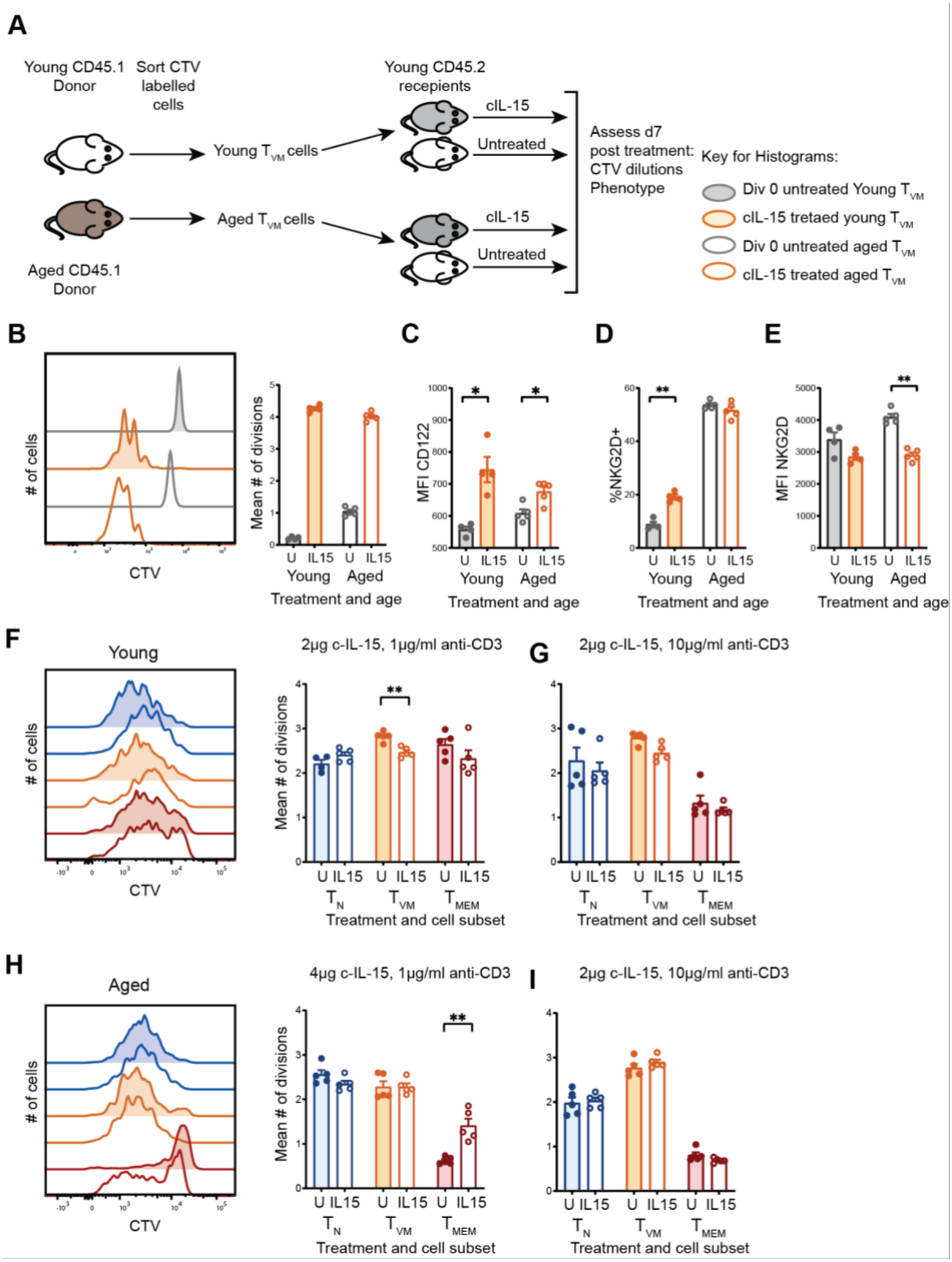

Figure 3.9. Intrinsic and functional comparison of young and aged TVM cell's response to IL-15. A) Schematic of the adoptive transfer of young and aged T_{VM} cells to untreated (U) or cIL-15 treated (IL15) mice. B) Representative histograms showing CTV dilution in transferred cells and bar graph showing mean number of divisions in transferred cells from each subset and treatment group. C) MFI of CD122 on transferred cells from each subset and treatment group. D) The proportion of transferred cells that

are NKG2D+ and E) the MFI of NKG2D on NKG2D+ transferred cells from untreated and cIL-15 treated recipients. Data are representative of 2 experiments with n=2-5 mice per group analyzed at d7 of 2 µg cIL-15 treatment. F) Representative histograms depicting CTV dilution in sorted CD8 T cell subsets after 65 hours of *in vitro* stimulation with anti-CD3/-CD8/-CD11a and bar graph showing the corresponding mean divisions. Experiment was performed once, using sorted cells from 4-5 pooled mice. Young mice were treated with 2 µg of cIL-15 and aged mice were treated with 2 µg or 4 µg of cIL-15. *p<0.05, **p<0.01 using Mann Whitney test. Bar graphs represent mean ± SEM.

3.2.5. *De novo* generation of T_{VM} cells from T_N cells

We have demonstrated that T_{VM} cells from young and aged mice are sensitive to increased IL-15, resulting in transient T_{VM} cell expansion. Other mechanisms that can contribute to an expanded T_{VM} cell subset is the acquisition, by T_N cells, of a T_{VM} phenotype in response to inflammatory cues, with such conversion being previously reported during lymphopenic conditions [87,223] and with IL-15 treatment [97]. We therefore assessed whether a more physiological perturbation, i.e., infection driven inflammation, could also induce an increase in T_{VM} cells and whether this was due to proliferation or conversion of T_N cells. Influenza A virus (IAV) infection of C57BL/6 mice is a model of acute, tissue-localised infection. A recent study has shown that T_{VM} cells respond rapidly to IAV by infiltrating the lungs[166]. To assess IAV-driven proliferation and/or conversion to a T_{VM} phenotype, CD8 T_N were sorted from young mice, CTV-labelled and adoptively transferred to congenically distinct recipients, which were then infected with the HKx31 strain of IAV one day later, T_{VM} cells were also adoptively transferred as a control in a separate experiment but CTV dilution was not used to assess IAV-driven T_{VM} cell proliferation (Figure 3.10 A). T_N cells were classified as either $CD44^{lo}$ or $CD44^{int}$ to determine any differential capacity of these subsets to undergo proliferation/conversion. This demarcation was based on observations that $CD44^{int}$ T_N cells appear to be partially differentiated with comparatively higher levels of Eomes and CD5 expression [20] than $CD44^{lo}$

cells, and higher CD5 has been reported to correlate with the capacity of T_N cells to adopt a T_{VM} phenotype [97].. We were unable to assess the proliferation of T_{VM} cells in this experiment due to different baseline CTV labelling prior to transfer. This resulted in our inability to correctly gate the undivided population of T_{VM} cells in the control and infected mice. However, we were able to address the aspect of proliferation and/or conversion of T_N cells to T_{VM} phenotype in these experiments. When proliferation was assessed based on CTV dilutions, only around 15-20% of the transferred T_N cells had divided (Figure 3.10 B). At 14d post-infection, less than 2% of the transferred T_N cells had converted to the T_{VM} phenotype and infection did not alter the rate of conversion (Figure 3.10 C, D) The rate of transition to a T_{MEM} phenotype, indicative of antigen-dependent activation, was increased by infection for all T cell subsets, and was significantly increased for $CD44^{int}$ T_N and $CD44^{hi}$ T_{VM} cells compared to $CD44^{lo}$ T_N cells (Figure 3.10 C, E).

In summary, IAV infection did not induce significant conversion of T_N cells to a T_{VM} phenotype, indicating that infection driven inflammation was not responsible for *de novo* generation of T_{VM} cells in the periphery.

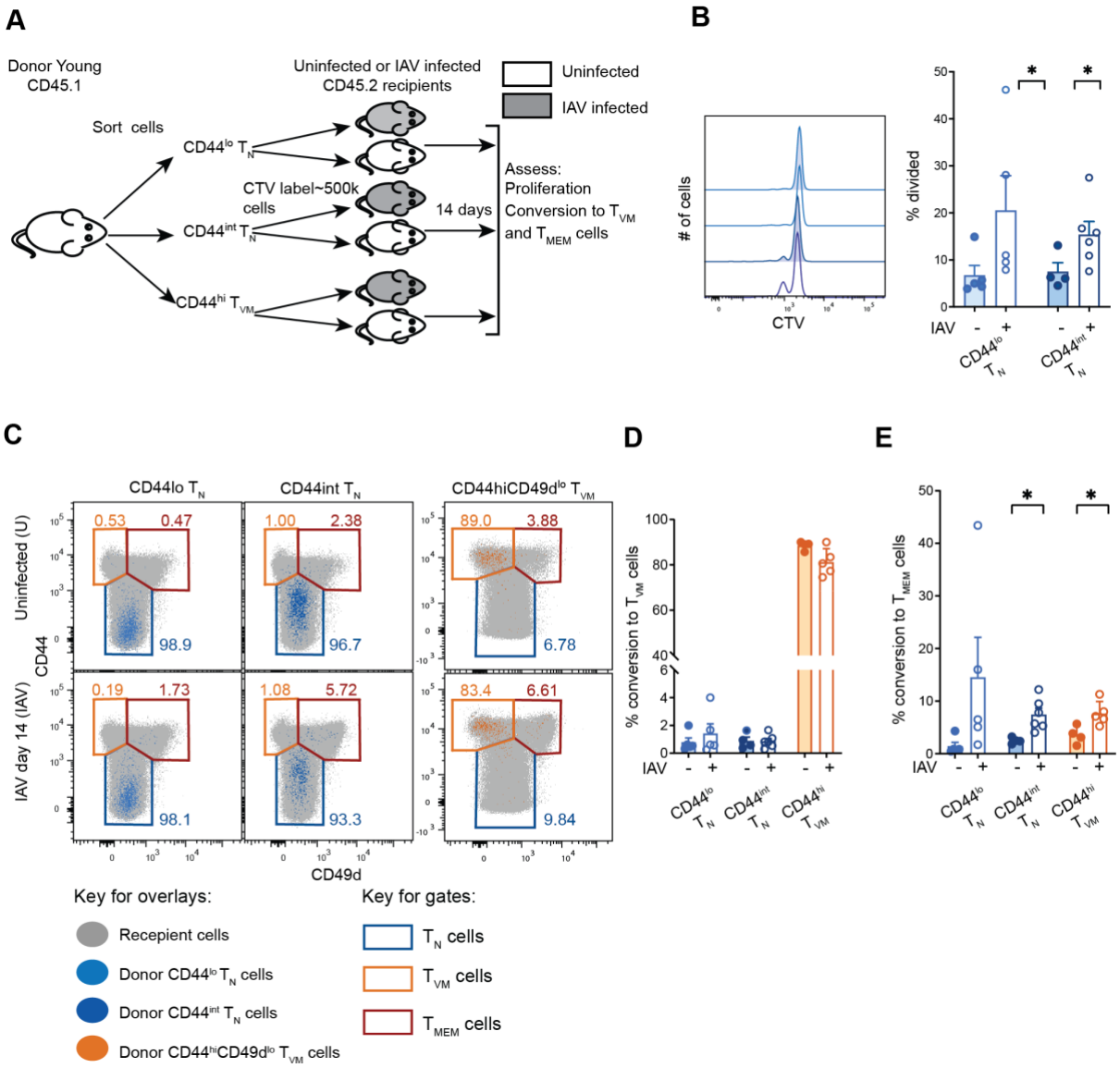

Figure 3.10. Proliferation and conversion of T_N and T_{VM} cells during IAV infection A) Schematic of adoptive transfer of young CD44^{lo} T_N, CD44^{int} T_N and CD44^{hi}CD49d^{lo} T_{VM} cells into recipient mice, followed by no infection or IAV infection and analyzed at d14 after infection. B) Representative histograms of CTV staining and bar graph of the mean number of divisions in transferred CD44^{lo} T_N, CD44^{int} T_N cells from uninfected and IAV infected recipients. C) Representative dot plots of live TCR+ CD8+ T cells, overlaying transferred cells (coloured) onto recipient cells (grey) in uninfected and IAV infected mice. Bar graphs of D) the proportion of transferred cells that exhibited a T_{VM} phenotype and E) the proportion of transferred cells that exhibited a T_{MEM} phenotype in uninfected and IAV infected recipients. T_N cell transfer data is representative of 3 experiments with n=2-4 mice per group. T_{VM} cell transfer data is from a separate experiment performed once. *p<0.05, **p<0.01 using Mann Whitney test. Bar graphs represent mean ± SEM.

3.2.6. TCR mediated responses of young T_{VM} cells

Our group and others have demonstrated that T_{VM} cells acquire a TCR-associated proliferative defect *in vitro* [95,186]. Aged T_{VM} cells have increased survival and heightened expression of senescence markers which may contribute to this loss of functionality with age [95]. In addition to the loss of TCR mediated proliferation in aged T_{VM} cells, we have also demonstrated a loss in antigen-specific cytotoxicity in aged T_{VM} cells *in vitro* [34]. To determine whether this age-associated loss in TCR mediated function observed *in vitro* would translate to poorer antigen specific responses *in vivo*, we required a system to compare contribution of T_{VM} cells to antigen specific responses. To this end, we compared the contribution of polyclonal T_N and T_{VM} cell subsets to an immune response in the IAV infection model. To validate our approach, we co-transferred $1x10^6$ CD45.2+ T_N and $1x10^6$ CD45.1+ T_{VM} cells into TCR$\alpha^{-/-}$ mice, which lack endogenous T cells, that were infected with IAV the previous day (Figure 3.11 A). Tissues were harvested at day 10 after transfer and the transferred CD45.2+ and CD45.1+ T cells were enumerated. We assessed the proportion of donor T_N and T_{VM} cells in the CD49d^{hi} CD8 T cells, to assess which subset had increased capability to undergo antigen-specific activation. We use CD49d as a maker to delineate T_{MEM} cells in this study as it denotes antigen-dependent activation and directs homing of pathogen-specific CD8 T cells to sites of infection [61,62,224]. We assessed if there was differential contribution of T_N and T_{VM} cells to the total CD49d^{hi} CD8 T cells in infected recipients. T_{VM} cells contributed significantly more to the CD49d^{hi} CD8 T cell response in the medLN and lungs and but were not significantly different in the spleens of the infected TCR$\alpha^{-/-}$ recipients (Figure 3.11 B). The results from spleen align with the previous experiment (Figure 3.10 E), where we evaluated capacity of T_N and T_{VM} cell to contribute to

antigen-specific response in separate lymphoreplete hosts and observed equal capacity of T_N and T_{VM} cells to become T_{MEM} cells in the spleen, evaluated by the acquisition of CD49d expression.

To assess the IAV-specific contribution of each donor subset, responses to immunodominant epitopes (H-2D^{b}NP$_{366}$ and H-2D^{b}PA$_{224}$) and sub-dominant epitopes (H-2D^{b}PB1-F2$_{62}$, H-2K^{b}PB1$_{703}$ and H-2K^{b}NS2$_{114}$) were assessed on day 10 using tetramer staining in the spleen. The composition of IAV-specific responses was very variable from mouse to mouse, likely due to the low frequency of epitope specific cells contained within the transferred cells (Figure 3.11 C). Thus, any differences in the relative contribution of T_N and T_{VM} cells to individual epitope-specific immune responses was inconclusive.

Our analysis of CD49d^{hi} cells after infection have shown that T_{VM} cells outcompete T_N cells in homing to sites of infection and have increased capacity to undergo antigen-dependent activation *in vivo*. These results lend support to our previous *in vitro* observations of enhanced TCR-mediated proliferation of T_{VM} cells compared to T_N cells [95]. A limitation of this kind of analysis is that a polyclonal population of T_{VM} and T_N cells will have less than 10 precursors of a particular epitope specificity per 10^6 cells [225]. Even when equal number of CD8 T cells are transferred across recipients, differences between engraftment efficiency and viability and can result in large variations in antigen-specific response after infection. Due to these limitations, we did not carry this analysis into aged mice where the isolation of 10^6 T_N cells would be a further challenge.

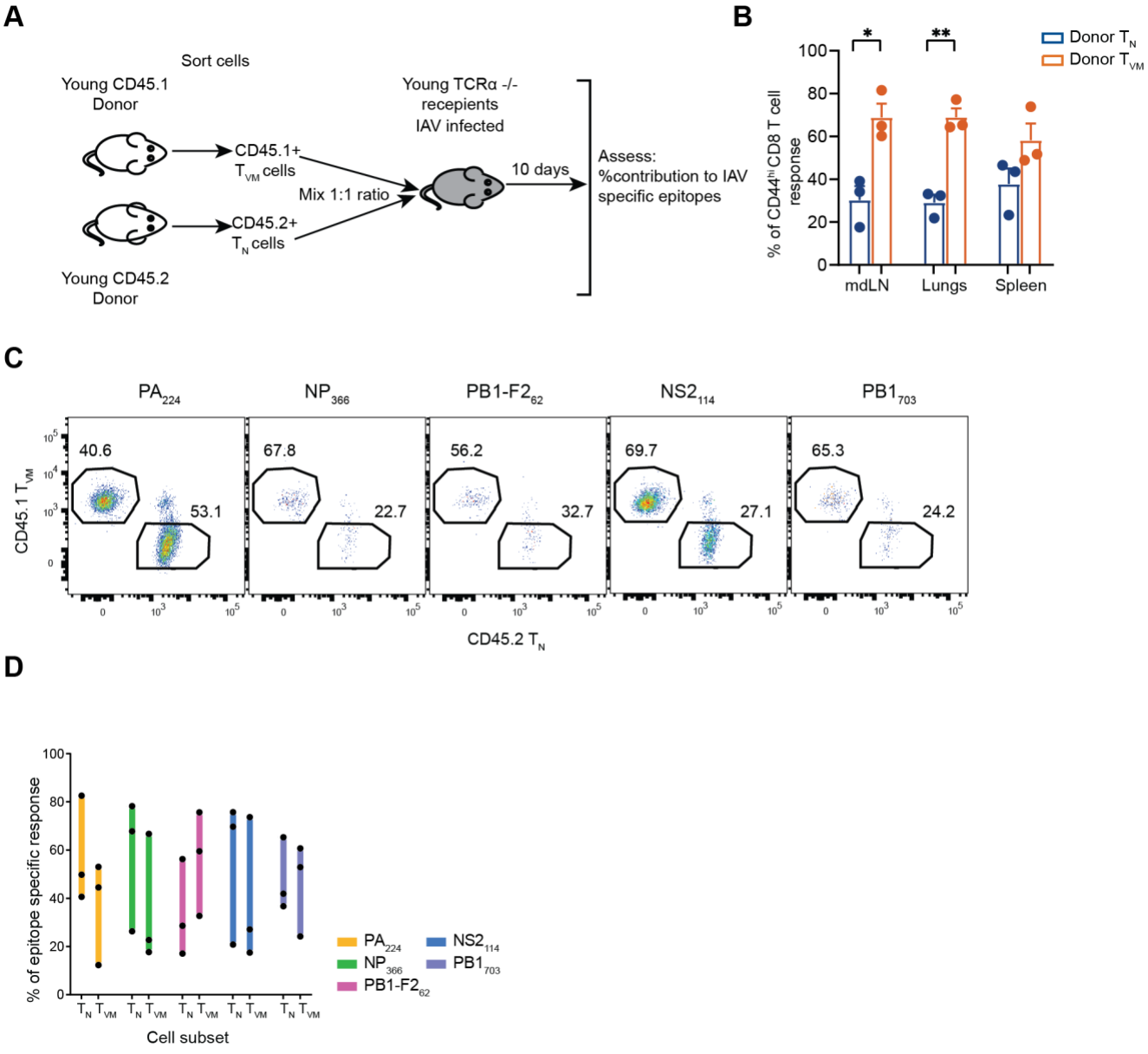

Figure 3.11: IAV-specific response of young T_{VM} and T_N cells. A) Schematic of the adoptive transfer of congenically distinct T_N and T_{VM} cells into IAV infected TCRα$^{-/-}$recipients and analyzed at d10. B) The percentage of contribution of transferred T_N and T_{VM} cells to total CD8 T cell response in different organs. C) Representative pseudocolor plots showing the percentage of contribution of transferred T_N and T_{VM} cells to each epitope-specific response in total CD44^{hi} tetramer+ cells in the spleen from each recipient. D) Bar graphs of the frequency of transferred T_N or T_{VM} cells contributing to each epitope specificity. * $p<0.05$, **$p<0.01$, using parametric test. Bar graphs represent mean ± SEM.

3.3. Discussion

In this study, we have extended our previous observations that assessed TCR mediated and IL-15 driven response of T_{VM} cells *in vitro* to an *in vivo* analysis. We observed that aged T_{VM} cells largely maintained their response to innate stimuli compared to young T_{VM} cells and this was evident in comparable levels of innate-like cytokine production, bystander killing capacity and similar levels of IL-15 mediated proliferation, observed in both young and aged T_{VM} cells. We also found that young T_{VM} cells were efficient at mediating antigen specific responses and outcompeted T_N cells in their ability to home to sites of infection.

The aged T_{VM} cells are marked by their retention of cytokine sensitivity, with the simultaneous loss of TCR-mediated capacity, as evidenced by previous studies [95,186]. Here we show that while ageing overall increases the innate-like appearance of T_{VM} cells, with increased cytokine, chemokine, and NK receptor expression on aged T_{VM} cells compared to young, it does not result in a proportional increase in innate-like functionality. There are some instances where aged T_{VM} cells appear unperturbed by innate stimuli whereas young T_{VM} cells respond robustly, such as observations of minimal increase in CD122 expression and no increase in frequency of NKG2D+ cells in aged T_{VM} cells after IL-15 treatment, indicating that aged T_{VM} cells may have reached the ceiling of their response.

T_{VM} cells become increasingly senescent with age, however they do not appear to be an end-stage dysfunctional population. The retention of cytokine response with a simultaneous loss in TCR activity with age [95] can indicate that T_{VM} cells have evolved to survive the increasingly inflammatory immune environment [178–180], which may not be conducive to effective TCR-mediated functions, by preserving their innate-like functionality. Given that ageing is

associated with a greater burden of chronic diseases, like viral infections [181,182,226] and cancer [183], this adaptation of function may be useful when other memory CD8 T cells may be restricted by exhaustion [227–229] but T_{VM} cells may still be able mediate protective functions in an innate-like manner, as they have lesser predisposition to become exhausted [95,230].

More evidence for this adaptation of T_{VM} cell function to an innate-like role comes from the increased proportion of T_{VM} cells expressing NKG2D with age. It was shown in a recent study that NKG2D can impede TCR activation in senescent human CD8 T cells [155]. NKG2D dampens TCR signaling in CD4 and CD8 T cells by co-localizing with DAP12 and sestrins, which then inhibits TCR signaling but promotes innate like killing functions in CD8 T cells in both humans and mice [155,191]. We observed reduced proliferation of NKG2D+T_{VM} cells in young mice compared to their NKG2D- counterparts, which would fit with the observation of reduced TCR signaling in NKG2D+ CD8 T cells [155]. However, we did not observe similar results in the aged setting where both NKG2D+/- subsets had equal proliferative response. Better understanding of the role of NKG2D in regulating TCR mediated functions of T_{VM} cells would require analysis of DAP12 and sestrin co-expression with NKG2D in young and aged T_{VM} cells.

Cytokine stimulation has the ability to modulate innate-like functions of T_{VM} cells. We observed that IL-15 exposure could induce an increased proportion of NKG2D+ T_{VM} cells in young mice, and this subset was maintained for weeks after low-dose cIL-15 exposure. Several pathogens that are relevant to human studies have been reported to induce increased IL-15 levels, such as *Salmonella* strains [231,232], CMV and HIV infections [143,144,169] where the response to subsequent infections could be improved due to an expanded T_{VM} cell population with increased bystander activity. The expansion in T_{VM} cells through IL-15 is also relevant in conditions that limit IL-15

production, such as that seen in immunological non-responders or treatment-naïve people with HIV [233,234]. Additionally, recent research on chimeric antigen receptor (CAR) T cell therapy has revealed that IL-15 priming enhances anti-tumor activity and longevity of response by preserving a stem cell memory phenotype that does not succumb to exhaustion [235,236]. This has led to the use of 'armoured' CAR therapies that co-express IL-15 and have been shown to have superior anti-tumor activity [237,238]. Given that T_{VM} cells are exquisitely sensitive to IL-15 and less prone to exhaustion [34,95,230], they may be ideal candidates for seeding the CAR-T cell population.

Previously we and others have shown that IL-15 sensitivity and dependence is a function of T_{VM} cells [29,34,35,37,97]. However, we observed a significant increase in T_N and T_{MEM} cell proliferation with *in vivo* administration of high dose cIL-15 in both young and aged mice. Higher concentrations of IL-15 are known to induce activation of proliferative MAPK pathways in NK cells, whereas lower concentrations of IL-15 only induce STAT5 phosphorylation [239]. It is possible that a similar threshold of response to low and high dose IL-15 also exists in T_N and T_{MEM} cells. Our results indicate that T_N and T_{MEM} cells require potent IL-15 stimulation which then results in activation of proliferative pathways, although it is unclear if these concentrations are commonly attained systemically or locally *in vivo.*

Current literature on T_{VM} cell biology lacks a comprehensive comparison of antigen-specific functionality of young and aged T_{VM} cells *in vivo*. When we evaluated capacity of T_N and T_{VM} cell to contribute to antigen-specific response in a co-transfer to mice lacking endogenous T cells, we observed that T_{VM} cells had a clear advantage over T_N cells in their ability to become activated in an antigen dependent manner, as evidenced by increased representation of T_{VM}

cells at the site of infection, which is in agreement with results from other studies that predict rapid antigen-specific response of T_{VM} cells during infections [85,166] . While we did not perform an analogous analysis for aged T_N and T_{VM} cells, another study has found that aged T_{VM} cells made a lower contribution to the total CD8 T cell response at the site of infection and in lymphoid organs, compared to T_N cells, when equal numbers of both subsets were co-transferred. They also observed a trend of reduced contribution of aged T_{VM} cells to epitope-specific responses compared to aged T_N cells in some recipients [168]. Our *in vivo* assessment of antigen-specific responses in young mice, and Blackman et al's assessment of aged mice, give support to the notion that our previous *in vitro* findings of reduced TCR mediated functions in aged T_{VM} cells [95] translate to *in vivo* loss of function. However, to better assess these age-related shifts in epitope-specific contributions, a simultaneous comparison of young and aged T_N and T_{VM} cells is required. Assessment of aged transgenic mouse models, that express fixed or semi-fixed TCRs, could overcome the challenge of having limited and/or variable frequencies of epitope-specific precursors observed in polyclonal wildtype CD8 T cell populations.

Collectively, our results reveal how young and aged T_{VM} cell response to innate stimulus is largely maintained with age. This adaptation of an innate-like role may be helpful in the increasingly senescent and inflammatory immune environment. Our findings also have relevance in understanding CD8 T cell response in infections or diseases driving increased cytokine signaling.

Chapter IV

4. Helminth infection-induced increase in virtual memory CD8 T cells is transient, driven by IL-15, and absent in aged mice.

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[2]**Grant Support:** This work was supported by the Australian Research Council (ARC) (DP170103631, DP200102776, FT170100174 to N.L.L.) and the National Health and Medical Research Council of Australia (NHMRC) (APP1182086 to N.L.L.).

[3]**Abbreviations:** T_{VM} cells, virtual memory T cells; T_N cells, naive T cells; T_{MEM} cells, conventional memory T cells; *Hp* or *H. polygyrus*, *Heligosomoides polygyrus*; *Tm* or *T. muris*, *Trichuris muris*; mesLN, mesenteric lymph nodes; Hp_{DC}, *H. polygyrus* infected and drug cured; CTV, Cell Trace Violet; IL-15c, complex of IL-15/IL-15Rα.

Running Title: T_{VM} cells after helminth infection in young and aged mice.

4.1. Abstract

Virtual memory CD8 T cells $(T_{VM})^3$ are antigen-naïve CD8 T cells that have undergone partial differentiation in response to common γ (γc) chain cytokines, particularly IL-15 and IL-4. T_{VM} cells from young individuals are highly proliferative in response to TCR and cytokine stimulation but, with age, they lose TCR-mediated proliferative capacity and exhibit hallmarks of senescence. Helminth infection can drive an increase in T_{VM} cells, which is associated with improved pathogen clearance during subsequent infectious challenge in young mice. Given the cytokine-dependent profile of T_{VM} cells and their age-associated dysfunction, we traced proliferative and functional changes in T_{VM} cells, compared to true naïve CD8 T (T_N) cells, after helminth infection in young and aged mice. We show that IL-15 is essential for the helminth-induced increase in T_{VM} cells, which is driven only by proliferation of existing T_{VM} cells, with negligible contribution from T_N cell differentiation. Additionally, T_{VM} cells were uniquely proliferative in response to helminth infections and IL-15 compared to other CD8 T cells. Furthermore, T_{VM} cells from aged mice did not undergo expansion after helminth infection due to both T_{VM} cell intrinsic and extrinsic changes associated with ageing.

4.2. Introduction

At any given time, a myriad of parasitic, fungal, bacterial and viral entities colonize different tissues in our body. The commensal microbiome and virome interacts with many cells within our immune system to play a key role in regulating immune homeostasis [240–242]. Various pathogenic microbes and viruses can also have an acute impact on subsequent immune responses[243] or engender more chronic changes in our immune system [192,193,244,245]. As an example, helminth infections are known to have an immunomodulatory impact on their host that can shift microbiome composition and induce prolonged changes to the homeostatic immune environment [246,247]. While helminth infections have largely been eradicated from many countries, they are still widespread in Africa and some Asian regions, resulting in a quarter of the world's population being affected [248]. Recent reports have found that helminth infections can provoke a rapid, sustained and antigen-independent increase in a subset of semi-differentiated, memory phenotype CD8 T cells called virtual memory T cells (T_{VM}) cells [84,167].

CD8 T_{VM} cells are found in mice and humans and, while antigen-naïve, appear to be semi-differentiated, with high expression of CD44 and CD122 (IL15-Rβ) in mice. In comparison to true naïve T (T_N) cells, T_{VM} cells are marked by their rapid proliferation and acquisition of effector functions after T cell receptor (TCR) stimulation, and exhibit heightened sensitivity to cytokines, which enables antigen-independent acquisition of T_{VM} effector function [94,96,100]. Recent studies have shown that T_{VM} cells can play a key protective role during infections and cancer, in both mice and humans. T_{VM} cells are increased after infections with helminths [84,167], human immunodeficiency virus (HIV) [169] and cytomegalovirus (CMV) [143] and have been shown to mediate protective functions mainly via bystander cytotoxic pathways [143,167,169]. T_{VM} cells

have also been found in tumor infiltrates [101,170] and elevated T_{VM} cell numbers have been associated with remission in CML patients [172], suggesting a possible role in immunity to cancer.

With ageing, T cell composition shifts dramatically. In both mice and humans, there is a dramatic loss (>70%) of T_N cells [184,185], while T_{VM} cell number remains remarkably stable, resulting in their dominance within the antigen-naïve T cell pool [95,98]. T_{VM} cell function is also known to shift dramatically with age. While T_{VM} cells from young individuals respond more rapidly to TCR stimulation, T_{VM} cells from aged individuals acquire a senescent phenotype and become largely unresponsive to TCR stimulation, although they retain responsiveness to cytokines [34,95].

Previous observations of a dramatic increase in memory phenotype CD8 T (T_{MP}) cells, and T_{VM} cells specifically, in response to helminth infection [84,99,167,249] showed that this increase was sustained over several weeks and correlated with improved protection to subsequent viral and bacterial infections [84,167]. Mechanistically, the increased T_{VM} cell number observed in both BALB/c and C57BL/6 mice was considered a consequence of elevated IL-4, produced as a result of type-2 helper T cell (Th2) mediated immunity during helminth infection [250], driving conversion of T_N cells to the T_{VM} lineage [84]. In BALB/c mice, T_{VM} cells arise in the thymus, expand in response to IL-4 produced by thymic natural killer T (NKT) cells [92], and therefore depend on IL-4 for survival in the periphery [91,93]. In contrast, T_{VM} cells in C57BL/6 mice have limited dependence on IL-4 for survival [93] (but do proliferate upon *in vivo* IL-4 administration [99]), and are instead critically dependent on IL-15, as T_{VM} cells do not develop in mice that lack either IL-15 or the IL-15 receptor [34,35,97].

Given the dramatic impact of helminth infection on T_{VM} cell number, and the consequences for subsequent viral or bacterial infection, we investigated the long-term impact of helminth infection on T_{VM} cells in aged mice, and whether T_{VM} cells from aged mice respond similarly to helminth infection and how this impacts their senescent phenotype. We describe a previously unrecognized role of IL-15 in driving T_{VM} expansion after helminth infection. Further, in contrast to previous studies, we show that the helminth-driven expansion of the T_{VM} cell compartment is due almost exclusively to T_{VM} cell proliferation and not differentiation from T_N cells. Finally, in contrast to *in vitro* findings, we show that aged T_{VM} cells have an intrinsic deficit in cytokine responsiveness, which, in combination with previously observed extrinsic changes in the aged response to helminth infection, results in no impact of helminth infection on T_{VM} cells in aged mice. Our findings broaden our understanding of T_{VM} cell response to infections and reveal deficits that may have a detrimental impact on T_{VM} cell mediated immunity in the elderly.

4.3. Methods

Mice and infections All C57BL/6 mice were from the colony bred at Monash Animal Research Platform (MARP), Victoria, Australia. We acquired CD45.1+ mice (B6.SJL-PtprcaPep3b/BoyJArc) from the Animal Resources Centre, Perth, Western Australia. Experimental procedures conducted during this study had prior approval from MARP Animal Ethics Committee (AEC). Young mice used in this study were between 8-12 weeks of age, whereas aged mice ranged from 18-20 months of age. All experiments used female mice.

For *Heligmosomoides polygyrus (H. polygyrus or Hp)* infection, *200 stage 3 larvae (L3) were administered via oral gavage. For Trichuris muris (T. muris or Tm)* infection, 200 eggs were administered orally. Mice were humanely euthanised at the indicated timepoints and tissues were collected.

Pyrantel treatment *To cure mice of H. polygyrus infection, pyrantel pamoate at 10 mg/kg of body weight was administered orally in water twice, one day apart. Mice were drug treated 2 weeks post infection and then rested for 14-21d before analysis.*

IL-15c preparation, anti-IL-15 treatment, and adoptive transfer of CD8 T cells. To block IL-15 signaling, an IL-15/Rα neutralising monoclonal antibody (Ab) (clone GRW15PLZ, eBiosciences) was administered (previously used in [34]). 40 µg of anti-IL-15/Rα Ab was administered twice intraperitoneally on d0 and d4 of *H. polygyrus* infection. For IL-15 administration and stimulation assays, a complexed form of IL-15 was used. Complexed IL-15 was selected as it persists longer *in vivo* and is efficiently trans-presented to CD8 T cells [97,199]. To generate this complex, carrier-free recombinant murine IL-15 (Peprotech) was combined with a recombinant

fusion protein, carrier-free mouse IL-15Rα:Fc (R&D systems). CD8 T cells were sorted from young and aged mice, and Cell Trace Violet (CTV) (ThermoFisher Scientific) labelled. Congenically distinct (CD45.1+) young recipients received $4x10^5$-$5x10^5$ cells intravenously. Within 18 hrs of transfer, 2 µg of IL-15c was injected intraperitoneally and proliferation was assessed 7d later.

Dendritic cell preparation To obtain an optimal yield of dendritic cells (DCs) from spleens, a digestion and light density gradient separation method was used. Spleens were digested in Type II Collagenase (Worthington Biochemical Corporation) and DNase 1 (Roche) for 20-30 minutes. After digestion, NycoPrep™ 1.077g/cm^3/EDTA (Axis SHIELD PoC AS/Ajax Finechem Pty Ltd) was used to separate DCs from the light density fraction. DCs were then stained with surface markers including a biotinylated anti-murine IL-15 [165] (Peprotech), Gating strategy used to define DC subsets is described in Supplementary file 1.

Identification, sorting and phenotyping of T cell subsets Tissues were processed and cell suspensions of splenocytes were either stained for phenotyping or enriched for adoptive transfer and in-vitro stimulation experiments. Cell counts were performed using ABX Micros 60, Hematology analyzer (HORIBA Medical). CD8 T cell were enriched using CD8α+ T cell isolation kit (130-095-236, Miltenyi). Gating strategy is described in Supplementary file 1. For sorting, $CD44^{lo}$ T_N cells were defined as the ~20% of total CD8 T cell with the lowest CD44 expression, whereas $CD44^{int}$ T_N cells were defined as the middle ~15-20% of CD8 T cells falling in T_N gate with intermediate expression of CD44. T_{VM} cells were sorted from the $CD44^{hi}CD49d^{lo}$ population whereas T_{MEM} were sorted from the $CD44^{hi}CD49d^{hi}$ population of CD8 T cells, the typical gating strategy used for sorting is described in Supplementary file 1. All antibodies and

their clone numbers are listed in Supplementary file 2. For intracellular cytokine staining Cytofix/Cytoperm kit (BD Pharmingen) was used, for staining of intranuclear markers, FoxP3 Fixation/Permeabilization kit (eBioscience) was used. Apoptosis Detection Kit (ThermoFisher Scientific) was used for Annexin-V staining.

In vitro stimulation and proliferation assay For proliferation assays cells were sorted and CTV labelled (ThermoFisher Scientific) according to manufacturer's instructions. For polyclonal stimulation for assessment of cytokine production sorted cells were stimulated for 36 hours in antibody-coated (anti-mouse(m)CD3ε 10 μg/ml, -CD8a 10 μg/ml, and -CD11a 5 μg/ml) for 36 hours with 10U/ml of recombinant human IL-2 (rhIL-2). For assessment of proliferation, CTV-labelled cells were stimulated, in a similar manner as described for cytokine production, for 65 hours, before viability staining and assessment of CTV dilutions.

For assessment of IFNγ production, sorted CD8 T cells were stimulated for 16 hours in culture containing 20 ng/ml mIL-12 (Peprotech), 20 ng/ml mIL-18 (Peprotech) and 100 ng/ml IL-15c (as described before). For IL-15 proliferation assay, CTV labelled sorted cells were stimulated with 100 ng/ml of IL-15c for 5d before viability staining and assessment of CTV dilutions.

Statistical analyses All analysis was performed in GraphPad (v9.3.1). Mann-Whitney non-parametric test was applied in most cases. Where a parametric t-test was applied, the data was first tested for normal distribution using Shapiro-Wilk test.

4.4. Results

4.4.1. Helminth infection leads to increase in T_{VM} cells in young mice and results in augmented responsiveness to IL-15

Previous studies have shown that helminth infections, such as *Schistosoma mansoni, Nippostrongylus brasiliensis,* or *H. polygyrus,* can induce a robust increase in T_{VM} cell numbers in BALB/c and C57BL/6 mice [84,167]. To determine whether this effect on T_{VM} cells is proportional to the strength of the Th2 response, we infected mice with either *H. polygyrus* or *T. muris*. A high dose of *T. muris* parasitic eggs causes an acute, self-limiting infection in C57BL/6 mice which is completely cleared around d30 after infection [251], whereas *H. polygyrus* establishes a chronic infection that can take more than 8 weeks to clear [252]. In addition, high dose *T. muris* triggers a Th2 response [253] in which the key cytokines are only detectable around d10 of infection [254,255], whereas *H. polygyrus* infection induces a rapid and robust Th2 response that is detectable within the first few days of infection [256,257].

Using these two characteristically distinct helminth infection models, we assessed CD8 T cell numbers in the spleens of helminth infected mice. At d21 and d10 after infection of C57BL/6 mice with *T. muris* (Figure 4.1A, B) or *H. polygyrus* (Figure 4.1C, D), respectively, we observed a robust expansion of T_{VM} cells in the spleen, with an 85% increase observed following *T. muris* and a 140% increase after *H. polygyrus* infection. This demonstrates that an increase in T_{VM} cells is a generalisable feature of helminth infections. *T. muris* infection also induced a modest but significant increase in T_{MEM} cells, likely due to antigen specific response, but no difference

in T_{MEM} cells was observed with *H. polygyrus* infection. For both infection models, the T_N numbers were negligibly impacted, indicating a T_{VM} cell-specific impact of helminth infection.

To determine how the magnitude and kinetic of the Th2 response relates to that of T_{VM} expansion, we tracked T_{VM} expansion over time after infection. T_{VM} cells started expanding earlier, peaked earlier (d12 vs d28) and reached a higher magnitude following *H. polygyrus*, as compared to *T. muris*, infection (Figure 4.1B, D). For example, splenic T_{VM} cell numbers did not differ significantly from uninfected mice at d14 after *T. muris* infection (Supplementary Figure S1A), but they were increased in the spleen, mesenteric lymph node (mesLN) and blood as early as d7 after *H. polygyrus* infection (Supplementary Figure S1B). Moreover, the increased number of T_{VM} cells was maintained out to d45 at least with *H. polygyrus* infection (Figure 4.1D). This illustrates that *H. polygyrus* drives a more rapid, and larger increase in T_{VM} cells, compared to *T. muris* infection, correlating with reports of its stronger induction of Th2 responses [256,257]. As a result, we used the *H. polygyrus* infection model for further analyses.

To determine whether the *H. polygyrus* infection-driven increase in T_{VM} cells was the result of proliferation, T_N, T_{VM} and T_{MEM} cells were isolated at d10 after infection stained for Ki67. All cell populations showed at least a trend toward increased proliferation (Figure 4.1E), with T_N and T_{VM} cells showing a significant increase ($p=0.0079$ for each analysis). However, T_{VM} cells showed the most striking shift, with a ~3-fold increase in Ki67+ cells, correlating with their elevated number and frequency at this timepoint (Figure 4.1C). We next attempted to define the key cytokine(s) driving T_{VM} cell proliferation. IL-15 is a key cytokine for T_{VM} cell development but recent reports have suggested that T_{VM} cells proliferate in response to the increased IL-4 produced in response to helminth infection [84,167]. We therefore analysed expression levels of

CD124 (a subunit of the IL-4 receptor), CD122 (the β-chain of IL-15 receptor) and the Eomes transcription factor, which is upregulated in T_{VM} cells with IL-4 stimulation [84,91,258] and directly acts to upregulate CD122 expression [158]. *H. polygyrus* infection drove a clear increase in CD124 levels in T_{VM} and T_N cells (Figure 4.1F), consistent with other studies where CD124 was reported to increase with *in vivo* IL-4 treatment[99]. We also observed an increase in Eomes expression across all T cells, with the most striking increase occurring in T_{VM} cells (Figure 4.1G). Most notably, CD122 expression increased markedly and exclusively on T_{VM} cells after infection (Figure 4.1H) and this was sustained over multiple timepoints (Figure 4.1I). While enhanced Eomes expression could support an increase in CD122, we have also observed a modest but reproducible increase in CD122 expression (~30%) on T_{VM} cells after acute *in vivo* exposure to IL-15 (Supplementary Figure S1C).

We also observed other striking phenotypic shifts with *H. polygyrus* infection, such as a ~70% increase in CCR2+ T_{VM} cells and a significant increase in expression of CCR2 exclusively on T_{VM} cells (Figure 4.1J). CCR2+ T_{VM} cells have previously been shown to traffic rapidly to lungs during influenza infection which was attributable to their high CXCR3 expression compared to CCR2- T_{VM} cell subset [166]. IL-15 was previously reported to induce multiple C-C chemokine receptors, such as CCR2 [166,259], but not CXC chemokine receptors, such as CXCR3, in human T cells [259]. Consistent with these data, we did not observe any change in CXCR3 expression after infection (Supplementary Figure S1D). Another striking observation was that CCR2+ T_{VM} cells expressed twice as much CD122 compared to the CCR2- T_{VM} subsets, and this was observed both in the uninfected and infected T_{VM} cells. CD122 expression was further increased by 20% on CCR2+ T_{VM} cells after infection (Supplementary Figure S1E).

Together, these data suggest that, due to the inflammatory environment induced by helminth infection, in addition to IL-4, T_{VM} cells show an increased sensitivity to IL-15 during *H. polygyrus* infection, which may drive the shifts in the T_{VM} cell population both in terms of number and phenotype.

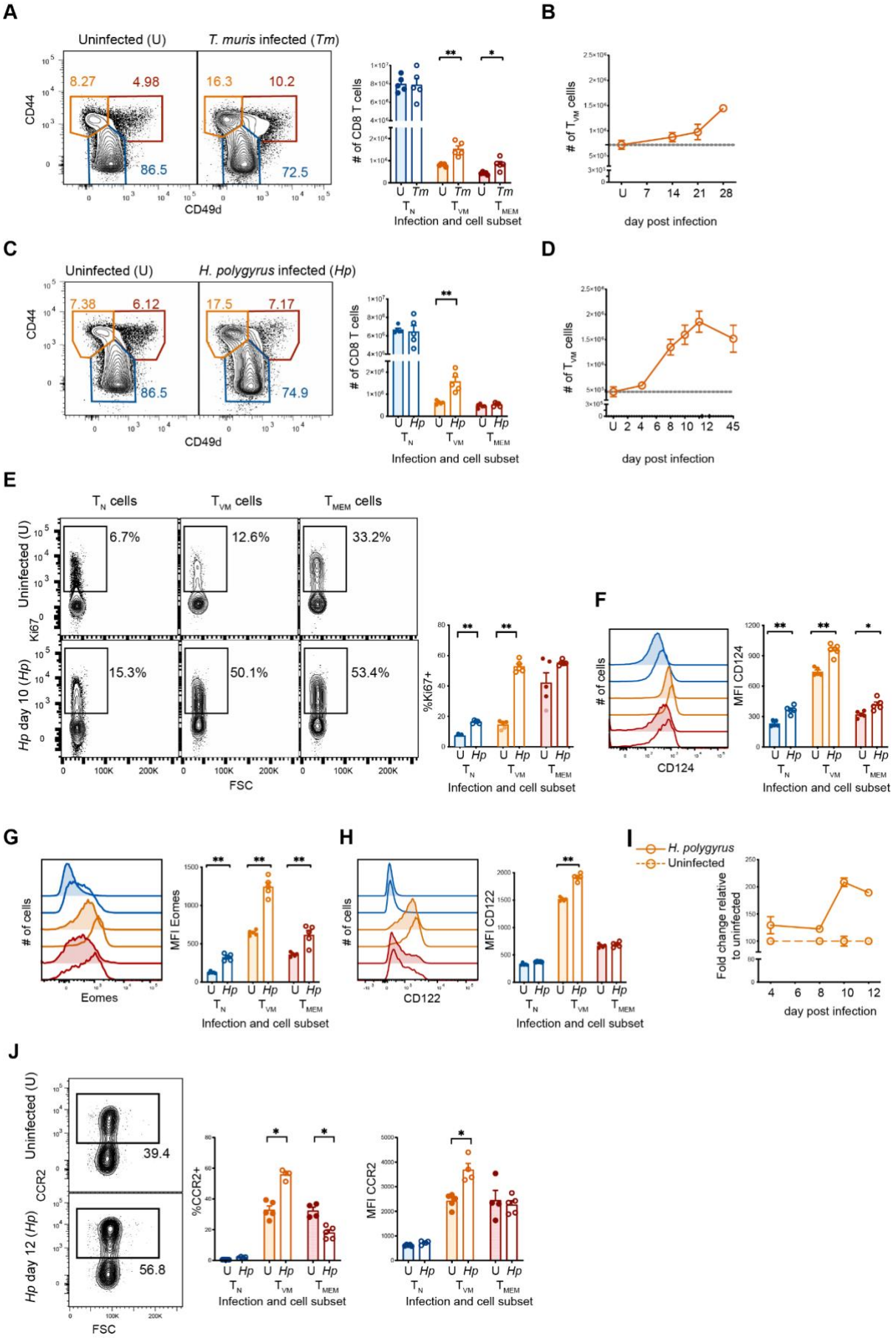

A
Uninfected (U)
T. muris infected (Tm)
8.27
4.98
86.5
16.3
10.2
72.5
CD44
CD49d
# of CD8 T cells
Infection and cell subset
B
# of T_VM cells
day post infection
C
Uninfected (U)
H. polygyrus infected (Hp)
7.38
6.12
86.5
17.5
7.17
74.9
D
E
T_N cells
T_VM cells
T_MEM cells
6.7%
12.6%
33.2%
15.3%
50.1%
53.4%
Ki67
FSC
%Ki67+
F
# of cells
CD124
MFI CD124
G
Eomes
MFI Eomes
H
CD122
MFI CD122
I
H. polygyrus
Uninfected
Fold change relative to uninfected
J
39.4
56.8
CCR2
%CCR2+
MFI CCR2

Figure 4.1. T_{VM} cell population is increased with helminth infection. A) Representative contour plots showing live/TCRBβ+CD8+ T cell subset from uninfected (U) and *Trichuris muris* (*Tm*) infected mice with bar graph showing number of CD8 T cells in each subset in the spleens at d21 post *Tm* infection and B) depicting change in *Tm*-induced T_{VM} cell numbers in the spleen at different timepoints C) representative contour plots showing TCRβ+CD8+ T cell subset from uninfected (U) and *H. polygyrus* (*Hp*) infected mice with bar graph showing number of CD8 T cells in each subset at d10 of *Hp* infection in the spleens and D) change in *Hp*-induced T_{VM} cell numbers in the spleen at different days post infection. E) representative contour plots showing T_N, T_{VM} and T_{MEM} cells depicting expression of Ki67 and the percentage of in each subset that are Ki67+ in uninfected and *Hp* groups. Histograms and graph showing median fluorescence intensity (MFI) of F) CD124 G) Eomes and H) CD122 on CD8 T cell subsets at 8d post *Hp* infection I) timecourse showing the percent change in MFI of CD122 on infected T_{VM} cells normalized to average MFI in uninfected T_{VM} cells at each timepoint J) Contour plots depicting CCR2 expression on T_{VM} cells at d10-12 post infection and bar graph showing frequency and MFI of CCR2 expression on CD8 T cell subsets. Data is representative of 2 different experiments with n=5 mice per group, except *Tm* d14 and *Hp* d4, d45 that was done once. *p<0.05 and **p<0.01 using a Mann Whitney test. Bar graphs represent mean ± SEM.

4.4.2. IL-15 plays a critical role in driving proliferation of T_{VM} cells during *H. polygyrus* infection

It has been proposed that the helminth infection-driven T_{VM} cell increase is driven by IL-4, based on the observation that T_{VM} cell numbers were reduced in IL-4 or IL-4Rα KO mice both before and after infection. However, helminth infection induced a similar fold increase in T_{VM} cells in IL-4/IL-4R KO compared to WT mice [167]. We interpret these data as indicating that while T_{VM} cells are partially dependent on IL-4 for steady state maintenance [93], their expansion after helminth infection occurs independently of IL-4, or at least does not require IL-4. Given the exquisite sensitivity of T_{VM} cells to IL-15 and the heightened CD122 expression after infection, we hypothesise that IL-15 contributes to their expansion.

To determine whether IL-15 was required for T_{VM} cell expansion during early *H. polygyrus* infection, we transiently blocked IL-15 signaling using a monoclonal antibody (mAb) directed against the IL-15/IL-15R complex. Since T_{VM} cells are dependent on IL-15 for survival, we first tested whether our mAb blockade regimen reduced T_{VM} cell survival. Mice were infected with

H. polygyrus and treated with mAb at d0 and d4 post-infection (Figure 4.2A). Splenocytes were harvested at d8 and stained for Annexin-V (Ann-V), a marker of early apoptosis. Ann-V staining in T_{VM} cells in the spleen and mesLN was unaffected by mAb blockade and infection itself reduced apoptosis in T_{VM} cells from the mesLN, independently of mAb blockade (Supplementary Figure S2A, B). Together, these data demonstrate that transient IL-15 mAb blockade does not reduce T_{VM} cell survival.

When T_{VM} cell numbers were assessed at d8 after infection, our transient IL-15 mAb blockade completely prevented the infection-mediated increase in T_{VM} cells, with similar numbers of T_{VM} cells observed in the spleens of infected/Ab treated mice and uninfected mice, and both significantly reduced compared to infection alone (Figure 4.2B, C). T_{VM} cells from mesLN were also reduced after mAb blockade, albeit not significantly ($p=0.06$, Figure 4.2D). The reduction in T_{VM} cell numbers with mAb blockade was accompanied by reduced proliferation as measured by Ki67 staining in treated, compared to untreated, infected mice (Figure 4.2E-G). Again, comparable results were observed for both the spleen and mesLN. Collectively, our results demonstrate that IL-15 is essential for the increase in T_{VM} cells after *H. polygyrus* infection and may explain previous data showing an unimpeded increase in T_{VM} cells after helminth infection in IL-4/IL-4Rα KO mice [167].

As discussed earlier, it is likely that IL-15-mediated T_{VM} cell expansion was due to increased IL-15 signaling during infection, as a consequence of increased T_{VM} sensitivity (Figure 4.1H, I), and possibly increased IL-15 availability after infection. To quantify IL-15 availability, trans-presentation of IL-15 on splenic cDC1 (CD8α+) and cDC2 (SIRPα+) subsets was assessed at d5 post *H. polygyrus* infection. We observed a >30% increase in the frequency of DCs that were

IL-15+ across both cDC subsets, and the MFI of IL-15 was marginally increased after infection, albeit not significantly in the cDC2 subset (p=0.15) (Figure 4.2H). The total number of IL-15+ cDC1s was also increased, (Supplementary Figure S2C).

Collectively, our results demonstrate that helminth infection-driven expansion of T_{VM} cells is mediated largely, if not exclusively, by IL-15 as a consequence of increased T_{VM} cell sensitivity to IL-15 and increased availability of IL-15 on DCs.

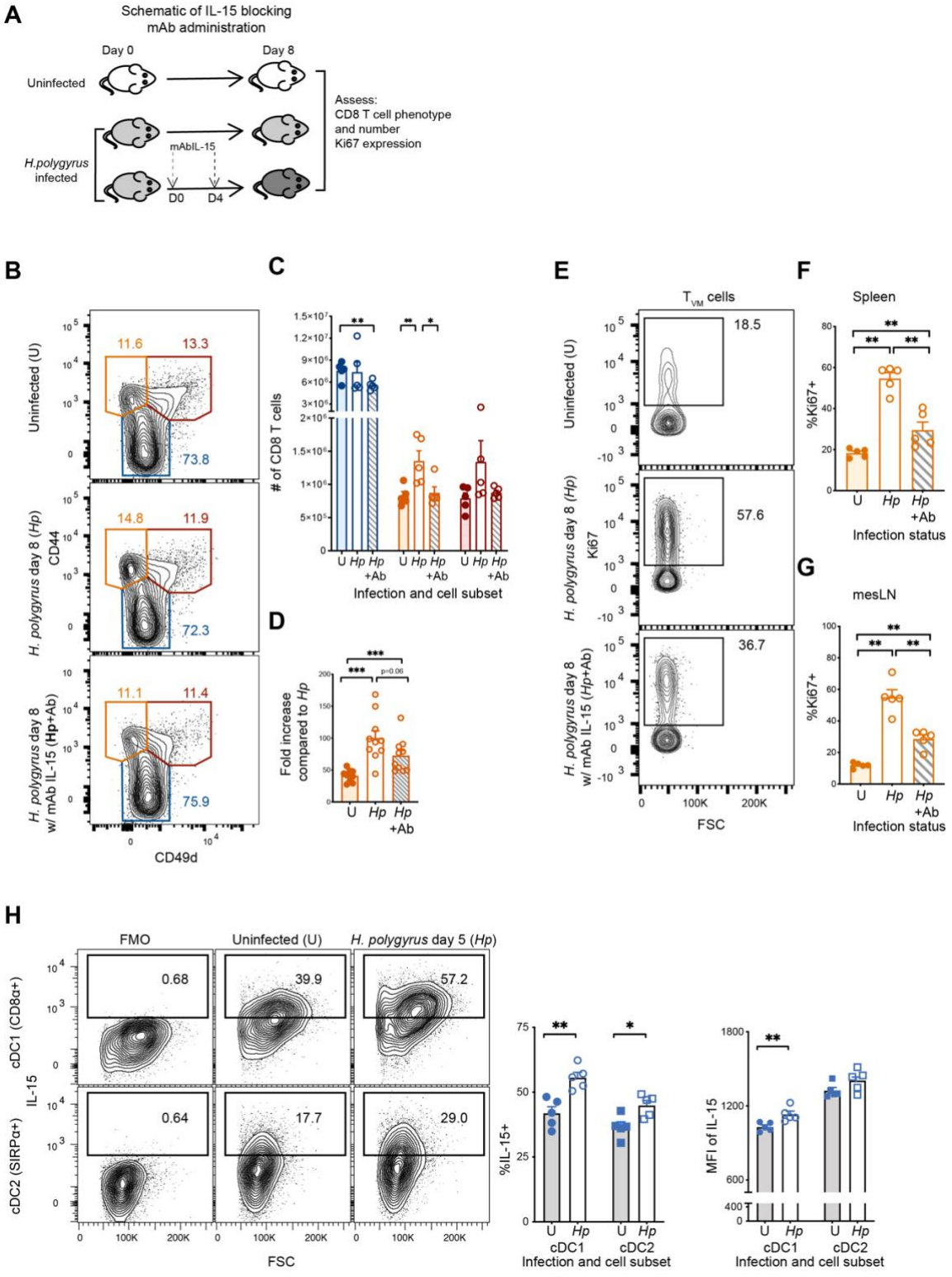


Figure 4.2. IL-15 plays a critical role in driving proliferation of T_{VM} cells during *H. polygyrus* infection. A) Schematic showing IL-15 blocking mAb treatment B) representative contour plots showing Live/TCRβ+CD8+ T cell subset from uninfected (U) *H. polygyrus* d8 (*Hp*) and *Hp* d8 treated with IL-15 blocking antibody (d0, d4) (*Hp*+Ab) with C) bar chart showing the number of CD8 T cells in each subset in spleens. D) Fold increase in T_{VM} cells in mesenteric lymph nodes (mesLN) of U, *Hp* and *Hp*+Ab mice

normalized to average number in *Hp* infected mice. E) Representative dot plot showing percentage of T_{VM} cells expressing Ki67 F) in spleen and G) in mesLN across the three groups of mice. H) Representative contour plots depicting expression of IL-15 on DC subsets with frequency of cDC1 and cDC2 subsets that express IL-15. Data is representative or combined from 2 different experiments with n=5 mice per group. *p<0.05 and **p<0.01 using a Mann Whitney test. Bar graphs represent mean ± SEM.

4.4.3. Infection mediated increase in T_{VM} cells occurs via proliferation and not conversion of T_N cells

Recent studies have demonstrated that T_{VM} cells are a distinct lineage whose development is initiated in the thymus [101], but some studies have suggested that cytokine signaling can trigger conversion of T_N cells to T_{VM} cells in the periphery [84,97]. For example, IL-15 treatment was reported to induce conversion of, in particular, $CD5^{hi}$ T_N cells to T_{VM} cells [97]. Similarly, it was suggested that the increase in T_{VM} cell numbers during helminth infection was due to conversion of T_N cells to a T_{VM} phenotype[84].

To determine whether T_{VM} cells can be derived from peripheral conversion of T_N cells in the context of helminth infection, we performed adoptive transfer experiments. CTV-labelled T_N or T_{VM} cells were transferred into congenically distinct recipient mice at d3 of *H. polygrus* infection and the rate of proliferation and conversion to a T_{VM} phenotype was assessed at d10 (i.e., 1 week post-transfer) (Figure 4.3A). T_N cells were further partitioned into either CD44 low ($CD44^{lo}$) or CD44 intermediate ($CD44^{int}$) cells to assess whether there was differential capacity within the T_N cell compartment to undergo conversion. $CD44^{int}$ T_N cells appear to be partially differentiated with higher levels of Eomes and CD5 expression [20].

Analysis of transferred cell phenotype at d10 revealed that a negligible proportion of T_N cells (only 0.7% of $CD44^{lo}$ T_N cells and around 1.5% of $CD44^{int}$ T_N cells) had converted to the T_{VM}

phenotype in the spleen (Figure 4.3B). Proliferation was also far lower in T_N cells compared to T_{VM} cells, irrespective of their level of CD44 expression (Figure 4.3C). In contrast, transferred T_{VM} cells largely retained their T_{VM} phenotype (Figure 4.3B) and showed high rates of proliferation (Figure 4.3C). We observed only marginally higher rates of T_N cell conversion in the mesLN (~5%; Supplementary Figure S3). These observations provide strong evidence that while T_N cells may undergo limited division in response to helminth infection, the increase in the T_{VM} cell number is primarily driven by proliferation of existing T_{VM} cells and not by *de novo* generation from T_N cells.

Adoptive transfer experiments with T_N ($CD44^{lo}$ T_N and $CD44^{int}$ cells) and T_{VM} cells were also performed into recipients treated with a complex of IL-15/ IL-15Rα (IL-15c) (Figure 4.3A), which potently activates IL-15 signaling. Similar to observations following *H. polygyrus* infection, IL-15c treatment induced <1% of $CD44^{lo}$ T_N cells and ~3.5% of $CD44^{int}$ T_N cells to adopt a T_{VM} phenotype (Figure 4.3D). These rare "converted" T_N cells acquired a $CD49d^{int}$ (rather than $CD49d^{lo}$) phenotype with relatively low levels of CD44, and thus may represent cells that were positioned on the margin of T_{VM} gate. $CD44^{int}$ T_N cells were also slightly more likely to have proliferated compared to $CD44^{lo}$ T_N cells with IL-15c treatment, although a similar proportion (~15-20%) underwent division following either infection or IL-15c treatment (Figure 4.3D, E). Finally, transferred T_{VM} cells again retained their T_{VM} phenotype with very high rates (~95%) of proliferation (Figure 4.3E).

T_{VM} cells are clearly sensitive to the cytokine environment induced by helminth infection. However, our data demonstrate that extensive conversion of T_N cells to T_{VM} cells is not seen in the periphery, even under the highly stimulatory conditions of IL-15c administration, and the

increase in T_{VM} cell numbers after helminth infection is therefore driven by proliferation of existing T_{VM} cells.

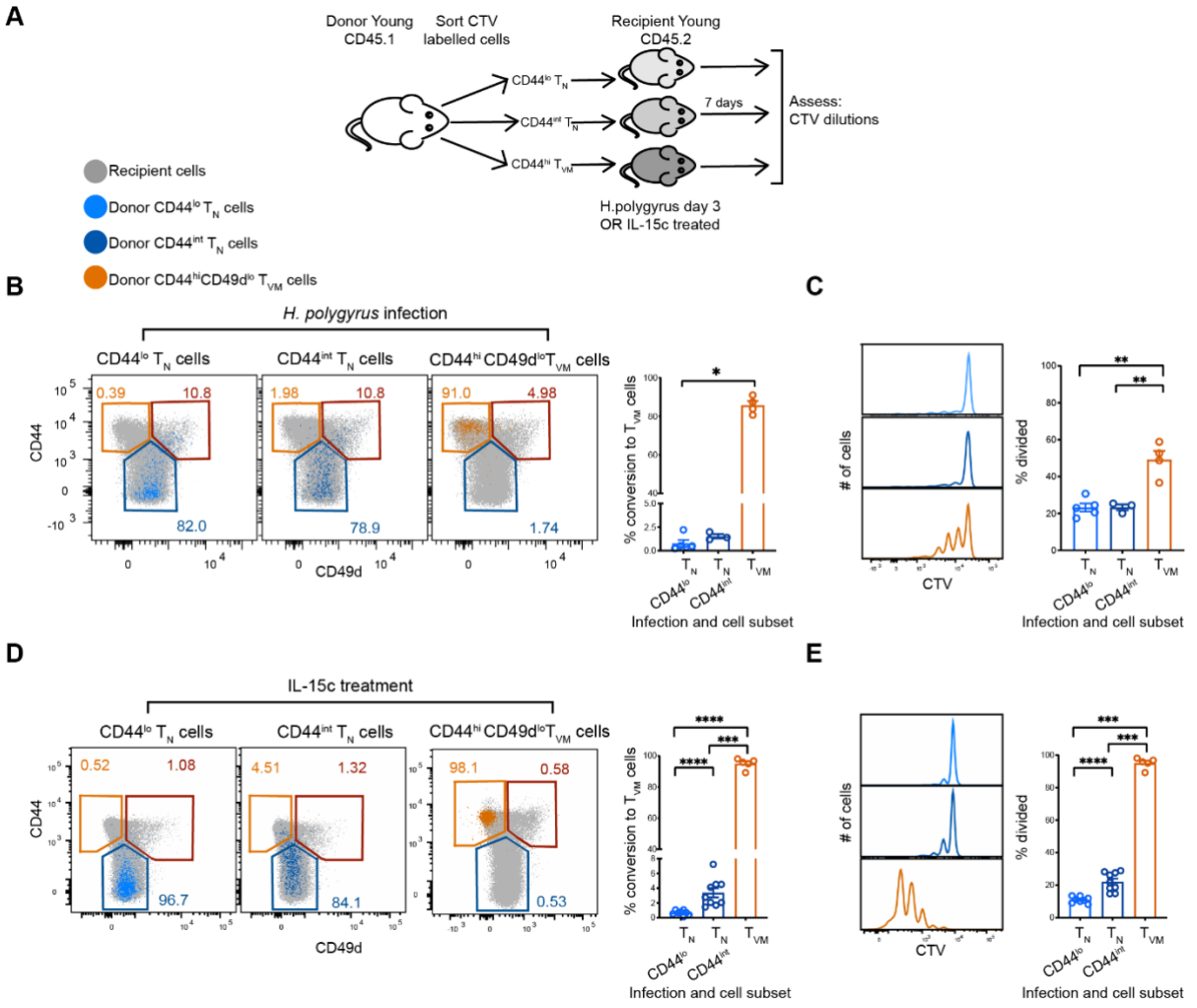


Figure 4.3. T_N cells have limited capacity to adopt the T_{VM} phenotype with *H.polygyrus* infection or IL-15 treatment. A) Schematic outlining the adoptive transfer of $CD44^{lo}$ and $CD44^{hi}$ T_N cells and T_{VM} cells to helminth infected or IL-15c treated mice. B) Representative dot plots showing CD45.1+ donor CD8 T cell subset overlayed on host CD45.2+ CD8 T cell population with bar graph plotting the respective frequency of donor cells adopting/retaining the T_{VM} cell phenotype in spleen at d10 of *H. polygyrus* infection. C) Representative histograms presenting CTV dilutions of each donor subset and total %age of donor population that proliferated in the *Hp* infected recipients. Data is pooled from 2 experiments for $CD44^{lo}$ T_N cells and representative of 2-3 experiments for T_{VM} cells, with n=2-4 per group. D) Representative dot plots showing donor CD8 T cell subset overlayed on host CD8 T cell population with bar graph showing the respective frequency of donor cells adopting/retaining the T_{VM} cell phenotype in spleen at d7 after IL-15c treatment. E) Representative histograms presenting CTV dilutions of each donor subset with total frequency of donor population that proliferated in the IL-15c treated recipients. Data is pooled from 2-3 different experiments with n=2-4 mice per group, T_{VM} cell transfers were done separately from T_N cell transfers. *p<0.05 , **p<0.01, ***p<0.001, ****p<0.0001 using a Mann Whitney

test, except in B and C where parametric t-test was applied after Shapiro-Wilk test of normality. Bar graphs represent mean ± SEM.

4.4.4. T_{VM} cells from *H. polygyrus* infected mice do not acquire increased TCR mediated functions

To assess whether the changes to T_{VM} cell numbers and phenotype were transient during acute infection or were maintained once infection was cleared, *H. polygyrus* infected mice were drug cured (Hp_{DC}) two weeks after infection (Figure 4.4A) and T_{VM} cell number and phenotype were assessed 2-3 weeks later. T_{VM} cell numbers were still more than two-fold higher in Hp_{DC} mice as compared to uninfected mice, indicating that the expanded T_{VM} cell population was maintained after infection was resolved (Figure 4.4B). However, phenotypic changes observed early post-infection in T_{VM} cells (Figure 4.1H-J) - including increases in CD122 (Figure 4.4C) and an increased proportion (Figure 4.4D) and level of CCR2 expression (Figure 4.4E) on T_{VM} cells - were still apparent but appeared attenuated in Hp_{DC} mice compared to observations at d8-12 post-infection (Figure 4.1H, J).

We next determined whether the helminth-induced increase in number and shift in phenotype of T_{VM} cells might improve antigen-specific functionality in Hp_{DC} mice. This was assessed by comparing TCR-mediated proliferation of sorted CD8 T cell subsets *in vitro*. In Hp_{DC} mice at 4-5 weeks post infection, we did not observe any difference in proliferation of CD8 T cell subsets from infected mice compared to uninfected controls (Figure 4.4F). Similarly, when CD8 T cell subsets from chronic (d45) *Hp* infected mice were sorted and stimulated *in vitro*, no change in proliferative capacity was observed in T_{VM} cells (Figure 4.4G). These results indicate that T_{VM} cells do not retain phenotypic changes for prolonged periods after helminth infection is

resolved, and helminth infection does not augment the TCR-mediated functionality of T_{VM} cells.

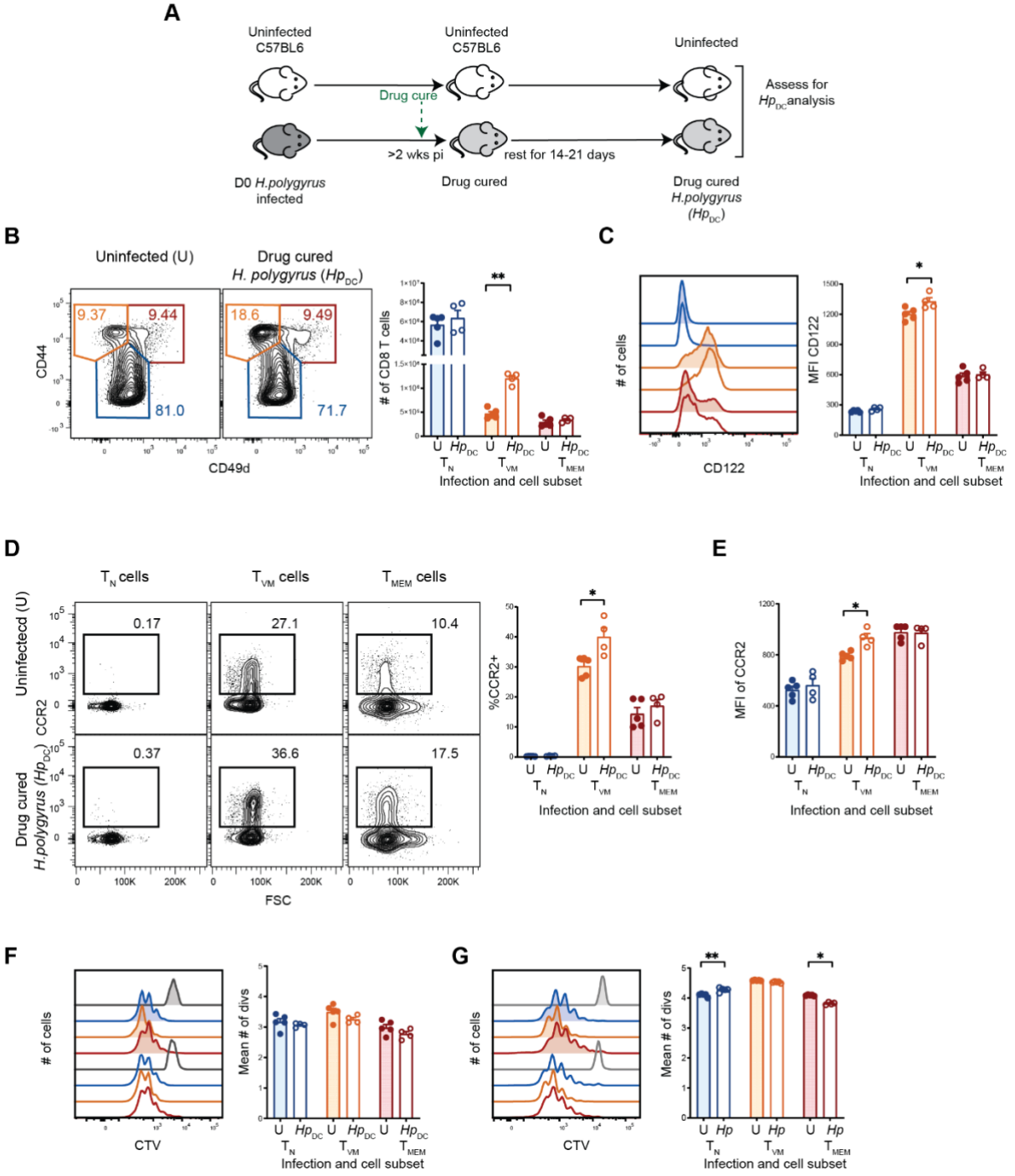


Figure 4.4. T_{VM} cells from *H. polygyrus* infected mice do not acquire increased TCR mediated functions. A) Schematic showing how mice were drug-cured (Hp_{DC}) and then rested before analysis B) Contour plots depicting Live/TCRβ+CD8+ subset in uninfected (U) and Hp_{DC} at 28d post infection with the respective number of cells in the T_N, T_{VM} and T_{MEM} subsets in the spleen. C) Representative histograms depicting expression of CD122 and MFI of CD122 and D) contour plots depicting CCR2+ cells and the respective frequencies and E) MFI of CCR2 in CCR2+ subsets across T_N, T_{VM} and T_{MEM} cells in uninfected and Hp_{DC} mice. F, G) Histograms depicting CTV dilutions and respective mean divisions of CD8 T cell subsets from F) uninfected and Hp_{DC} mice G) and in uninfected and chronic *Hp* d45 infected mice after 65h of anti-CD3/8/11a stimulation. Data is representative of 2-3 experiments with n=4-5 mice per group, except d45 (G) was done once. *p<0.05 and **p<0.01 using a Mann Whitney test. Bar graphs represent mean ± SEM.

4.4.5. The shift in T_{VM} cell number and function after helminth infection is not maintained in advanced age

While the T_{VM} cell population remained elevated, even 4-5 weeks after infection in drug-cured mice, it was unclear whether this expanded population was permanently maintained into advanced age. To address this, mice were infected with *H. polygyrus* at 8-12 weeks of age and T_{VM} cell number and phenotype were assessed 6-7 months (9 month old mice) or 15-18 months later (18-20 month old mice).

While we had observed increased T_{VM} cell number at 45 days after infection (Figure 4.1D), in 9 mo infected mice we only observed a slight trend of increased T_{VM} cells when compared to age-matched uninfected mice (Figure 4.5A). In addition, T_{VM} cells did not retain any of the phenotypic characteristics acquired after helminth infection, with no difference in Eomes or CD122 expression in previously infected mice compared to uninfected mice (Figure 4.5B, C). Similarly, mice that were previously infected then aged to 18-20 mo showed no significant difference in the number of T_{VM} cells compared to uninfected aged controls, although this analysis was confounded by substantial variation in T_{VM} cell numbers within groups (Figure 4.5D).

Analysis of functional parameters revealed no difference in TCR-mediated proliferation or cytokine production *in vitro* with cells from previously infected and aged mice compared to uninfected age-matched mice (Supplementary Figure S4A-C). Additionally, there was no difference in IL-15-mediated proliferation or IFNγ production after IL-12, IL-18 and IL-15 stimulation, which are both hallmarks of non-antigen-specific T_{VM} cell function (Figure S4D, E). Our results therefore show that while the increased number and altered phenotype of helminth-expanded T_{VM} cells persists beyond the duration of the infection, it is not maintained indefinitely.

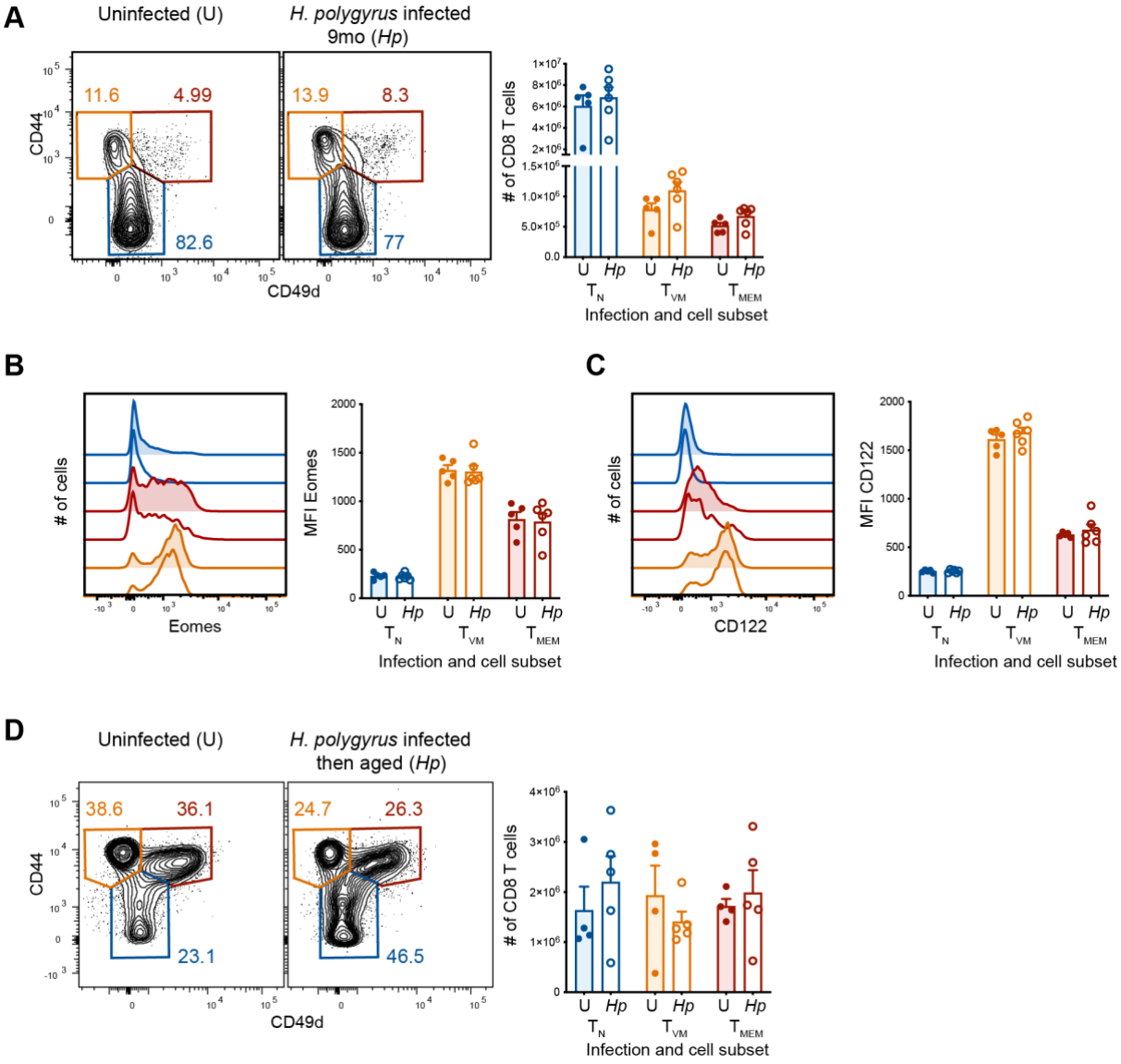


Figure 4.5. Increase in T_{VM} cell population after helminth infection is not maintained in advanced age. A) Representative contour plots showing TCRβ+CD8+ T cell subset from uninfected (U) and *H. polygyrus* (*Hp*) infected 9 month old (9mo) mice with the respective number of CD8 T cells in each subset. B) Bar charts showing MFI of Eomes and C) CD122 in uninfected and 9 mo *Hp* infected mice. Data is representative of 1 experiment with n=5 mice per group. D) Representative contour plots showing TCRβ+CD8+ T cell subset from aged uninfected (U) and previously *H. polygyrus* (*Hp*) infected then aged 18-20 mo mice with the respective number of CD8 T cells in each subset in uninfected aged and previously *Hp* infected aged mice. Data is representative of 3 experiments with n=4-5 mice per group. Bar graphs represent mean ± SEM.

4.4.6. Aged T_{VM} cells do not proliferate in response to helminth infection

Work from our group and others has shown that T_{VM} cells accumulate with age and acquire TCR-associated dysfunction [95,186], but their ability to proliferate in response to IL-15 stimulation is maintained [95]. To determine whether cytokine stimulation during helminth infection can expand the T_{VM} cell population in aged mice, we infected 18-20 mo mice with *H. polygyrus* and analysed T_{VM} cells from the spleen, blood, mesLN and liver at d10 post-infection. In contrast to young mice, we observed no change in the frequency or number of T_{VM} cells in the spleen, mesLN or the liver of infected aged mice (Figure 4.6A-D), nor was there any change in the number of T_{VM} cells in the blood (Figure 4.6E). Similar results were observed at d28 post infection (Supplementary Figure S5A-D), except for modest increase seen in the liver (Supplementary Figure S5D). These findings demonstrate that helminth infection of aged mice does not induce the substantial alterations to the T_{VM} cell compartment seen in young mice.

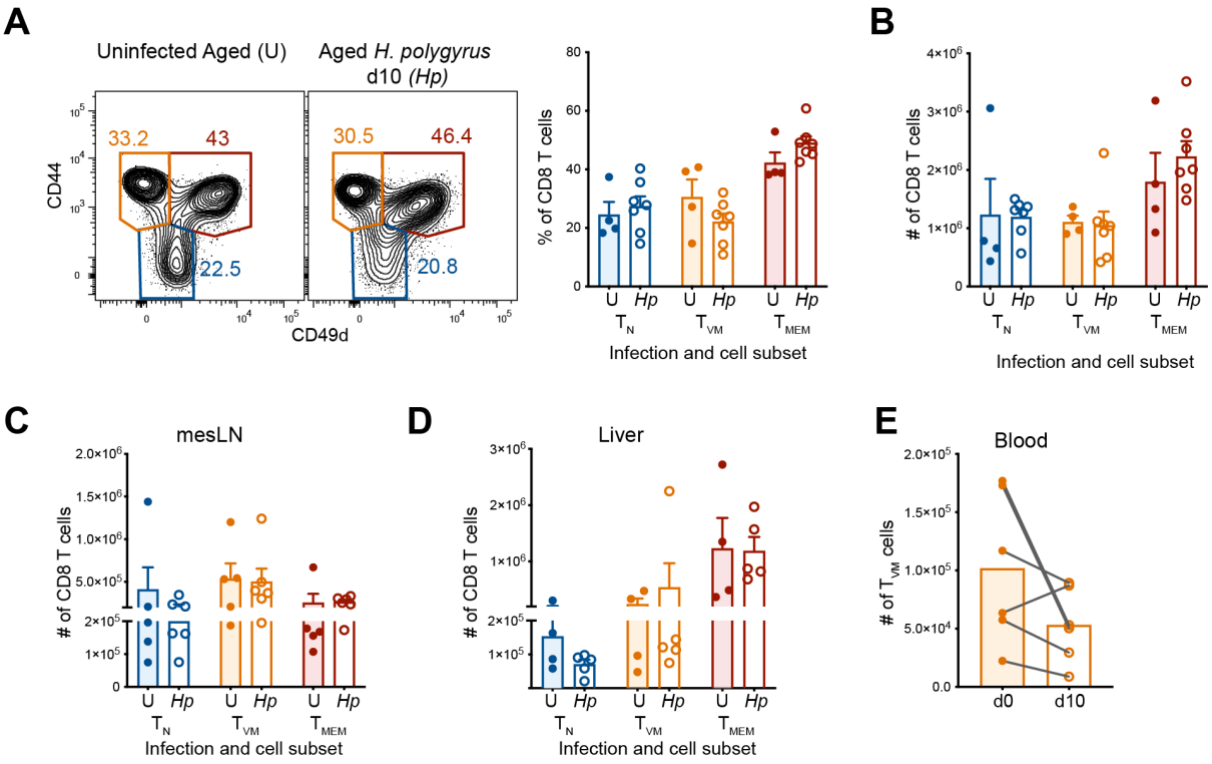


Figure 4.6. Aged T_{VM} cells do not proliferate in response to helminth infection. A) Representative contour plots showing Live/TCRβ+CD8+ T cell subset from uninfected (U) and d10 *H. polygyrus* (*Hp*) infected aged mice with bar graph showing frequency of CD8 T cell subsets and B) showing number of CD8 T cells in each subset in the spleen C) in the mesenteric lymph nodes (mesLN) D) in the liver and E) showing change in number of T_{VM} cells in blood from d0 to d10 post infection in a paired analysis in the aged mice. Spleen and mesLN data are representative of 2 experiments, liver and blood data is from one experiment with n=4-7 mice per group. *p<0.05 and **p<0.01 using a Mann Whitney test. Bar graphs represent mean ± SEM.

4.4.7. The lack of responsiveness of aged T_{VM} cells to helminth infection is due, in part, to an intrinsic defect.

The lack of T_{VM} cell expansion in aged mice could be due to extrinsic factors, such as dysregulation of Th2 immunity in aged mice, and/or intrinsic factors, such as reduced sensing of cytokines by aged T_{VM} cells during helminth infection. Given that there was already evidence of deficit in extrinsic Th2 response in helminth infections of aged mice, observed in *H. polygyrus* [260] and *T. muris* [261] infections, we focused on dissecting intrinsic defects in aged T_{VM} cell's response to cytokines.

Our previous data suggests that there may be minimal intrinsic deficits with IL-15 sensing. Aged T_{VM} cells have increased expression of CD122 in the steady state and increased phosphorylation of STAT5 (p-STAT5) after IL-15 stimulation [34]. When we have previously assessed *in vitro* proliferation, aged T_{VM} cells did not exhibit any deficit in proliferative capacity with IL-15 stimulation, compared to young T_{VM} cells [95]. Here, we assessed intrinsic cytokine responsiveness of T_{VM} cells *in vivo* by adoptively transferring young and aged CTV-labelled T_{VM} cells into young mice, treating with IL-15c and assessing proliferation (Figure 4.7A). Aged T_{VM} cells exhibited a modest but significant and reproducible reduction in proliferative capacity with *in vivo* IL-15c stimulation (Figure 4.7B, C). We then assessed the impact of helminth

infection on intrinsic proliferative capacity. Young and aged CTV-labelled T_{VM} cells were adoptively transferred into young, helminth infected, congenically distinct mice and proliferation in the spleen and mesLN was tracked 7 days later (Figure 4.7A). The mean number of divisions in aged T_{VM} cells was significantly lower than in young T_{VM} cells, both in the mesLN and spleen (Figure 4.7D), indicating an intrinsic, age-related deficit in response to helminth infection. This suggests that aged T_{VM} cells may have a moderate intrinsic deficit in their response to IL-15, which could contribute to their reduced response to helminth infection.

We also noted that other phenotypic differences observed during young helminth infection (Figure 4.1F-I) were absent after infection of aged mice. Young helminth infected T_{VM} cells exhibit increased CD122 expression after infection (Figure 4.1H), but CD122 appeared to be reduced on aged T_{VM} cells with infection (Figure 4.7E). CD124 was downregulated by nearly 40% on aged T_{VM} cells after helminth infection (Figure 4.7F). No difference was observed in the expression of Eomes in CD8 T cells following aged helminth infection (Figure 4.7G). Collectively, the proliferative profile and phenotypic changes in aged T_{VM} cells suggests that they have an intrinsic deficit in cytokine sensing *in vivo* which is a major cause of their inability to proliferate after helminth infection. Of note, it is possible that extrinsic deficits also contribute, whereby the helminth-induced cytokine environment in aged mice is not robust enough to promote significant IL-15 production. We speculate that a combination of possible extrinsic deficits and our demonstrated intrinsic defects in aged T_{VM} cells combine to result in a complete inability of T_{VM} cells to proliferate following helminth infection of aged mice.

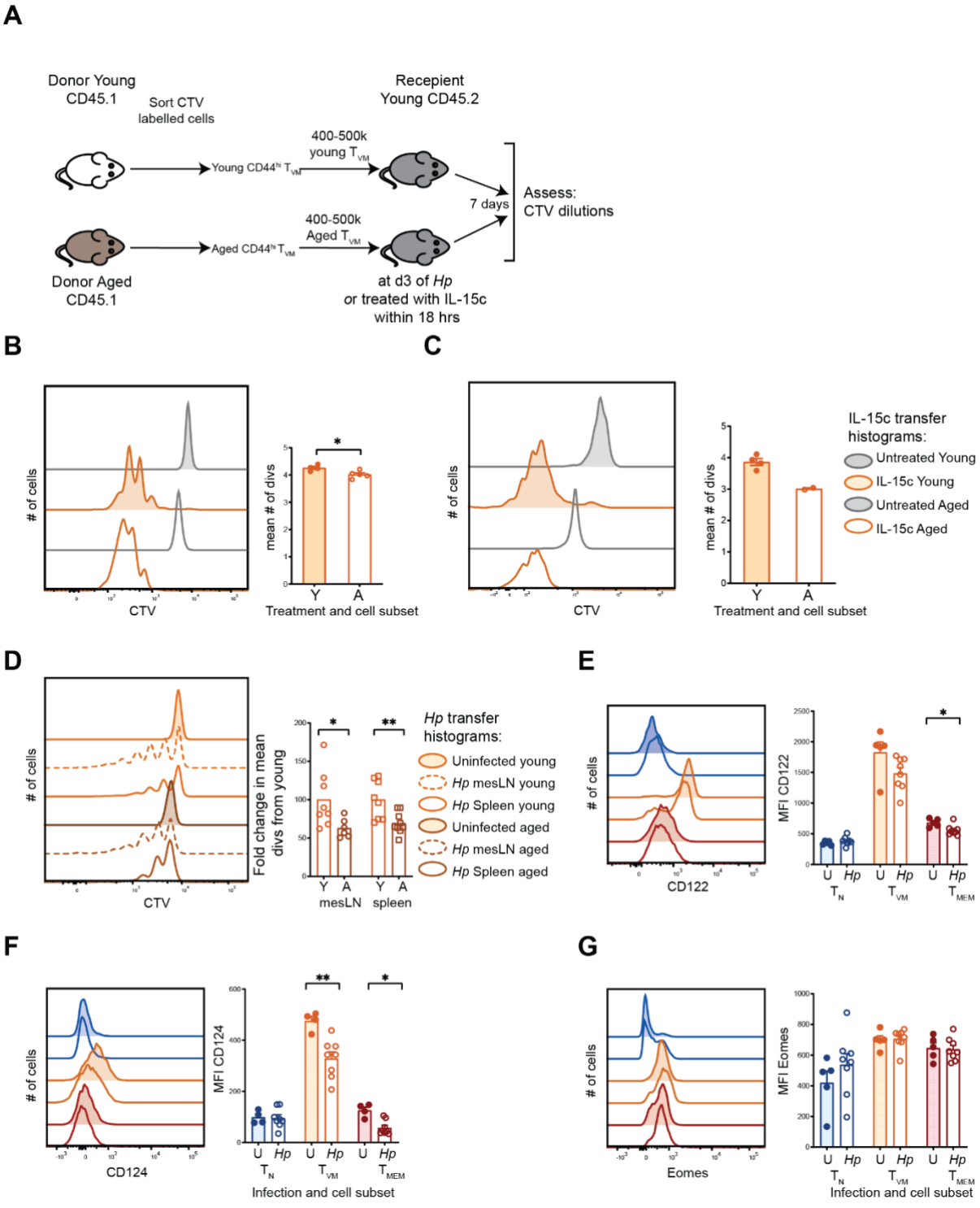


Figure 4.7. The lack of responsiveness of aged T_{VM} cells to helminth infection is due, in part, to an intrinsic defect. A) Schematic outlining the adoptive transfer of young and aged CD44^{hi} T_{VM} cells to helminth infected young mice or in mice that treated with IL-15c post-transfer. B,C) Data from two different experiments is being shown in B and C with representative histograms depicting CTV dilutions of transferred young and aged T_{VM} population in spleens of young IL-15c treated mice, 7d after transfer with the respective mean number of divisions in bar graphs (n=2-5 per group). D) Representative

histograms depicting CTV dilutions of transferred young and aged T_{VM} population in spleens of *H. polygyrus* infected host at d10 post infection with mean number of divisions of young and aged subset in spleen and mesenteric lymph nodes (mesLN) of infected host. Data is combined from two different experiments, with n=3-5 per group. Representative histograms depicting expression of E) CD122, F) CD124, and G) Eomes along with bar graphs of MFI of each marker in CD8 T cell subsets in uninfected and at d10 of *H. polygyrus* infection in aged mice. Data is representative of 2 experiments with n=4-8 mice per group. *$p<0.05$ and **$p<0.01$ using a Mann Whitney test. Bar graphs represent mean ± SEM.

4.5. Discussion

While we have known that helminth infections can drive an expanded T_{VM} cell subset, the pathways underpinning this proliferation remained vaguely defined. Here we have demonstrated for the first time that IL-15 supports T_{VM} cell expansion during helminth infection. We observed that IL-15 presentation was increased during helminth infection and that T_{VM} cell expansion was abrogated when IL-15 was blocked during *H. polygyrus* infection. We also found that infection with *H. polygyrus* or strong IL-15 stimulation is not sufficient to drive widespread acquisition of T_{VM} phenotype by T_N cells and instead drove proliferation of pre-existing T_{VM} cells to expand the T_{VM} cell compartment. This expanded compartment could persist after resolution of infection but was not maintained indefinitely into advanced age. In addition, helminth infection was unable to induce a similar expansion in aged T_{VM} cells, in part due to an intrinsic deficit in aged T_{VM} cells that is likely mediated, in part, by reduced IL-15 sensing. Collectively, these results demonstrate that T_{VM} cells are a distinct CD8 T cell subset that is highly responsive to cytokine cues during helminth infections, however such perturbations are corrected over time and do not manifest in major alterations in T_{VM} cell number or function in old age.

IL-4 sensing can clearly have an impact on T_{VM} cell generation and maintenance, but previous studies have suggested that the impact may be somewhat limited in scope. For example, loss of IL-4 signaling has only a modest impact on T_{VM} cell population in C57BL/6 mice [86,93]. Helminth infection is unable to induce an increase in T_{VM} cells in IL-4KO or IL-4RKO mice on the BALB/c background [84,167], which indicated the absolute dependence of T_{VM} cells on IL-4 in this strain. However, helminth infection of C57BL/6 mice lacking either IL-4 or IL-4R was still

able to drive a 2-fold increase in the frequency of T_{VM} cells [167]. Lin et al suggested that helminth driven expansion of T_{VM} cells may be compensated for by another signal in the absence of IL-4 in C57BL/6 mice. Given that the impact of IL-4 appears to be relatively modest in certain contexts, it was reasonable to hypothesise that additional cytokines to IL-4 were augmenting helminth-driven proliferation of T_{VM} cells. We demonstrated that T_{VM} cell expansion during helminth infection was IL-15-dependent. Of note, there have been studies that have shown that IL-4 can drive an increase in IL-15 from macrophages [262], and IL-4 can increase Eomes which can directly regulate CD122 expression. It is possible that T_{VM} cell expansion during helminth infection is supported by the observed increase in IL-15+ cDC1s, which are the most efficient DC subset for IL-15 transpresentation to T_{VM} cells [35,165]. Indeed, our observation of increased CD122 and CCR2 expression on T_{VM} cells in helminth infected mice also indicates increased IL-15 stimulation [259,263]. We therefore conclude that that IL-4 is not the direct driver of T_{VM} cell proliferation during helminth infection. However, whether this IL-15-driven increase in T_{VM} cells occurs as a consequence of IL-4 increasing production of, or sensitivity to, IL-15, is yet to be explored.

Of note, our observations may be strain-specific and may depend on the relative abundance of IL-4 and/or IL-15 during the generation of T_{VM} cells. In C57BL/6 and BALB/c mouse model, T_{MP} cells follow distinct developmental pathways based on differential signaling through IL-4 or IL-15. In BALB/c mice T_{MP} cells, also called innate memory T (T_{IM}) cells, first arise in the thymus in response to IL-4 production by NKT cells [92]. However, in C57BL/6 mice, T_{VM} cell precursors arise in the thymus with increased Eomes expression [101] and possibly heightened reactivity to self-antigen [205], but they proliferate and adopt the T_{VM} phenotype ($CD122^{hi}$) only

in the periphery where IL-15 is available [35]. Consequently, T_{MP} cells (i.e. T_{IM} cells) are more dependent on IL-4 in BALB/c mice, whereas T_{MP} cells (i.e. T_{VM} cells) are wholly dependent on IL-15 in C57BL/6 mice. In helminth infection of BALB/c mice, no proliferation was observed in T_{MP} cells in the absence of IL-4 [84,99], while helminth-driven proliferation of T_{VM} cells was unimpeded in C57BL/6 mice [167]. Therefore, it is possible that dependence on IL-15 is only relevant to helminth-infection in C57BL/6 mice, given their developmental history.

T_{MP} cells have been discussed in literature for some time as a population induced by lymphopenia [87] or homeostatic proliferation [159], but the pathways leading to their generation have only begun to surface recently. The current model of T_{VM} cell development is that they arise as a distinct lineage in the thymus in a TCR-directed process [101] from precursors that have high expression of Eomes and higher affinity for self-peptide-MHC[101,157,203,205]. They later acquire the $CD44^{hi}CD122^{hi}$ phenotype in the periphery where they homeostatically proliferate at slightly higher levels than T_N cells [101]. They are known to proliferate in response to both IL-15 [35] and it is thought that homeostatic TCR signaling also contributes to this increase [97,205]. Some studies have suggested that T_N cells can seed the T_{VM} cell population in the periphery. IL-15 stimulation, high CD5 expression on T_N cells, and helminth infections are all reported to promote T_N cell conversion to T_{VM} cells [84,97]. In contrast, we observed minimal proliferation and conversion capacity of T_N cells in response to infection or even after potent IL-15c stimulation. These results are consistent with other studies that report marginal capacity of T_N cells to acquire $CD44^{hi}$ phenotype as a result of IL-15 treatment [29,165]. Again, we should consider whether there is a species-specific effect as Rolot et al [84] have suggested that T_N cells may be converting to T_{VM} cells during helminth infection of BALB/c mice. While it may be possible that

the responses of CD8 T cells in the BALB/c mice are very different from the ones observed in our study, it would be surprising if T_{VM} cells were not proliferating during helminth infection in a BALB/c mouse where their thymic development is mainly dependent on IL-4 and no increase in T_{VM} cells is observed in IL-4KO/ IL-4RKO helminth infected mice on the BALB/c background [84]. Morris et al also showed that *in vivo* IL-4 treatment in BALB/c mice did not induce a conversion of T_N cells to the T_{VM} phenotype [99]. T_N cells proliferated after treatment with an IL-4c (IL-4/anti-IL4 complex), but they retained their $CD44^{lo}$ phenotype [99]. Given these results, we conclude that T_{VM} cells population does not increase via stochastic processes in the periphery and overt cytokine exposure does not appear to drive substantial acquisition of the T_{VM} phenotype by T_N cells.

Studies on T_{VM} cells and helminth infection in the past have focused on demonstrating the beneficial impact of helminth infection in a subsequent challenge. Despite that, we did not observe evidence of altered TCR-driven *in vitro* functionality in T_{VM} cells, or any other CD8 T cells, during helminth infection, which could have resulted in improved antigen-specific responses. Our observations are also supported by previous studies, when equal number of T_{VM} cells from helminth infected and uninfected mice were transferred to murine gammaherpesvirus (MuHV)-4 infected recipients, and when antigen-specific response was assessed, both T_{VM} cell populations made equal contribution [84]. We therefore did not delve further into dissecting antigen specific responses to another infection. However, our observation of improved cytokine and chemokine receptor expression could suggest augmented trafficking of T_{VM} cells to sites of infection. It was recently reported that increased CCR2 expression on T_{VM} cells can lead to improved recruitment to lungs during respiratory

infections [166]. Additionally, a 2-fold increase in T_{VM} cells with helminth infection is likely to impart a numerical advantage when mediating non-antigen-specific, bystander effector functions in subsequent pathogen encounters.

We also observed that the helminth-induced T_{VM} cell expansion was maintained for a few weeks beyond the clearance of an infection but not out to very extended timepoints and does not result in an expanded T_{VM} cell subset in aged mice. Additionally, aged T_{VM} cells fail to proliferate in response to helminth infection. This is possibly partly due to a defective immune response to helminth infection in aged mice. Previous studies have reported that aged mice do not establish a Th2 response to helminth infection as well as young mice, with lower levels of IL-4 and IL-13 during *T. muris* infection [260] along with a defect in the activation of alternatively activated macrophages during *H. polygyrus* infection [261]. There is also evidence of skewing towards an inflammatory Th1 response in aged helminth infection, with observation of increased levels of TNF, IFNγ, IL-12 at the site of infection [260,261]. However, it is clear that T_{VM} cells from aged mice also exhibit an intrinsic defect in cytokine sensing that is evident even when they are transferred to a young host. *In vivo* administration of IL-15c revealed a slight but significant reduction in T_{VM} cell proliferation. Our previous data suggest that aged and young T_{VM} cells exhibit similar proliferation when stimulated with IL-15 *in vitro* [95]. However, it is possible that the strong signaling induced by IL-15 cytokine stimulation *in vitro* and IL-15c stimulation *in vivo* may mask a reduced sensitivity to physiological levels of IL-15, such as those induced by helminth infection. Collectively, these indices of defective intrinsic T_{VM} cell cytokine responsiveness, combined with dysregulated anti-helminth responses in aged mice - which further compromises CD122 and CD124 upregulation on T_{VM}

cells - provide an explanation for the absence of T_{VM} cell expansion after helminth infection of aged mice.

Taken together, our results not only define mechanisms of T_{VM} cell proliferation during helminth infection but also identify intrinsic differences in the young and aged T_{VM} cell response to helminth infection. These findings challenge the previous understanding of cytokine responsiveness in young and aged CD8 T cells and therefore have implications on infections driving a broad cytokine response.

4.6. Acknowledgements

The authors wish to thank Prof Mariapia Degli-Esposti for provision of reagents, and staff at Monash FlowCore and Animal Research Platforms (MARP).

4.7. Author Contributions

TH performed majority of the experiments and analysed the results, NLL., KMQ., designed the study and supervised the research, AN and DT performed key aged experiments, JL performed adoptive transfers, AZ set up *T. muris* infections, CD maintained *H. polygyrus* lifecycle, ESP and MO'K collaborated on DC experiments, CZ and NLH provided helminths parasites, CZ, NLH and MO'K provided subject matter expertise. NLL and TH wrote the initial draft of the manuscript and all authors participated in writing the final manuscript.

4.8. Competing interests

The authors declare no competing interests.

4.9 Supplementary data

4.9.1. Supplementary Figure S1:

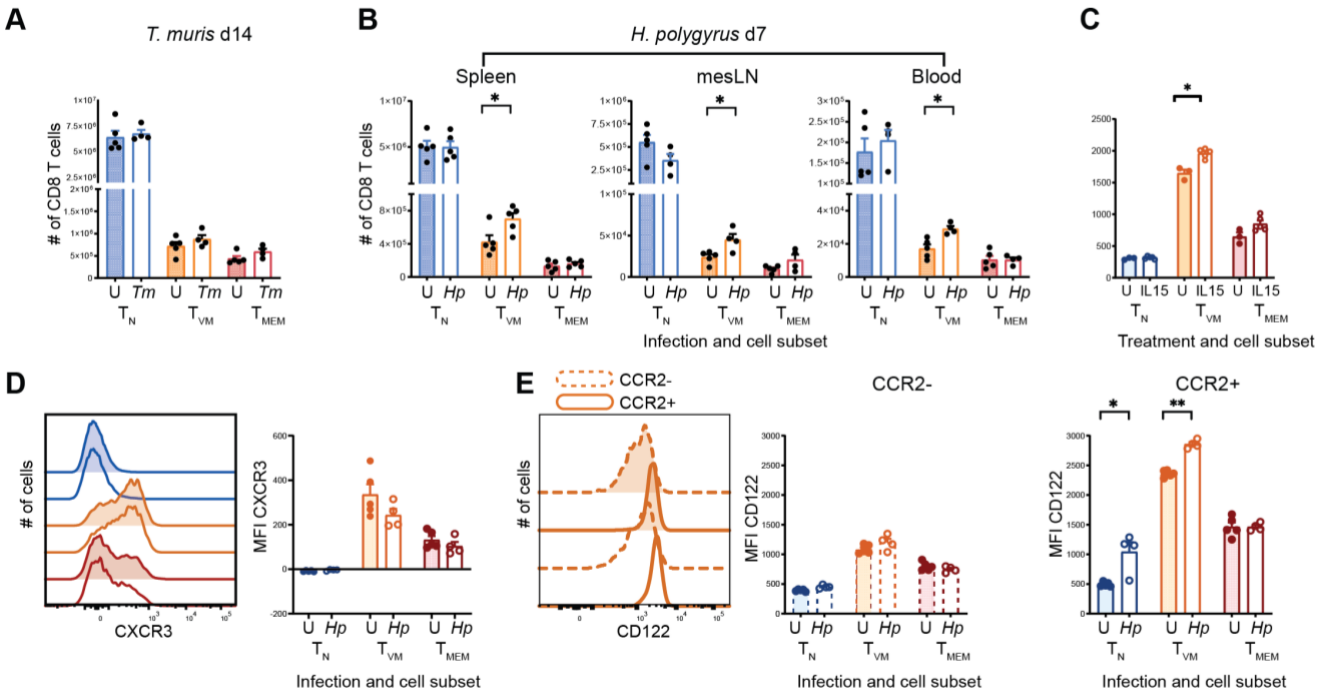


Figure 4.8. Early timepoints of *T. muris* and *H. polygyrus* infection. A) Number of T_N, T_{VM} and T_{MEM} cells at d14 of T.muris (*Tm*) infection and in uninfected (U) mice. Number of T_N, T_{VM} and T_{MEM} cells in B) spleen, mesenteric lymph nodes and blood on d7 of H. polygrus (*Hp*) and in uninfected (U) mice. C) median fluorescence intensity (MFI) of CD122 at d7 after *in vivo* treatment with IL-15c (IL-15) and in untreated (U) mice. D) Representative histograms showing expression of CXCR3 and bar graph showing MFI of CXCR3 on T_N, T_{VM} and T_{MEM} cells E) representative histograms showing expression of CD122 on CCR2+ and CCR2- T_{VM} cells and bar graph showing expression of CD122 in the CCR2- and CCR2+ cells among T_N, T_{VM} and T_{MEM} subsets, at d10-12 of *Hp* infection and in uninfected mice. Data is representative of 2 experiments with n=3-5 mice per group. * and ** indicates $p<0.05$ and $p<0.01$, (Mann Whitney test). Bar graphs represent mean ± SEM.

4.9.2. Supplementary Figure S2:

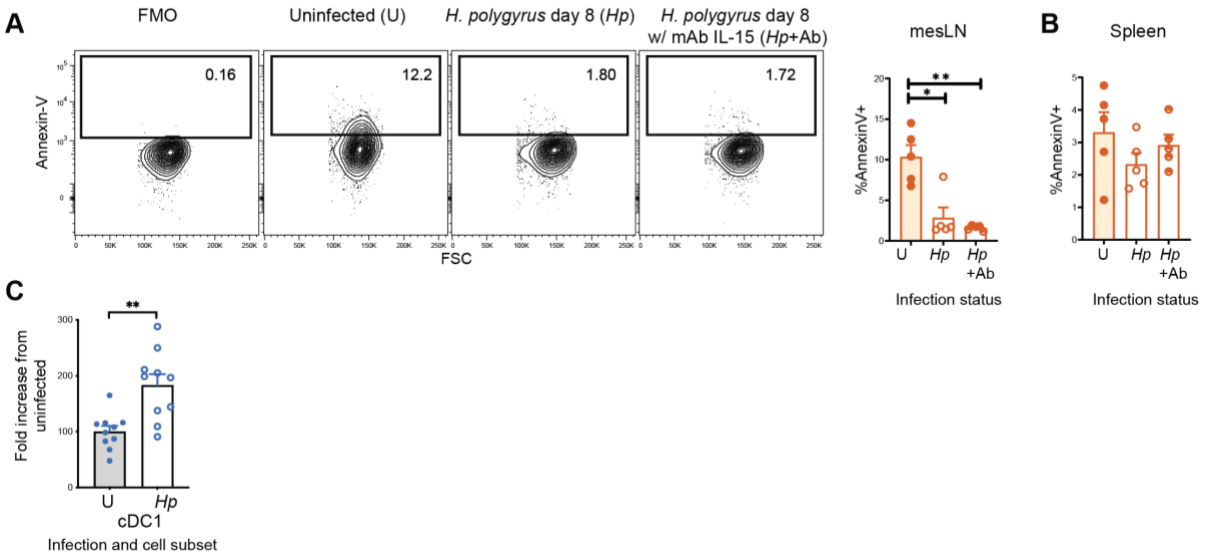


Figure 4.9. Apoptosis detection after IL-15 blocking and IL-15 expression on DCs. A) Representative contour plot gated on T_{VM} cells showing expression of Annexin-V on T_{VM} cells in mesenteric lymph nodes (mesLN) along with bar graph showing frequency of T_{VM} cells that are Annexin V+ in mesLN and B) spleen at d8 of *H. polygyrus* (*Hp*) infection, in IL-15 blocking antibody treated (d0, d4) mice at d8 of *Hp* infection (*Hp*+Ab) and in uninfected (U) mice. C) Fold increase in number of IL15+ cDC1s at d8 of *Hp* infection and in uninfected as a ratio of mean number of cDC1s in uninfected mice. Data is representative of 2 experiments with n=5 mice per group. * and ** indicates $p<0.05$ and $p<0.01$, (Mann Whitney test). Bar graphs represent mean ± SEM.

4.9.3. Supplementary Figure S3:

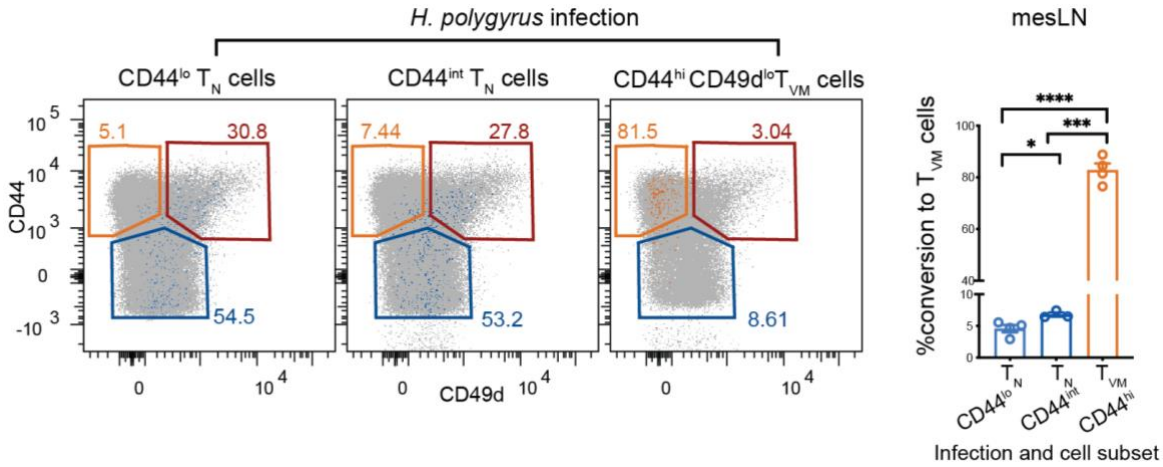


Figure 4.10. Conversion of T_N cells to T_{VM} cells in mesLN after *H. polygyrus* infection. A) Representative dot plots showing CD45.1+ donor CD8 T cell subset overlayed on host CD45.2+ CD8 T cell population with bar graph plotting the respective frequency of donor cells adopting/retaining the T_{VM} cell phenotype in mesenteric lymph nodes (mesLN) at d10 of *H. polygyrus* infection. Data is pooled from 2 experiments for $CD44^{lo}$ T_N cells, one experiment for $CD44^{int}$ T_N cells and representative of 2-3 experiments for T_{VM} cells, with n=2-4 per group. *, ***, **** indicates $p<0.05$, $p<0.001$ and $p<0.0001$ (parametric t-test was applied after Shapiro-Wilk test of normality), Bar graphs represent mean ± SEM.

4.9.4. Supplementary Figure S4:

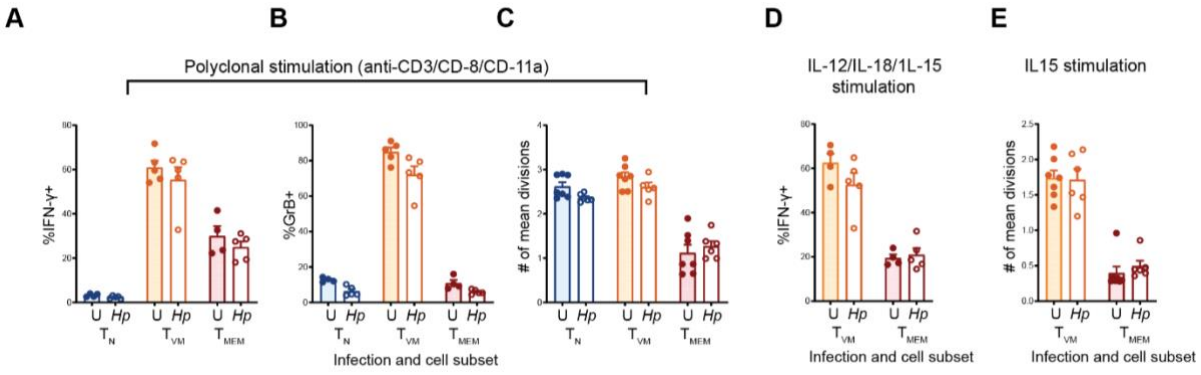


Figure 4.11. Assessment of function in aged, previously *H. polygyrus* infected mice. A) Frequency of T_N, T_{VM} and T_{MEM} cells producing A) IFN-γ and B) granzyme B (GrB) after 36 hours of *in vitro* stimulation with anti-CD3/-CD8/-CD11a C) mean divisions of T_N, T_{VM} and T_{MEM} cells after 65 hours of *in vitro* stimulation with anti-CD3/-CD8/-CD11a D) frequency of IFN-γ+ cells after 16 hours of stimulation with IL-12/IL-18/IL-15 and E) proliferation of T_{VM} and T_{MEM} cells after 5 days of culture with 100ng of cIL-15. All data is from CD8 T cells sorted from aged, previously H. polygyrus (*Hp*) infected and uninfected (U) mice. A, B, D were performed once, C, E were performed 2-3 times with n=4-6 per group. Bar graphs represent mean ± SEM.

4.9.5. Supplementary Figure S5:

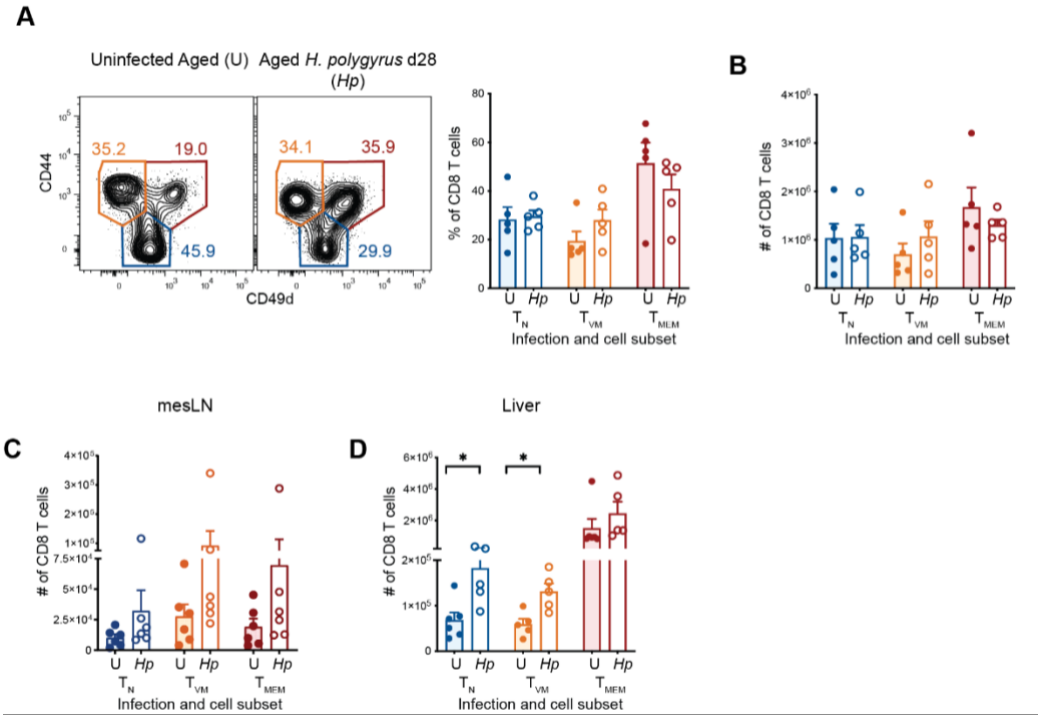
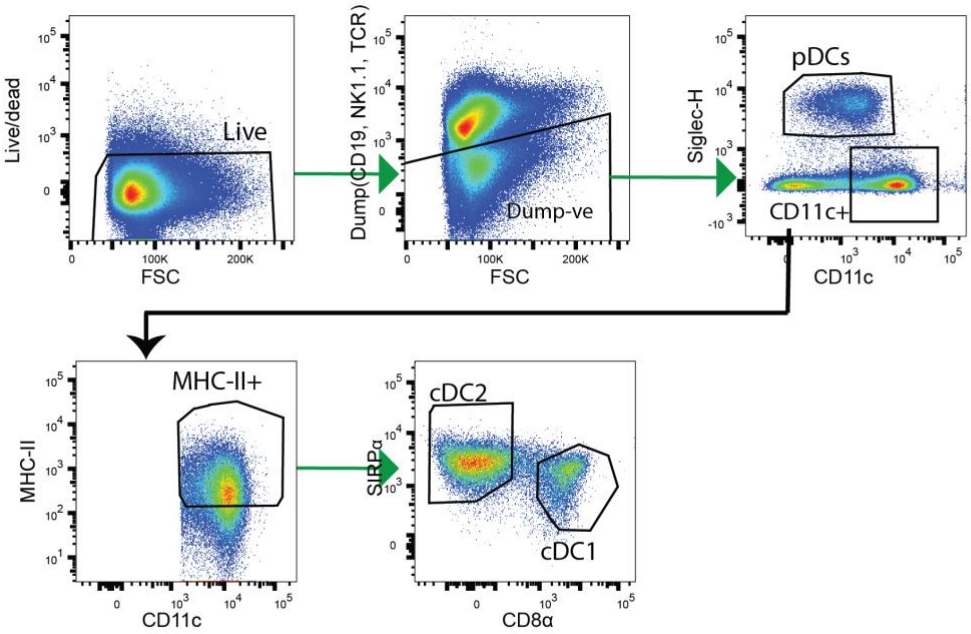


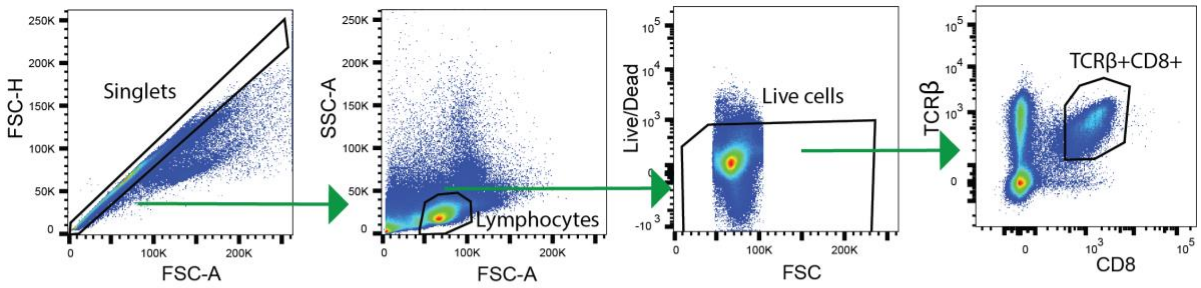
Figure 4.12. Day 28 of aged *H. polygyrus* infection. Representative contour plots showing Live/TCRβ+CD8+ T cell subset from uninfected (U) and day 28 *H. polygyrus* (*Hp*) infected aged mice with bar graph showing frequency of CD8 T cell subsets and B) showing number of CD8 T cells in each subset in the spleen C) in the mesLN D) in the liver. Spleen d*ata* is representative of 2 experiments, mesLN and liver data is from one experiment, with n=5-6 mice per group. * indicates $p<0.05$, (Mann Whitney t-test). Bar graphs represent mean ± SEM.

4.9.5. Supplementary File 1:

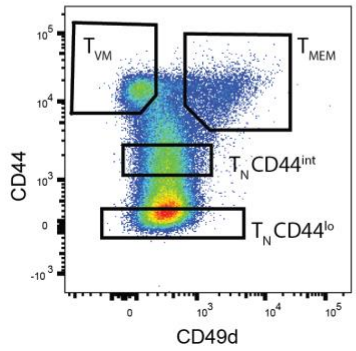
Gating strategy for DC subsets:



Gating strategy for CD8 T cell phenotyping:



Typical gating strategy for sorts:



4.9.6. Supplementary File 2:

	Conjugate	Clone	Isotype	Manufacturer	Catalogue #	Dilution
Surface markers						
CD8α	BUV395	53-6.7	Rat IgG2a, k	BD Biosciences	563786	400
CD44	PE-Cy7	IM7	Rat IgG2b, k	ebioscience/Jomar	25-0441-82	800
CD44	FITC	IM7	Rat IgG2b, k	BD Biosciences	553133	400
CD45.1	APC-Cy7	A20	Mouse IgG2a, κ	Biolegend	110716	200
CD45.2	PE	30-F11	Rat IgG2b, k	eBioscience	12-0454-83	200
TCRβ	AF700	H57-597	Ar Ham IgG	Biolegend	109224	400
TCRβ	BV421	H57-597	Ar Ham IgG	Biolegend	109229	400
CD49d	AF647	R1-2	Rat IgG2b, k	Biolegend	103614	400
CD49d	PE-Cy7	R1-2	Rat IgG2b, k	Biolegend	103618	400
CD124	PE	mIL4R-M1	Rat IgG2b, k	BD Biosciences	552509	100
CD122	BV421	TM-b1	Rat IgG2b, k	BD Biosciences	566301	200
CD122	FITC	TM-b1	Rat IgG2b, k	eBioscience	11-1222-82	200
CXCR3	APC	220803	Rat IgG2a	In Vitro Technologies	FAB1685A025	20
CCR2	unconjgated	EPR20844-15	Rabbit IgG	Biolegend	ab273050	200
CD172α (SIRPα)	BUV496	P84	Rat IgG1	BD Biosciences	741131	400
CD11c	FITC	HL3	Ar Ham IgG_1, l2	BD Biosciences	557400	400
Siglec H	AF647	551	Rat IgG1, k	Biolegend	129607	1000
I-A (b/d/q), I-E(d/k)MHC Class II	APC-Cy7	M5/114.15.2	Rat IgG2b, k	Biolegend		800
NK1.1	PacBlue	PK137	Mouse IgG2a, k	Biolegend	108722	400
CD19	PacBlue	6D5	Rat IgG2a, k	Biolegend	115523	400
IL-15	Biotin		Polyclonal rabbit	Peprotech	500-P173BT	100

	Conjugate	Clone	Isotype	Manufacturer	Catalogue #	Dilution
Intracellular/intranuclear markers						
Eomes	PE-Cy7	Dan11Mag	Rat IgG2a, κ	eBioscience	25-4875-82	200
TNF	PE	MP6-XT22	Rat IgG1, k	Biolegend	506306	400
IFN-Gma	FITC	XMG1.2	Rat IgG1, k	BD Biosciences	554411	400
Granzyme B	PacBlue	GB11	Mouses IgG2b, k	Biolegend	515407	200
Streptavidin	PE			Life Technologies	S866	500
Anti-Rabbit IgG F(ab')2 Fragment	PE		Goat	Cell signaling Technologies	8885	500
Viability markers						
Fixable Viability dye AquaBlue				Life Technologies	L34966	400
Fixable Viability dye Near Infra red				Life Technologies	L10119	800
Fixable viability stain R700				BD Biosciences	564997	800

Chapter V

5. Impact of LCMV infection on T_{VM} cells in young and aged mice

5.1. Introduction

T_{VM} cells are of interest in immune ageing, not only due to their accumulation relative to T_N cells with age in mice and humans, comprising up to 50% of all antigen-naïve CD8 T cells [95,96], but also because they exhibit disproportionate age-related dysfunction relative to T_N cells. Aged T_{VM} cells become senescent, lose TCR-mediated proliferative capacity *in vitro* and become increasingly monofunctional in terms of cytokine production[95]. This age-related defect has also been shown *in vivo*, with T_{VM}-derived effector cells comprising a reduced proportion of influenza-specific responses with age as compared to T_N cells[168] .

As most studies on T_{VM} cells have focused on SPF mice, there is some uncertainty as to whether the accumulation and dysfunction in T_{VM} cells would still occur after life course exposure to normal pathogens. A recent study highlighted that SPF mice may be a better model for the neonatal immune system rather than a more mature human immune system due to ultra-hygienic conditions[193]. While the importance of SPF environment in studying fundamental and mechanistic roles of gene function cannot be underestimated, the value of SPF mouse model as a readout of vaccine response has been questioned in the past[192]. Variable environmental conditions, as well as repeated pathogen exposure that occurs during the lifespan in humans and feral mice can impart extended effects on the immune system which is evident in the vastly

different immune cell distribution observed in petstore/feral mice and SPF mice, and in human cord blood when compared to adult humans[192,193]. With regard to T_{VM} cells, specifically, a recent study exploring differences in the T_{VM} subset between SPF and feral mice found an increased frequency of T_{VM} cells in feral mice. This indicates that the T_{VM} compartment is a significant immune cell subset that is actively maintained in real-world conditions, and that infections may mediate changes to the T_{VM} cell population in an antigen-independent manner[102]. However, it is still unclear how the T_{VM} cell subset responds to repeated exposure to different pathogens in a controlled environment.

Others have shown transient and sustained changes in the number and function of T_{VM} cells after HIV and CMV infections[143,169] and we have demonstrated similar findings in our assessment of T_{VM} cells following helminth infection (Chapter 4). However, not only were these numerical differences in cell number transient, but they did not impart any functional difference on T_{VM} cells. Apart from helminth infection and its induction of a strong Th2-biased cytokine profile, it is not clear how T_{VM} cells respond to the varied cytokine signals induced by other viral or bacterial infections, and if fluctuations in the inflammatory environment can modulate the age-related accumulation and/or functionality of T_{VM} cells. We and others have shown that the expression level of CD122 as well as the number of $CD122^{hi}$ T_{VM} cells is increased with ageing[34,102], coincident with robust cytokine responsiveness even as the capacity for TCR-driven proliferation and effector function is lost [34,95]. This led us to hypothesize that infection driven inflammation may shape the T_{VM} cell compartment and alter antigen-dependent and -independent immune responses in advanced age.

In this study, we explored whether infections that are known to induce T_{VM}-associated cytokines such as type I IFN, IL-15 and IL-12 can impact T_{VM} cell numbers and functionality, and the extent to which such changes are maintained long-term, influencing the immune senescent phenotype in mice. We assessed the impact of both bacterial (*Salmonella (S.) typhi*) and viral (IAV, lymphocytic choriomeningitis virus (LCMV)) infections. Salmonella serovars are known to induce IL-12 and IL-18 production in mouse studies[264,265] and IL-15 in human studies[231,232] , whereas IAV and LCMV infections induce a type I IFN response and robust CD8 T cell activation[266–268]. These cytokines have been shown to either impact T_{VM} cell function, by mediating enhanced innate-like functions[94,96,97] or proliferation[35,97,202]. Therefore, we examined how these infections, alone and in combination, have an effect on T_{VM} cell number and function, both immediately following infection and long-term after resolution of infection. Importantly, T_{VM} cells have the capacity to act in a non-antigen-specific manner but the functional consequences of multiple infections on non-antigen-specific T_{VM} cell function have not been explored. To dissect the impact of these infections on future antigen-dependent and independent immune responses, we assessed TCR-associated and cytokine-driven functions side by side in the *S. typhi*, IAV and LCMV infection models. Our findings demonstrate that LCMV alone was able to permanently reduce the frequency and improve TCR associated functions of T_{VM} cells, resulting in an attenuation of the senescent phenotype with age. This is predicted to have cumulative and ongoing impacts on how individuals respond to infections across the lifespan, possibly contributing to the heterogeneity in responses to infection observed across individuals and variable kinetics of biological ageing.

5.2. Results

5.2.1. Infection driven changes to T_{VM} cells are pathogen-specific

While dramatic shifts have been observed in CD8 T cell populations when SPF mice were co-housed with wild or pet store mice[193], a later study focussing on T_{VM} cells did not observe any differences after co-housing with dirty mice but did report a major increase in T_{VM} cells in feral mice compared to SPF mice[102]. To assess the impact of environmental microbes on T_{VM} cells, we examined the impact of "dirty" housing conditions on both young and aged mice.

Through colleagues at James Cook University (JCU), Townsville, Queensland, we were able to source aged (20 mo) dirty male C57BL/6 mice that had been housed in an unsealed building. These aged dirty mice had exposure to wild rodents and had norovirus and pinworm infections as indicated in their health report (Appendix 9.3). Young (5 mo) dirty male mice were also sourced from the JCU's C57BL/6 colony and received bedding from the aged dirty mice weekly but were not co-housed. The young dirty mice were age- and gender-matched with SPF control mice from the JCU C57BL/6 colony. Aged dirty mice were unable to be age- and gender-matched with SPF control mice; the aged dirty mice were all male, there were no aged SPF mice available at JCU and the MARP aged SPF colony is ex-breeder female mice. Therefore, we were just able to do a three-way analysis between young SPF, young dirty and aged dirty male mice.

Analysis of young dirty mice alongside their SPF housed controls did not reveal any difference in frequency, number and TCR mediated proliferative capacity of T_{VM} cells from the two groups

(Figure 5.1 A-D). These results are consistent with the findings of Moudra et al, where no difference in the T_{VM} cell population was observed after co-housing SPF mice with feral mice[102]. However, they contrast with other studies, which observed major differences in immune cell distribution with environmental exposure to pathogens[192,193]. A notable finding here is that the number and frequency of T_{VM} cells appeared to be higher in both the young SPF and young dirty mice from JCU (~35%) (Figure 5.1 A-C) compared to what we typically observe in young mice of the same age from our MARP colony (~20%). This implies general housing conditions in different facilities could influence T_{VM} cell frequency or there could be sub-strain differences in T_{VM} cell frequency across different breeding programs.

As we did not have appropriate controls to assess with aged dirty mice, we only compared differences between young and aged dirty mice to see if environmental conditions can alter the accumulation and phenotype of young and aged T_{VM} cells. In aged mice, we observed a significantly reduced number and frequency of T_{VM} cells when compared to T_{VM} cells from young dirty mice (Figure 5.1 A, B, C), which contrasts with what we have observed in our comparisons of young and aged mice where no difference in T_{VM} cell number is observed (Chapter 3, Figure 3.1 A). We also observed a reduction in T_N cells in dirty aged mice compared to dirty young (Figure 5.1 C), which is expected to occur with ageing as T_N cells undergo attrition[185]. While T_{MEM} cells were increased in frequency in aged dirty mice compared to young dirty mice (Figure 5.1 B), this increase did not translate to a greater number of T_{MEM} cells in aged dirty mice, instead they were reduced (Figure 5.1 C). This was surprising considering that the aged dirty mice had significant exposure to environmental pathogens through the life

course, like norovirus and pinworms, as indicated in their health report (Appendix 9.3) and were expected to be increased (Figure 5.1 C).

We have demonstrated that aged T_{VM} cells have a loss in TCR mediated proliferation compared to young T_{VM} cells[95], we now wanted to assess if exposure to infections has any impact on modulating the functionality of T_{VM} cells in aged mice. TCR mediated proliferation was assessed in a single cell proliferation assay, using sorted T_N, T_{VM} and T_{MEM} cells stimulated for 4 days with anti-CD3/8/11a Ab stimulation (section 2.8.3). Our previous work has shown that in young mice, T_{VM} cells have the highest rate of proliferation, followed by T_N and then T_{MEM} cells. However, within each group of mice, we did not observe this distribution of proliferative capacity, instead T_N, T_{VM} and T_{MEM} cells appeared to have the same magnitude of proliferation (Figure 5.1 D). Young T_{VM} cells did not to have an advantage over aged T_{VM} cells in proliferation, as mean burst size was similar across all groups (Figure 5.1 D).

When phenotypic differences like CD122 and CD5 expression were assessed, no difference was observed between young clean and dirty cohorts in any subset (data not shown). When young and aged dirty cohorts were assessed for these markers, we did not observe an increase in CD122 expression in T_{VM} cells which was expected to occur with ageing (Figure 5.1 E) as previously described (Chapter 3, Figure 3.1 C). When CD5 expression was assessed, it was significantly reduced on T_{VM} cells from aged dirty mice, whereas T_{MEM} cells had significantly increased CD5 expression in the aged dirty mice compared to young dirty mice (Figure 5.1 F). Our data from SPF mice parallels these changes in CD5 expression (Chapter 3, Figure 3.1 G).

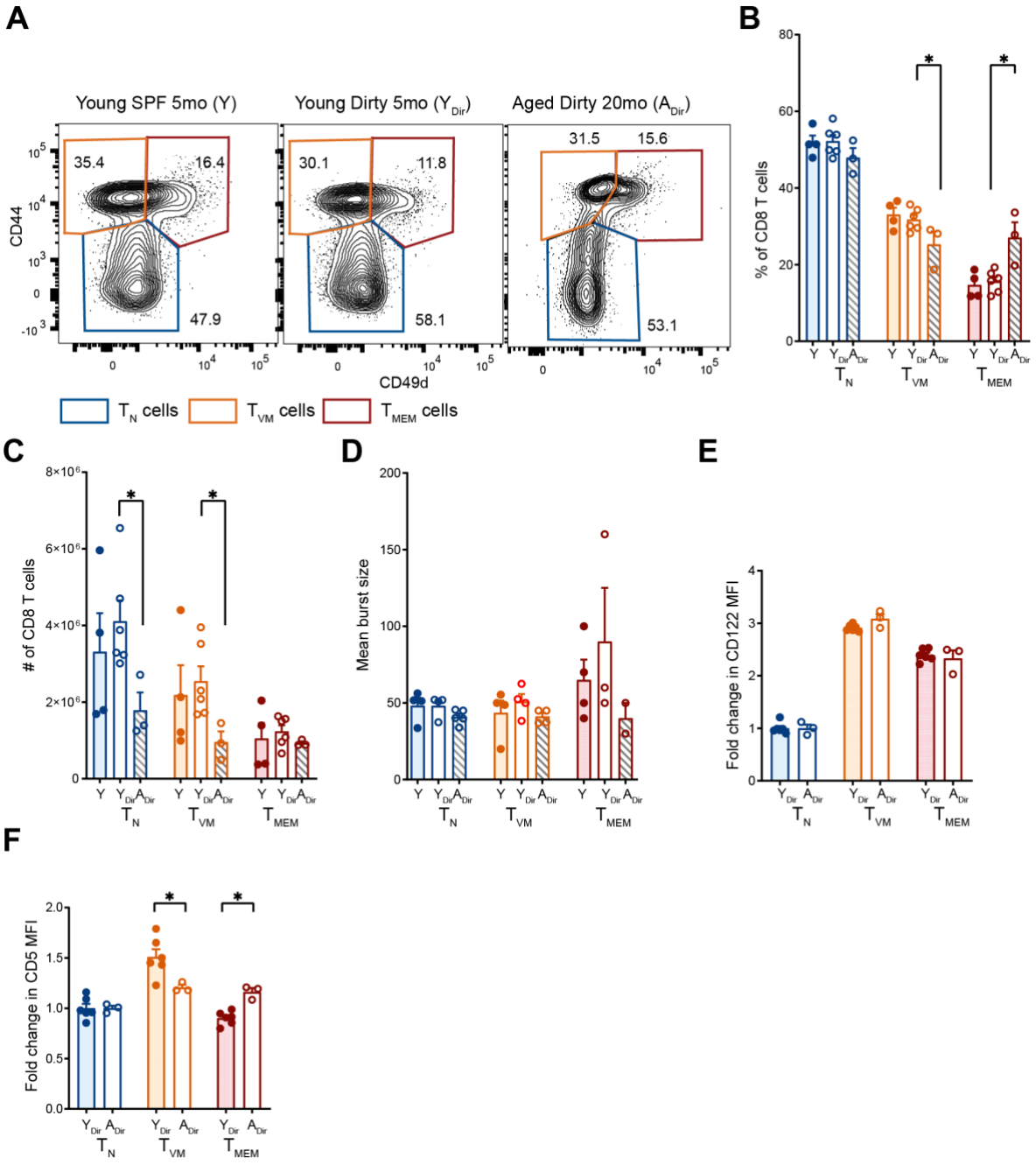


Figure 5.1. Impact of dirty housing conditions on CD8 T cells in young and aged mice. A) Representative contour plots gated on Live/TCR+/CD8+ cells and depicting T_N, T_{VM} and T_{MEM} populations and bar graph depicting B) frequency, C) number, D) mean burst size of single cell colonies after 4 days of culture with anti-CD3/8/11a stimulation E) fold change in median fluorescence intensity (MFI) of CD122 and D) fold change in MFI of CD5, normalized to T_N cells in each group, depicted for all subsets from young SPF (Y), young dirty (Y_{Dir}) and aged dirty (A_{Dir}) mice. Data is from 1 experiment with n=3-5 mice per group. *p<0.05 using Mann Whitney test. Bar graphs represent mean ± SEM.

Dirty mouse models have been enlightening as a resource to study broad differences in immune cell populations, however the precise nature or severity of infections that lead to the observed immune perturbations in dirty against SPF mice are undefined. To provide a more defined analysis of the impact of infections, we proceeded to experimental models, including bacterial and viral infections, where these variables can be controlled. To assess the short- and long-term impact of these infections, mice were analysed at different timepoints.

We first examined the impact of IAV infection. Mice infected i.n with 10,000 pfu of IAV strain, HKx31, were humanely killed and CD8 T cell subset number and proportions were measured (described in section 2.2.1). T_{VM} cell numbers and proportions remained the same, both during acute infection (d10) (Figure 5.2 A, B) and after recovery (d60) in the spleens (Figure 5.2 C), compared to uninfected mice. $T_{EFF/MEM}$ cell numbers and proportions increased significantly at d10, as expected due to the antigen-specific CD8 T cell response. IAV infection therefore does not appear to have any impact on the T_{VM} cells population. We did not assess mediastinal lymph nodes and lungs in these experiments as we were interested in persistent differences that could be observed systemically and were not driven by inflammation at the site of infection. However, in other experiments, we have assessed mediastinal lymph nodes and did not observe an increased number of T_{VM} cells after two weeks of infection (data not shown).

We next assessed the effect of a bacterial infection, using an attenuated strain of *S. typhi*, strain BRD509. *S. typhi* is known to induce a Th1 response, with increased production of IL-12 to facilitate bacterial clearance[265]. We have previously shown that T_{VM} cells respond to IL-12 stimulation with robust IFN-γ production. Mice infected i.v. with 200 cfu of *S. typhi* (described in section 2.2.2) were humanely killed and CD8 T cell subset number and proportions were

measured. In addition to the expected increase in $T_{EFF/MEM}$ cells with infection, T_{VM} cell numbers were also significantly and substantially (>2-fold) increased compared to uninfected mice and d20 post-infection (Figure 5.2 C). T_{VM} cell numbers returned to baseline by d60 (Figure 5.2 D). These data suggest that the acute increase in inflammatory cytokines induced by *S. typhi* was able drive a transient, coincident increase in T_{VM} cells, however this was not maintained long term.

Finally, we examined the impact of LCMV infection on T_{VM} cells as it induces a strong CD8 T cell memory response and a robust yet acute type I IFN response[266], to which T_{VM} cells are particularly sensitive[202] . Mice were infected i.v. with 3000 pfu of an acute strain of LCMV (WE) (described in section 2.2.3) and were humanely killed and CD8 T cell subset number and proportions were measured. An acute LCMV strain was chosen to assess differences that could persist beyond the duration of active viral replication. Strikingly, we observed a dramatic (>60%) loss of both T_N cells and T_{VM} cells (Figure 5.2 E). This loss occurs a dramatic increase in the T_{EFF} cells at d10 (Figure 5.2 E), which is a consequence of the large LCMV-specific CD8 T cell response[269,270]. Notably, neither the T_{VM} cell or T_N cell populations were recovered at d60 after infection (Figure 5.2 F), despite the fact that LCMV-WE viral load is cleared by day 8-10 post-infection[269,271]. Given the sustained phenotype induced by LCMV infection, we investigated the impact of this infection further.

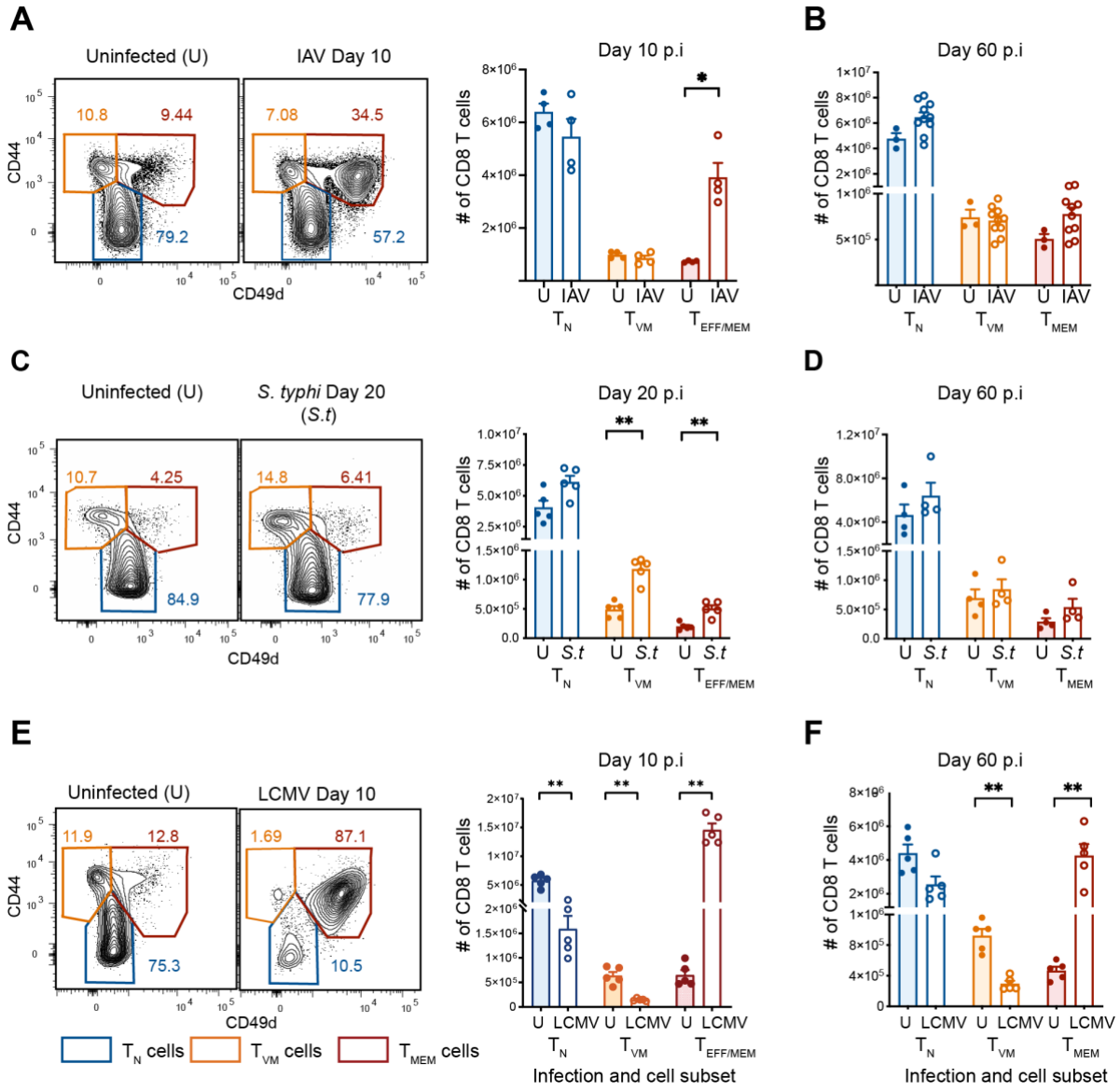


Figure 5.2. Impact of different infections on CD8 T cells at early and late timepoints. A) Representative contour plots gated on Live/TCR+/CD8+ T cells and depicting splenic T_N, T_{VM} and T_{MEM} cells and bar graph depicting number of each subset at day(d) 10 and at B) d60 of IAV infection (IAV) and uninfected (U) controls. C) Representative contour plots gated on Live/TCR+/CD8+ T cells and depicting splenic T_N, T_{VM} and T_{MEM} cells and bar graph depicting number of each subset at d20 and D) d60 of *S. typhi* (*S.t*) infection and uninfected controls E) Representative contour plots gated on Live/TCR+/CD8+ T cells and depicting splenic T_N, T_{VM} and T_{MEM} cells and bar graph depicting number of each subset at d10 and F) d60 of LCMV WE (LCMV) infection and uninfected controls. Data is representative of 2-3 experiments with n=4-9 mice per group. *p<0.05, **p<0.01 using Mann Whitney test. Bar graphs represent mean ± SEM.

5.2.2 LCMV infection rapidly depletes the antigen-naïve CD8 T cells compartment

To begin to dissect mechanisms driving the profound and prolonged T cell depletion observed with LCMV infection, we explored the kinetics of T cell loss after infection. We saw a marked, early loss of all T cell populations to less than 50% of their original number at d2 post infection (Figure 5.3 A, B, C). For T_N and T_{VM} cells, this loss continued with a nadir of T_N and T_{VM} cells at d10 (~30% of original number). T_N and T_{VM} cells recover modestly at later timepoints (>d21), however even after recovery they were still only half of their original populations (Figure 5.3 A, B). In contrast $T_{EFF/MEM}$ cells recover rapidly after their early loss, equalling pre-infection numbers by d5 and then exceeding these numbers by >10-fold by d10 (Figure 5.3 C) due to the massive antigen-specific T cell expansion observed in response to LCMV[269]. Collectively, following acute LCMV infection, we observed a profound early loss of all CD8 T cells, followed by a sustained reduction of T_N and T_{VM} cells but recovery and accumulation of T_{MEM} cells.

We next examined recent T cell proliferation using Ki67 staining. This analysis demonstrated that relatively few T_N, T_{VM} and T_{MEM} cells were proliferating at d2 post-infection. In contrast, around 20% of T_N cells, 70% of T_{VM} cells and 80% of T_{MEM} cells were proliferating at d5 (Figure 5.3 D, E). This proliferation was short-lived, dropping dramatically by d10, and, surprisingly, did not correspond to an increase in T_N and T_{VM} cell numbers. Previous studies have shown that LCMV infection drives a rapid type I IFN response that peaks at 24h with the level of IFN-α elevated out to at least 48h after infection[266]. The induction of type I IFN was associated with attrition in the CD8 T cell compartment within the first two days of infection[272], while blockade

of type I IFN rescued CD8 T cells from apoptosis and enhanced control of persistent LCMV infection[266,272]. In agreement with these studies, we observed elevated serum levels of IFN-α and IFN-β within the first day of infection which then peaked at 42 hours after infection (Figure 5.3 F, G).

Type I IFNs play a key role in regulating the T_{VM} cell subset and are required for age-related accumulation of T_{VM} cells[202] . Type I IFNs regulate Eomes expression which is a transcription factor required for lineage differentiation of T_{VM} cells[101,202] and can also maintain T_{VM} cells indirectly by upregulating production of IL-15 during infection and inflammation[113]. Type I IFNs and IL-15 have both been shown to mediate proliferation of bystander memory T cells in different studies[32,273–275], and this could be inducing the increase in Ki67 expression on d5.

Foxp3+CD25+ CD4 T_{REG} cells suppress IL-15 presentation from CD11b+/SIRPα+ DCs (cDC2s) in the steady state[165]. We observed a twofold reduction in T_{REG} cells with LCMV infection (Figure 5.3 H), which may result in an opportunity for T_{VM} cells to access IL-15 from cDC2s during LCMV infection. We therefore propose a model where high levels of type I IFNs immediately after LCMV infection lead to attrition of a range of immune cells, including T_N, T_{VM} and T_{MEM} CD8 T cells, and T_{REG} cells, leading to lymphopenic conditions[276,277]. The substantial increase in Ki67 expression at day 5 (Figure 5.3 D, E) may be a direct result of type I IFN and IL-15 production. However, this does not result in a considerable increase in T_{VM} cells (Figure 5.3 B) possibly due to the high IFN-α levels that were maintained even after 4 days of infection (Figure 5.3 F) that may still be leading to apoptosis of cells that are proliferating. In addition, bystander CD8 T cells proliferating due to type I IFN stimulus could be undergoing proliferation induced apoptosis in this highly inflammatory environment. As a result of these

possible factors, both T_{VM} and T_N cell numbers remain significantly decreased compared to uninfected controls.

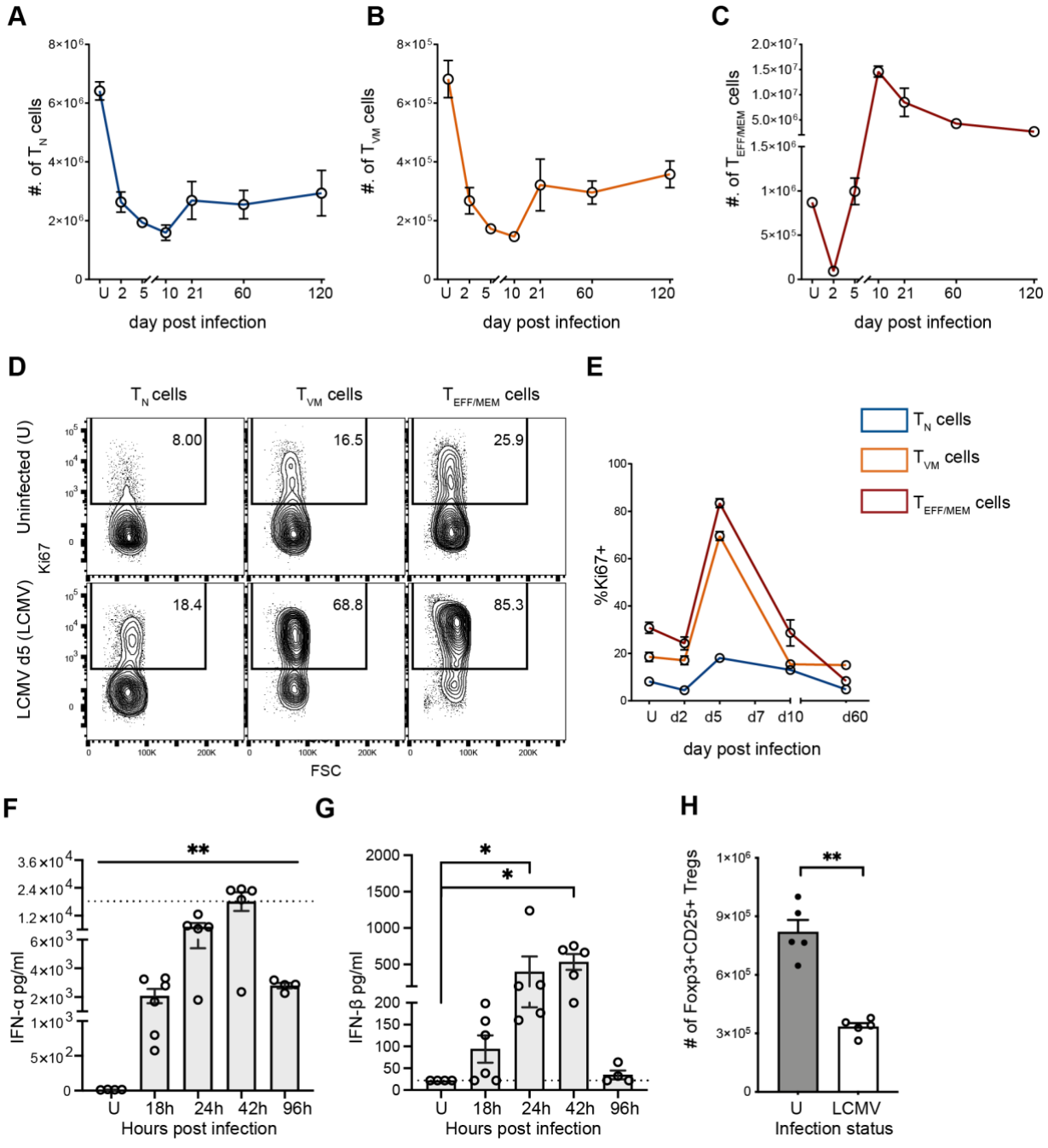


Figure 5.3. Kinetics of T cell loss and division during LCMV infection. Number of splenic A) T_N, B) T_{VM}, and C) $T_{EFF/MEM}$ cells from d2-d120 of LCMV infection and in uninfected (U). Representative contour plots gated on T_N and T_{VM} and $T_{EFF/MEM}$ cells showing frequency of Ki67 expression and E) line graphs depicting frequency of Ki67+ cells in uninfected and from d2-d60 of LCMV infection. F) Level of IFNα and G) IFNβ

in serum from 0-96 hours post infection. H) Number of T_{REG} cells at d5 of LCMV infection. Data is representative of 2-3 experiments with n=4-5 mice per group, except d21, d120 were analysed once. *p<0.05, **p<0.01 using Mann Whitney test. Bar graphs represent mean ± SEM.

5.2.3 T_{VM} cells adopt a distinct phenotype following LCMV infection, which is maintained long term

As described earlier, we observed significant depletion in both T_N and T_{VM} cell subsets both at early and extended timepoints post LCMV infection, indicating that these changes in antigen-naïve CD8 T cells were sustained (Figure 5.3). The $T_{EFF/MEM}$ population after LCMV infection is largely comprised of LCMV-specific CD8 T cells that have expanded in an antigen-specific manner, and the activation and function of this subset has previously been described in detail[269,270,278,279] and over an extended period of time[280,281], therefore our further analysis focuses only on cells impacted by LCMV via antigen-independent mechanisms, i.e T_N and T_{VM} cells. T_{VM} cells are unique in their capacity to mediate both antigen-dependent and -independent, innate-like effector functions. We therefore assessed the expression of markers that reflect the unique functionality of T_{VM} cells; CD122, Eomes and NKG2D. T_N cells are included as a control in our analyses, as these markers are not typically expressed by T_N cells. As described in previous chapters, T_{VM} cells have the highest expression of Eomes and CD122 among CD8 T cells and both these factors are required for the development of the T_{VM} cell subset[35,101].

We found a small but significant upregulation of CD122 on T_{VM} cells at d10 post-infection (Figure 5.4 A), followed by a modest (~20%), but significant downregulation of CD122 on T_{VM} cells by d60 post-infection compared to uninfected controls (Figure 5.4 B). Eomes expression was significantly reduced at d10 post-infection and this was maintained out to d60 (Figure 5.4 C, D). Previous studies[263] as well as our work has shown that CD122 can be increased in the presence of elevated levels of IL-15 (Chapter 3, Figures 3.7 A, 3.9 C). Thus, the initial increase

in CD122 expression could be the result of increased levels of IL-15 that may be induced by type I IFNs. However, the persistent suppression of Eomes from d10 to d60 (Figure 5.4 C, D), which directly binds to CD122 promoter and regulates its expression[158], may be responsible for driving down the expression of CD122 at later timepoints.

We next analysed expression of the activating NKR, NKG2D. As described in earlier chapters, we had observed that a small subset (5-10%) of T_{VM} cells from uninfected mice expressed the activating receptor (Chapter 3, Figure 3.3 A) and that T_{VM} cells acquire increased expression of NKG2D with age and with IL-15 exposure (Chapter 3, Figures 3.7 D, 3.9 D). NKG2D can also mediate innate-like functionality in human CD8 T cells while simultaneously dampening TCR signaling[155]. At d10 post-LCMV infection, the proportion of NKG2D+ T_{VM} cells increased dramatically (3-fold), and this increase was largely maintained at d60 post-infection (Figure 5.4 E, F).

These results strongly indicate that LCMV infection and the resulting cytokine environment induces a distinct phenotype, indicative of altered function in T_{VM} cells, that is evidenced by the skewed expression of classical T_{VM} cell markers such as CD122, Eomes and NKG2D.

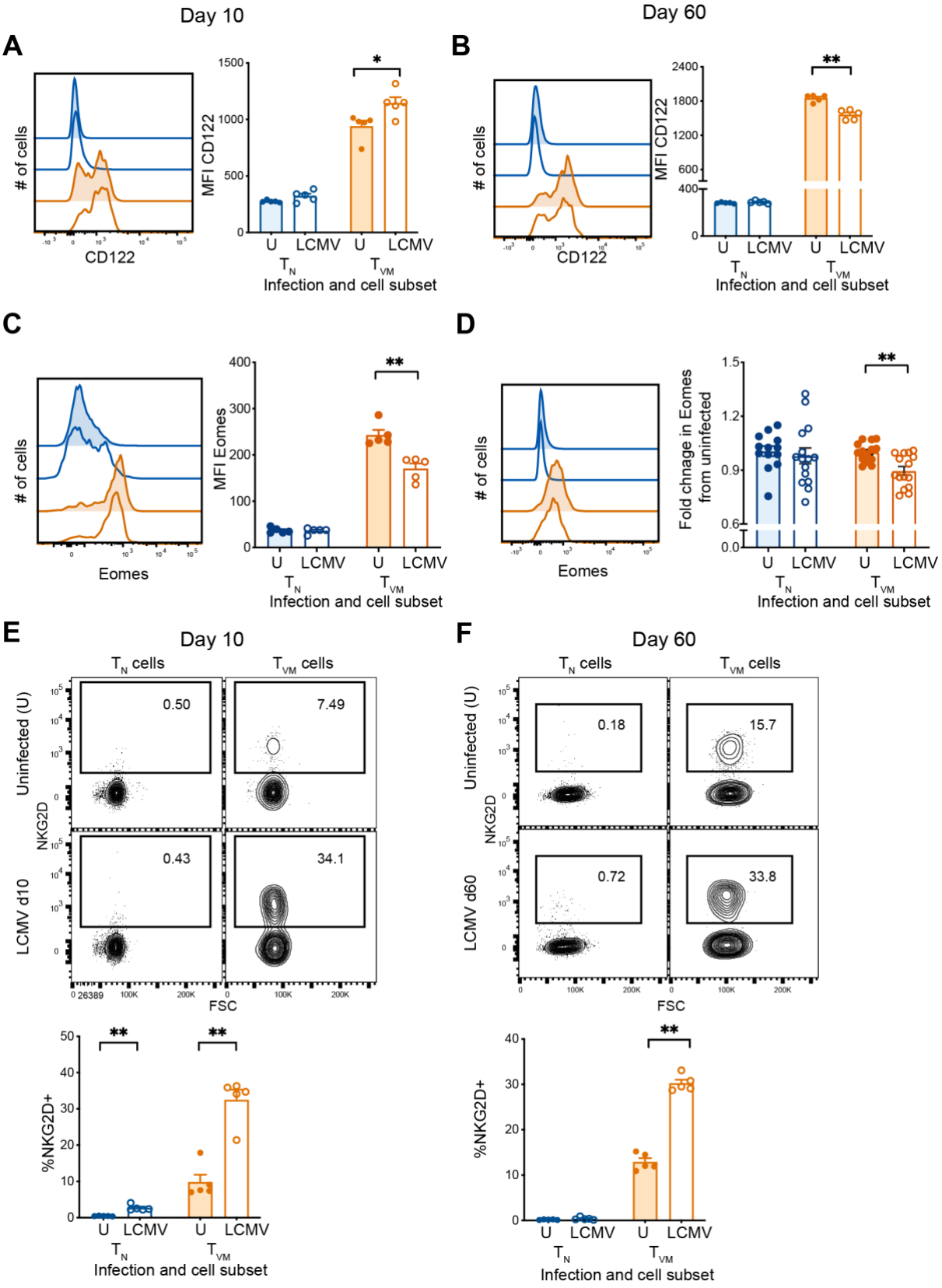

Day 10
Day 60
A
B
C
D
E
F
# of cells
CD122
MFI CD122
Eomes
MFI Eomes
Fold chnage in Eomes from uninfected
U
LCMV
T_N
T_{VM}
Infection and cell subset
T_N cells
T_{VM} cells
Uninfected (U)
LCMV d10
LCMV d60
NKG2D
FSC
0.50
7.49
0.43
34.1
0.18
15.7
0.72
33.8
%NKG2D+

Figure 5.4. T_{VM} cells present a skewed phenotype after LCMV infection. Representative histograms showing CD122 expression and bar graph showing median fluorescence intensity (MFI) of CD122 in T_N and T_{VM} cells at A) d10 and B) d60 after LCMV infection and in uninfected (U) mice. Representative histograms showing Eomes expression and bar graph showing MFI of Eomes in T_N and T_{VM} cells at C) d10 and D) d60 after LCMV infection and in uninfected (U) mice. Representative contour plots of T_N and T_{VM} cells gated on NKG2D+ subset and bar graphs depicting total frequency of NKG2D+ cells in each subset at E) d10 and F) d60 after LCMV infection and in uninfected (U) mice. Data is representative 2-3 experiments with n=4-5 mice per group. *p<0.05, **p<0.01 using Mann Whitney test. Bar graphs represent mean ± SEM.

5.2.4. LCMV infection of young mice results in improved T_{VM} cell responses to TCR-mediated stimuli

Given the qualitative changes in T_{VM} cell phenotype, we next assessed the impact of LCMV infection on the responsiveness and functionality of the remaining T_N and T_{VM} cells to both TCR-mediated and innate stimuli. These functional analyses were performed on CD8 T cell subsets from mice that had been infected with LCMV >2 months earlier and compared to uninfected mice. We first assessed the cytokine production profiles of T_N and T_{VM} cells after TCR-mediated stimulation. T cell polyfunctionality, or the ability to produce multiple cytokines and/or effector molecules, is associated with increased cytokine production on a per cell basis and augmented viral and bacterial clearance[282–284]. Analysis of CD8 T cell cytokine production profiles after polyclonal TCR stimulation revealed a significant increase in the proportion of IFN-γ+ cells that produce TNF, as well as a slightly increased expression of TNF per cell, in T_{VM} cells from infected mice compared to uninfected mice (Figure 5.5 A, B). This enhanced functionality was not observed in T_N cells from infected mice.

We next assessed the proliferative capacity of T_N and T_{VM} cells in response to polyclonal TCR stimulation (described in sections 2.7, 2.8.2). While the mean number of divisions was unchanged in T_N cells from infected compared to uninfected mice, T_{VM} cells from infected mice

showed a significantly increased proliferative capacity compared to their uninfected counterparts (Figure 5.5 C) after 65h of *in vitro* polyclonal TCR stimulation. We also observed enhanced phosphorylation of ERK in T_{VM} cells from infected mice (Figure 5.5 D) after TCR stimulation (described in 2.9.4), indicating that enhanced TCR signaling strength is contributing to the increased TCR-driven proliferation and cytokine production.

Given that we had observed downregulation of CD122 expression on T_{VM} cells at extended timepoints, i.e d60 after LCMV infection (Figure 5.3 A, B), we assessed the proliferative capacity of T_N and T_{VM} cells isolated at d60 in response to stimulation with IL-15. Sorted CD8 T cell subsets were cultured in the presence of IL-15/IL-15Rα complexes (cIL-15) (described in sections 2.3.1, 2.8.4) and proliferation was assessed at d5. In contrast to the significantly increased sensitivity to TCR mediated stimulation, T_{VM} cells from LCMV infected mice showed reduced proliferation after *in vitro* IL-15 stimulation (Figure 5.5 E), correlating with the lower CD122 expression on T_{VM} cells (Figure 5.5 F). Together, these results suggest that LCMV infection is having a selective impact on T_{VM} cells that results in a functionally superior population of T_{VM} cells after infection, and even though T_N cells remain depleted these changes are not observed in T_N cells. While we observed increased responsiveness to adaptive stimuli and a decrease in responsiveness to innate stimuli in T_{VM} cells after infection, it is not clear if these changes are mechanistically coupled.

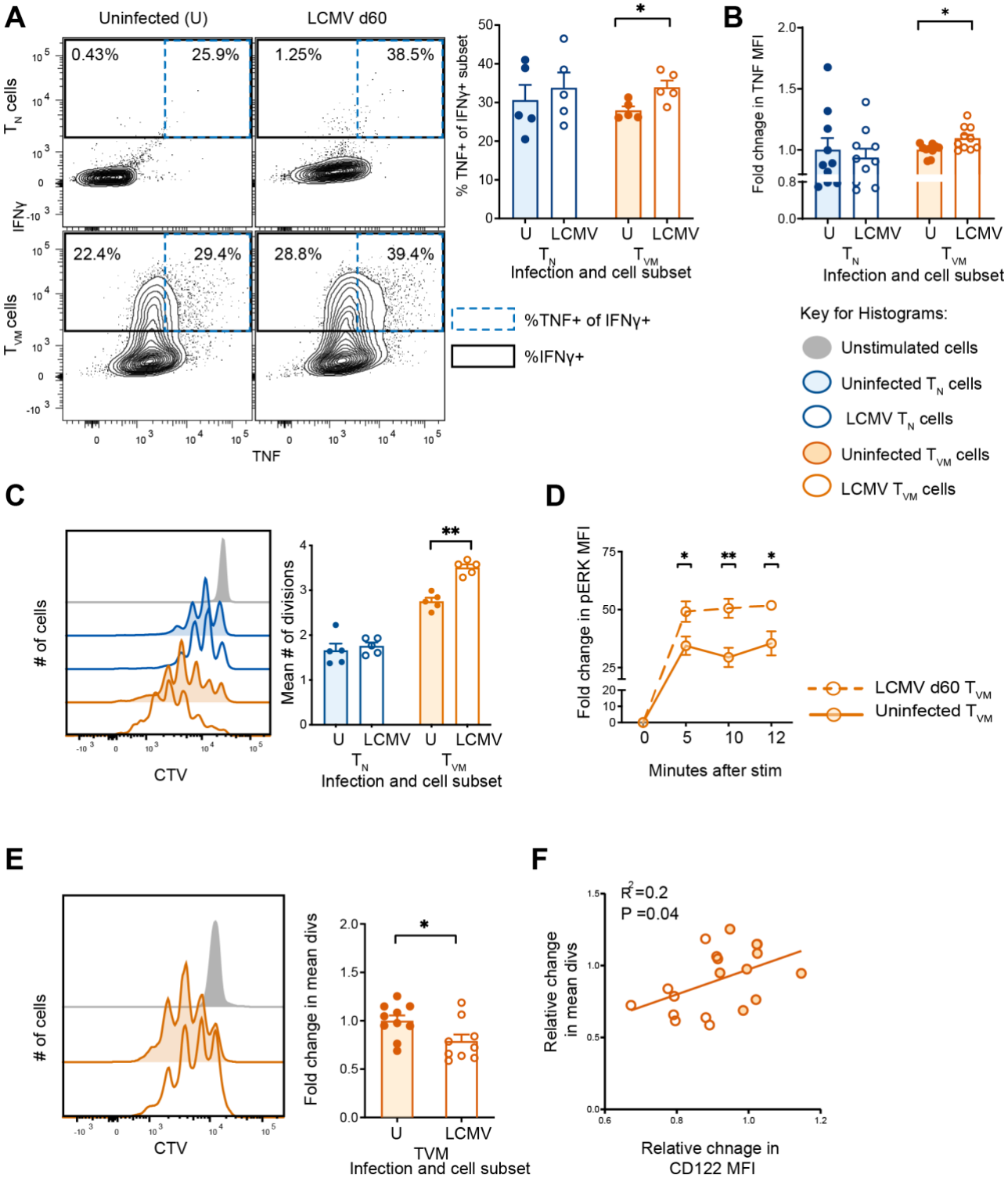


Figure 5.5. LCMV infection results in a functionally superior T_{VM} subset. A) Representative contour plots gated on T_N and T_{VM} cells and showing the proportion of cells producing IFNɣ, TNF or both cytokines after 36 hrs of polyclonal stimulation (anti-CD3/CD8/CD11a Ab). B) Fold change in TNF MFI relative to average TNF expression in each subset from uninfected (U) mice. C) Representative histograms

depicting CTV dilution of sorted T_N and T_{VM} cells after 65 hours of polyclonal stimulation (anti-CD3/8/11a) and bar graph showing the corresponding mean divisions. D) Change in p-ERK expression relative to unstimulated controls from 0 to 12 minutes of polyclonal (anti-CD3/CD8/CD28 Ab) stimulation. E) Representative histograms depicting CTV dilution of sorted T_{VM} cells after 5d stimulation with 100ng of cIL-15 and bar graph showing change in mean divisions relative to average divisions in uninfected T_{VM} cells, with data pooled from two different experiments. F) Simple linear regression analysis of relative change in CD122 expression against relative change in Eomes expression in T_{VM} cells from mice at d60 post-infection and in uninfected (U) mice. Data is representative of 2-3 experiments or combined from 2 different experiments in case of B-F with n=4-5 mice per group. * $p<0.05$ ** $p<0.01$ using Mann Whitney test. Bar graphs represent mean ± SEM.

5.2.5. T_{VM} cell number is not recovered with ageing in previously LCMV infected mice

We have previously shown that in the increased number of T_{VM} cells observed because of helminth infection were not maintained for extended periods and out to advanced age (Chapter 4). We now wanted to assess if impact of LCMV infection, which clearly alters T_{VM} cell number, phenotype and function over the short- and medium-term, can persist out to the old age. To determine whether these changes were stable over the lifespan, 8-12 week old mice were infected with LCMV, as previously described, and then aged to 18-19 mo (hereafter referred to as $LCMV_{AGED}$ mice). Remarkably, the loss of T_{VM} cells observed early after infection (Figure 5.3 B) was maintained out to 18-19 months of age, with the T_{VM} cells in $LCMV_{AGED}$ mice still reduced to half of the number of T_{VM} cells in uninfected aged mice (Figure 5.6 A, B). The increased number of T_{MEM} cells observed in young mice after LCMV infection (Figure 5.3 C) persisted in aged mice as well, albeit to a reduced level, and were increased by more than twofold compared to their number in uninfected aged mice (Figure 5.6 A, B). There was no significant difference in the number of T_N cells between aged uninfected and $LCMV_{AGED}$ mice (Figure 5.6 A, B).

These data indicate that, in contrast to helminth infection where dramatic yet transient changes to T_{VM} cell numbers are observed (Chapter 4), early life LCMV infection resulted in a permanent reduction in T_{VM} cell numbers (Figure 5.6 A, B). In terms of phenotype, T_{VM} cells from $LCMV_{AGED}$ mice also maintained downregulated Eomes and CD122 expression, by about 30% (Figure 5.6 C, D) but the difference in proportion of NKG2D+ cells observed at earlier timepoints was not maintained into old age, with both uninfected and $LCMV_{AGED}$ T_{VM} cells showing a similarly high prevalence of NKG2D+ cells (Figure 5.6 E). Our previous data on the impact of IL-15 treatment on NKG2D expression in aged mice indicates that there may be ceiling of NKG2D+ cells within the T_{VM} subset (Chapter 3, Figures 3.7 F, 3.9 D). Given that the proportion of NKG2D+ T_{VM} subset was already at 30% at d60 post infection (Figure 5.4 F), it is possible that the T_{VM} subset cannot sustain a further increase than beyond what is observed in uninfected aged mice.

Interestingly, T_N cells normally undergo marked attrition with age, down to nearly 20% of T_N cell in young mice and humans[185,194], however we did not observe further loss of T_N cells over the course of ageing (Figure 5.6 A, B) beyond that induced early after LCMV infection (Figure 5.3 A).

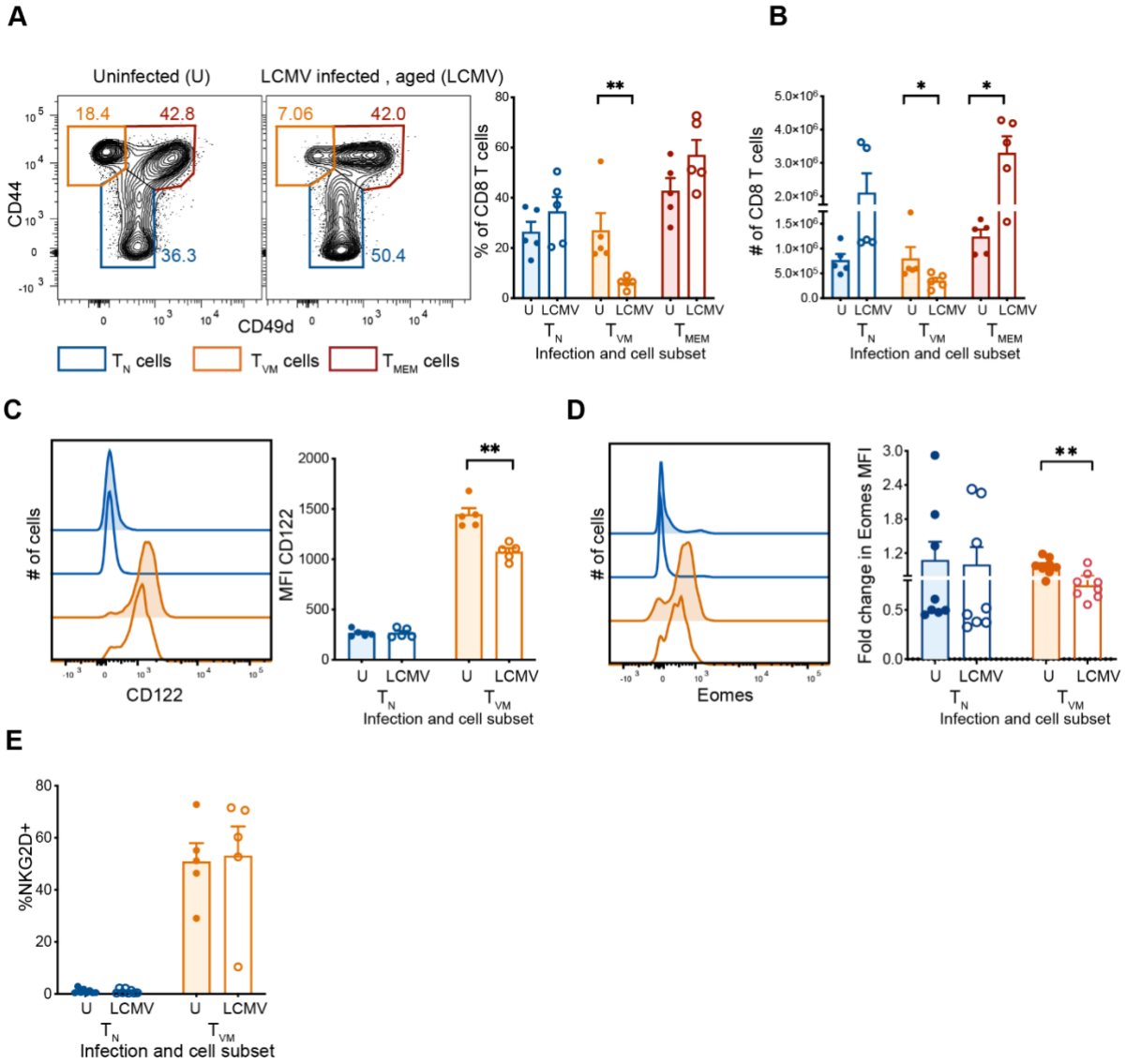


Figure 5.6. T_{VM} cells from $LCMV_{AGED}$ mice maintain the quantitative and qualitative changes observed early after infection. A) Representative contour plots gated on Live/TCR+/CD8+ T cells and depicting T_N, T_{VM} and T_{MEM} cells from LCMV infected and then aged (LCMV) and uninfected (U) mice and bar graph depicting frequency and B) number of cells in each subset. C) Representative histograms depicting expression of CD122 on T_N and T_{VM} cells and MFI of CD122 in each subset. D) Representative histograms depicting Eomes expression and fold change in Eomes MFI relative to average Eomes expression in each subset from uninfected (U) mice. E) Bar graph depicting frequency of NKG2D expression in T_N and T_{VM} cells from LCMV infected and then aged ($LCMV_{AGED}$) and uninfected (U) mice. Data is representative of 2 experiments with n=3-5 mice per group, *p<0.05, **p<0.01 using Mann Whitney test. Bar graphs represent mean ± SEM.

5.2.6. T_{VM} cells exhibit reduced senescence with age in LCMV infected mice

An established characteristic of T_{VM} cells is their development of a senescent phenotype with age, uncharacteristic of antigen-naïve T cells, with acquisition of deficits in TCR-mediated proliferation and higher levels of γH2Ax, Bcl-2 and basal phosphorylation of p38[95]. The presence of phosphorylated γH2Ax in the cytosol is an indicator of DNA damage and is commonly used as a marker of senescence[285]. Bcl-2 is upregulated in senescent cells to support survival[286,287] and basal phosphorylation of p38 is observed in senescent T cells,, where p-p38 can form complexes to inhibit TCR signaling, as observed in CD4 T cells[191,288] .

Given the marked alteration in T_{VM} cell number and phenotype with prior LCMV infection that is sustained through the life course, we explored whether function and senescence-related markers were also altered. When markers of senescence were assessed, significant reductions were observed in the level of γH2Ax, Bcl-2 and basal phosphorylation of p38 in $LCMV_{AGED}$ mice compared to uninfected, aged controls (Figure 5.7 A, B, C). This suggests that prior LCMV infection reduces the development of a senescence phenotype in T_{VM} cells with age.

We next determined whether the augmented TCR sensitivity alongside the reduced IL-15-mediated proliferation observed shortly after LCMV infection of young mice (Figure 5.5) were maintained in advanced age. We found that T_{VM} cells from $LCMV_{AGED}$ mice had a small but significant increase in TCR-driven proliferation compared to uninfected, aged mice (Figure 5.7 D), suggesting that this functional advantage acquired during early life exposure to LCMV (Figure 5.5) was maintained over the life course. T_{VM} cells from $LCMV_{AGED}$ mice also trended

towards higher TCR-driven proliferation in a single cell proliferation assay, in terms of burst size as well as average clone size compared to uninfected, aged controls (Figure 5.7 E, F). We did not observe any difference in cytokine production after polyclonal stimulation, and frequency of T_{VM} cells producing IFN-γ and TNF were similar in uninfected aged and $LCMV_{AGED}$ mice (Figure 5.7 G, H). This is in contrast to the observations made in young infected mice, where we observed increased frequency of TNF producers in T_{VM} cells from infected mice (Figure 5.5 A, B). We also did not observe any change in IL-15 mediated proliferation in $LCMV_{AGED}$ compared to uninfected aged mice (Figure 5.7 J), which indicates that the reduced cytokine responsiveness observed in young infected mice (Figure 5.5 E, F) may not be actively maintained through the course of ageing.

These data demonstrate that T_{VM} cells that survive LCMV infection exhibit enhanced TCR mediated proliferation and cytokine production, and this superior proliferative capacity is maintained out in old age along with a suppression of senescent phenotype in T_{VM} cells from $LCMV_{AGED}$ mice. Ultimately, it suggests that LCMV infection in young mice purges the T_{VM} cell population of cells that are likely to become dysfunctional with age.

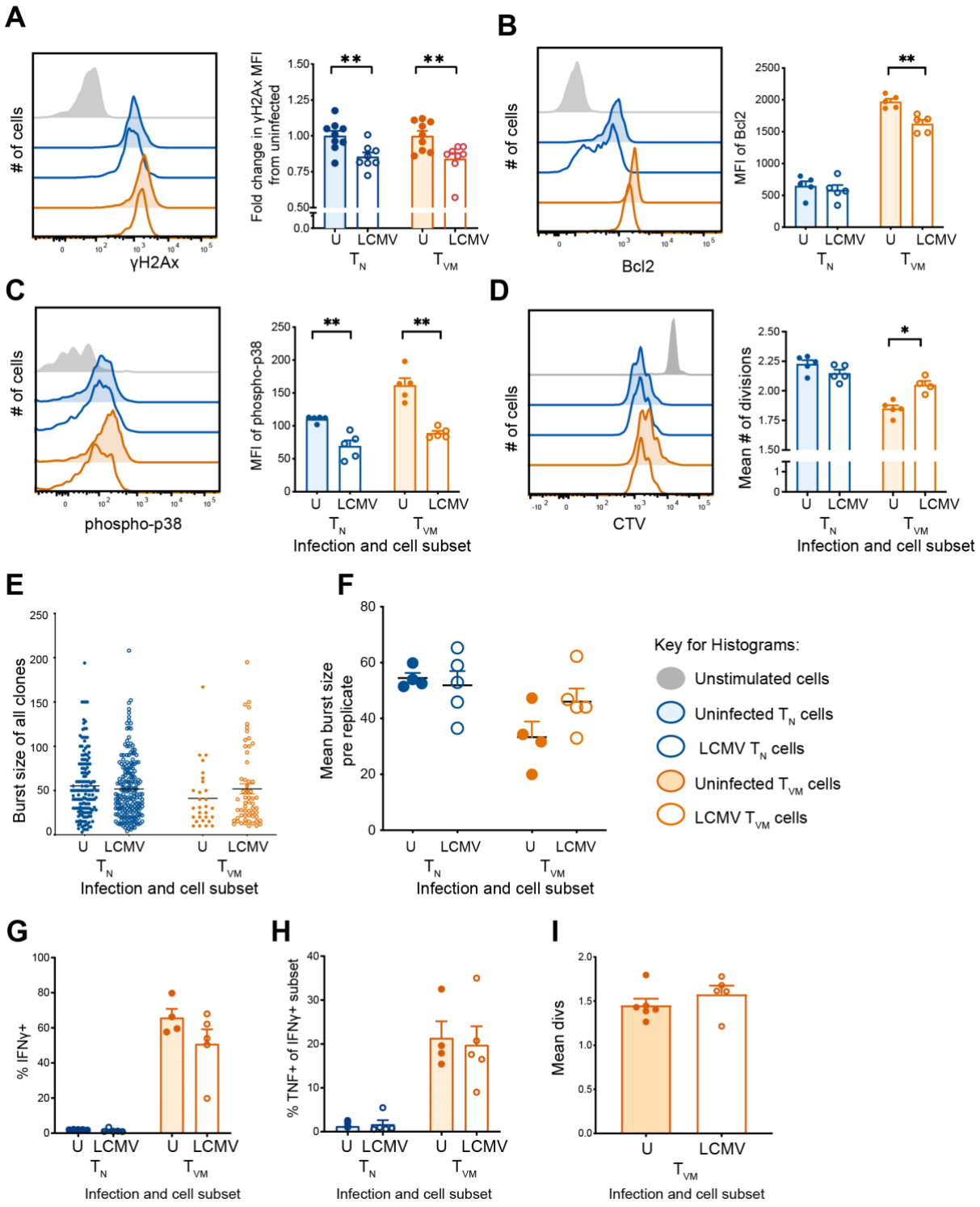


Figure 5.7. Assessment of senescence and function in LCMV$_{AGED}$ mice. A) Representative histograms showing γH2Ax expression in T_N and T_{VM} cells and bar graph showing fold change in MFI of γH2Ax compared to average of MFI in uninfected subsets. B) Representative histograms showing Bcl2 expression in T_N and T_{VM} cells and bar graph showing fold change in MFI of Bcl2 compared to average MFI in uninfected subsets. C) Representative histograms showing basal phosphorylated (p)-p38 levels

in T_N and T_{VM} cells and bar graph showing MFI of p-p38 in T_N and T_{VM} cells from aged, previously infected (LCMV) and uninfected (U) mice. D) Representative histograms depicting CTV dilution of sorted T_N and T_{VM} cells after 65 hours of polyclonal stimulation (anti-CD3/8/11a) and bar graph showing the corresponding mean divisions E) Burst size of colonies originating from single cells sorted from each mouse and stimulated for 4 days (anti-CD3/8/11a) in a single cell proliferation assay. F) Average burst size of colonies of T_N and T_{VM} cells from each mouse in aged, previously infected (LCMV) and uninfected (U) mice. G) IFN-γ expression H) TNF expression as proportion of total IFN-γ+ subset in T_N, T_{VM} cells after 36 hrs of polyclonal stimulation (anti-CD3/8/11a) and I) mean divisions of sorted T_{VM} cells after 5 days of in-vitro stimulation with 50 ng cIL-15 from previously LCMV infected and aged and uninfected mice. Data represents one experiment with n=4-5 mice per group. *p<0.05, **p<0.01 using Mann Whitney test. Bar graphs represent mean ± SEM.

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5.2.7. Additional infections do not alter T_{VM} cell loss induced by LCMV infection

We have shown that early life exposure to LCMV infection drove a reduction in T_{VM} cells that was sustained over a life course. To determine whether these LCMV-related changes in T_{VM} cell number could be mitigated or modulated by other infections which are likely to drive a strong inflammatory signal, we sequentially infected young adult mice with *S. typhi*, LCMV and IAV (3Seq model) and analysed T cells at 18-19 months of age (Figure 5.8 A). Intriguingly, we found that LCMV, even in the context of other proinflammatory infections, was able to cause a severe (~3-fold) and prolonged depletion of T_{VM} cells (Figure 5.8 B). T_{VM} cells from 3Seq mice also exhibited downregulation of Eomes and CD122, by 40%, compared to age-matched uninfected mice (Figure 5.8 C, D). A similar phenotypic profile was seen in mice at d10 and d60 following LCMV only infection (Figure 5.2 E, F, Figure 5.4) and in $LCMV_{AGED}$ mice (Figure 5.6), signifying that LCMV-driven phenotype cannot be masked by other infections.

Of note, the loss of T_{VM} cells and the associated phenotypic changes were not seen in mice infected with only *S. typhi* and IAV (2Seq model) and then aged out to 18-20 months (Figure

5.8 E, F, G), which would be expected as these infections could not independently alter T_{VM} cell phenotype in young mice (5.2 A, B, C, D). Collectively, these data demonstrate that the long term phenotypic and functional changes in T_{VM} cells were pathogen-specific, and that LCMV infection has a dominant impact on T_{VM} cells that cannot be ameliorated by additional infection-driven inflammation.

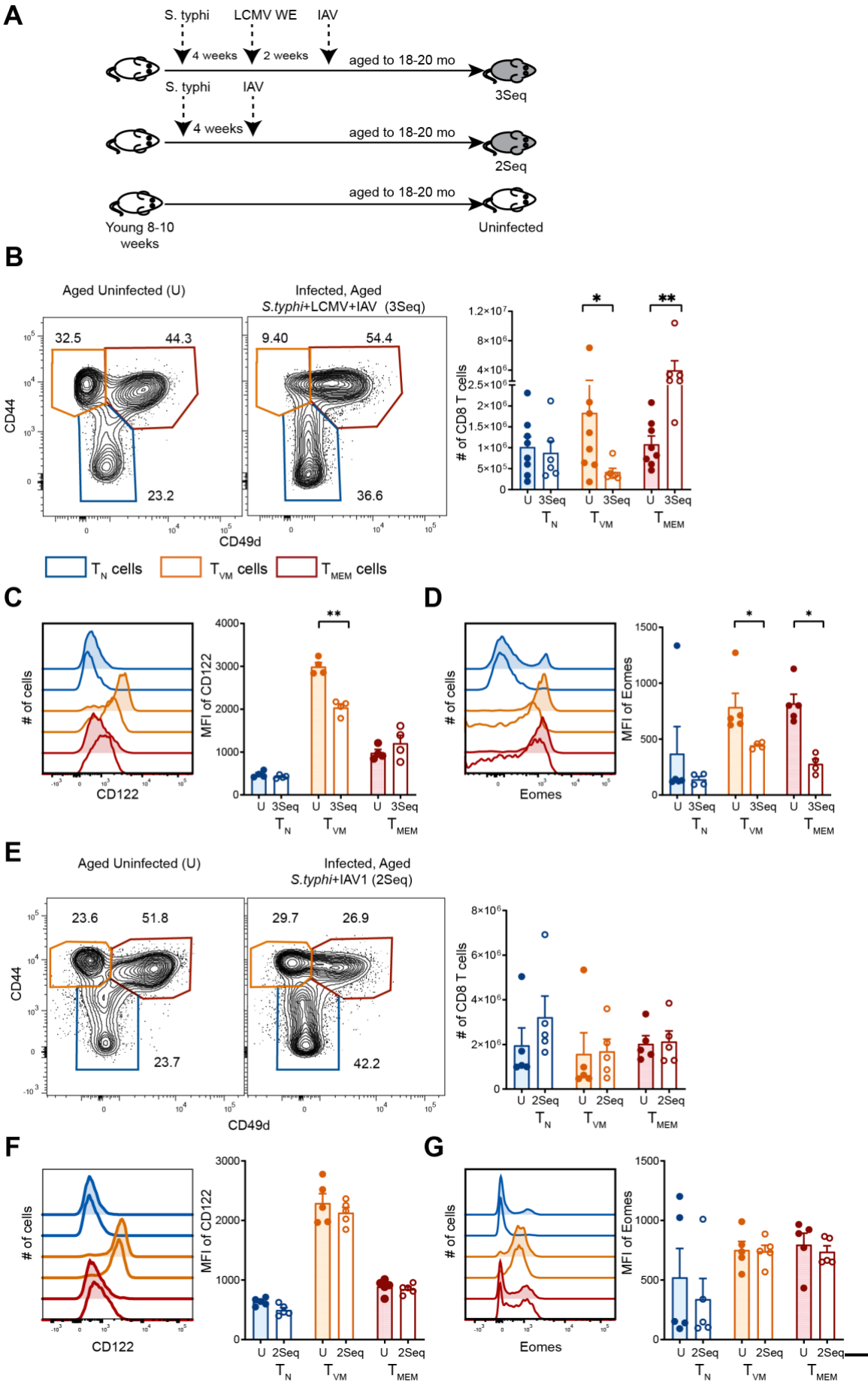
A
S. typhi
LCMV WE
IAV
4 weeks
2 weeks
aged to 18-20 mo
3Seq
S. typhi
IAV
4 weeks
aged to 18-20 mo
2Seq
aged to 18-20 mo
Young 8-10 weeks
Uninfected
B
Aged Uninfected (U)
Infected, Aged S.typhi+LCMV+IAV (3Seq)
32.5
44.3
23.2
9.40
54.4
36.6
CD44
CD49d
# of CD8 T cells
U 3Seq
T_N cells
T_VM cells
T_MEM cells
C
# of cells
CD122
MFI of CD122
D
Eomes
MFI of Eomes
E
Aged Uninfected (U)
Infected, Aged S.typhi+IAV1 (2Seq)
23.6
51.8
23.7
29.7
26.9
42.2
U 2Seq
F
G

Figure 5.8. LCMV-driven reduction in T_{VM} cells is unperturbed by other infections. A) Schematic depicting sequential infection of mice with 3 (*S. typhi*, LCMV, IAV) or 2 pathogens (*S. typhi* and IAV) and then aged. B) Representative contour plots gated on Live/TCR+/CD8+ T cells and depicting T_N, T_{VM} and T_{MEM} cells from *S. typhi*, LCMV and then IAV infected and then aged (3Seq) and uninfected (U) mice and bar graph depicting number of cells in each subset C) Representative histograms depicting expression of CD122 on T_N and T_{VM} cells and the MFI of CD122 in each subset. D) Representative histograms depicting Eomes expression and the respective MFI of Eomes across T_N and T_{VM} cells from 3Seq and uninfected mice. E) Representative contour plots gated on Live/TCR+/CD8+ T cells and depicting T_N, T_{VM} and T_{MEM} cells from *S. typhi*, and then IAV infected and then aged (2Seq) and uninfected (U) mice and bar graph depicting number of cells in each subset F) Representative histograms depicting expression of CD122 on T_N and T_{VM} cells and the median fluorescence intensity (MFI) of CD122 in each subset G) Representative histograms depicting Eomes expression and the respective MFI of Eomes across T_N and T_{VM} cells from 2Seq and uninfected mice. Data is representative of 2-3 experiments with n=3-5 mice per group, *, **, indicates $p<0.05$, $p<0.01$ respectively (Mann Whitney test), Bar graphs represent mean ± SEM.

5.3. Discussion

In this study, we have demonstrated that LCMV infection can significantly impact the T_{VM} cells, resulting in a permanent reduction of this subset where the surviving T_{VM} cell population has improved TCR mediated functionality that is retained out to old age and ultimately attenuates the dysfunction observed with ageing[95] . We also describe that lower cytokine sensitivity in T_{VM} cells may be correlated to improved TCR functionality in young infected mice. However, the dampened innate responses do not persist in T_{VM} cells from $LCMV_{AGED}$ mice, but T_{VM} cells maintain a largely skewed expression of classical markers associated with memory phenotype CD8 T cells.

Additionally, the age-associated accumulation of T_{VM} cells is not enhanced with a history of pathogen-exposure which was seen in both our dirty mouse analysis as well as sequential infection models. We found that even infections like *S. typhi,* which can induce IL-12 and IL-15 mediated protective pathways [232,264,265] *,* a cytokine signature that favours T_{VM} cells[35,94,97], do not lead to a major expansion in T_{VM} cell population. We also observed that varied infectious exposure does not impart a different phenotype or functionality in T_{VM} cells and that only select type of infection i.e., LCMV, can cause a major and permanent shift in T_{VM} cells. We propose that this shift is caused by selective death of a subset of T_{VM} cells due to excessive exposure to type I IFNs but further study is needed to define the precise mechanism of T_{VM} cell loss.

We found that T_{VM} cells, along with other CD8 T cells, underwent attrition during early LCMV infection alongside an exaggerated type I IFN response, and this cytokine-induced cytotoxicity

of T cells following LCMV has been described previously[272] . Other studies have also shown that type I IFNs can be detrimental during an immune response to *Listeria monocytogenes* and can lead to apoptosis of lymphocytes[289]. Indeed, blockade or absence of type I IFN signaling can lead to better outcomes during LCMV infection[266,272], as well as during *L. moncytogenes* and malarial infections[289,290]. One interpretation of our results could be that T_{VM} cells are functionally heterogenous and that LCMV infection selectively targets T_{VM} cells with greater cytokine sensitivity, leaving a less differentiated subset behind, thus purging T_{VM} population of cells that have greater potential to become senescent over time[95]. Indeed, heterogeneity in the T_{VM} cell population has been described before. Multiple studies have reported that subsets within T_{VM} cells have differential capacity to mediate effector-like responses, with heightened cytokine sensitivity[166,291], as opposed to mediating memory-like responses where genes associated with differentiation appear suppressed[291].

T_{VM} cells have rapid TCR mediated proliferation and cytokine-driven responses in young mice, with ageing there is loss in TCR functionality and increase in senescence, however cytokine responsiveness is largely retained. With LCMV infection we observed that the remaining T_{VM} cell subset had improved TCR mediated responses but at the same time they became less sensitive to IL-15 stimulus in young mice. Similar observations, where TCR signaling and bystander functions appear to have an inverse relationship, were made in terminally differentiated human CD8 T cells where it was observed that activation of bystander cytotoxicity via NKG2D in human and mice CD8 T cells is coupled with a loss of TCR signaling. This was mediated by inhibitory complexes formed by sestrins, that localized with NKG2D in the cytosol, and disrupted activation of TCR signaling complex[155]. We also observed that T_{VM}

cell population did not recover back to its original number, which is expected considering its high reactivity to self-peptide/MHC [157,205], increased homeostatic turnover[101] (Chapter 3, Section 3.1) and high sensitivity to homeostatic cytokines, like IL-15 and IL-7, described in this study (Chapter 3, Section 3.1) and earlier [34,35] . This indicates that the T_{VM} cell subset remaining after depletion, with downregulated Eomes and CD122 expression, may not be as receptive to homeostatic proliferation, and therefore less sensitive to cytokine stimulus. While the reduction in IL-15 response was not evident in $LCMV_{AGED}$ mice, it is possible that the overall modulation of cytokine sensitivity that occurs in T_{VM} cells in young LCMV infection, positions the surviving T_{VM} cell subset at an advantage by making them oblivious to the increasingly inflammatory environment induced through the course of ageing. The T_{VM} cells that survive LCMV infection with intrinsically suppressed sensitivity to the 'inflammaged' environment could therefore give rise to aged T_{VM} cells with less senescence and improved TCR functions, that we observed in $LCMV_{AGED}$ mice. This of course has implications for understanding how age-associated senescence of CD8 T cells is regulated and how aged CD8 T cell responses can be improved to infections and other diseases. We have previously shown that the impact of ageing is heterogeneous, with T_{VM} cells showing a greater deficit than T_N cells[95]. This data indicates that the age-associated defect may also be variable within T_{VM} cells, with a subpopulation that is able to endure age-associated stress without succumbing to senescence.

While earlier studies have reported that LCMV-mediated attrition of T_N cells is not as severe as for $CD44^{hi}$ T_{MP} cells [272], we observed a similar reduction in both T_N cells and T_{VM} cells as early as d2 after infection. The greater than two-fold reduction in T_N and T_{VM} cells after LCMV infection that is maintained for long periods reduces the antigen-naïve CD8 T cells down to

level of attrition that is observed in old age. The loss in antigen-naïve CD8 T cells, which includes both T_N and T_{VM} cells has important implications on future immune responses, where the drastically reduced diversity and abundance of epitope-specific precursors can limit the ability to respond to future infections. However, we did not observe a further reduction in T_N cells with ageing in $LCMV_{AGED}$ mice, and indeed T_N cells trended towards being higher in $LCMV_{AGED}$ mice compared to uninfected mice. A tempting inference here would be that the remaining T_N cell subset that survives after LCMV infection is "fitter" and is better able to survive the age-induced immune environment. The rapid reduction in naïve CD8 T cells is also observed in HIV infection[292–294], which is known to induce an immune-ageing phenotype with an increase in terminally differentiated CD8 T cells [292,295,296]. It would therefore be pertinent to explore whether other viral infections in humans that induce attrition in the T cell compartment can also impart similar changes to T_{VM} cells as observed during LCMV infection in mice.

In this study we were unable to assess if the improvement in TCR mediated functions that we see in young LCMV infected mice also leads to a similar enhanced functionality *in vivo*. A transgenic mouse model with semi-fixed TCR could be used to determine whether LCMV infection results in the formation of a T cell population that has superior capacity to respond to a subsequent unrelated pathogen, thus demonstrating a tangible consequence of early life LCMV infection.

We and others have shown that helminth infection also has a distinct impact on T_{VM} cells and leads to a twofold increase in the T_{VM} cell subset, however there was no evidence of enhanced TCR functionality in other studies [84] and in our findings (Chapter 4). The phenotype of T_{VM} cells driven by helminth infection contrasted with LCMV infection, while helminth infection

induced an increase in Eomes and CD122, these markers were downregulated during LCMV infection. It may be predicted that increased expression of CD122 and Eomes may not have a beneficial impact on TCR driven responses, whereas their downregulation with LCMV infection favours TCR mediated functions. To assess if these outcomes can be generalized to other infections that induce excessive type I IFNs, the pathways that induce the emergence of such a distinct cellular and functional phenotype of T_{VM} cells during LCMV infection need to be investigated further and may improve our understanding of heterogeneity within the T_{VM} cell subset.

In this study, we focused on observing systemic changes in T_{VM} cells that can have an impact on subsequent infections that occur at different sites or can alter age-related accumulation of T_{VM} cells. T_{VM} cells may be undergoing rapid changes at sites of infection which was not captured in our infection studies. Recent evidence suggests that T_{VM} cells are recruited to lungs after IAV infection and can mediate antigen specific and resident memory responses [166]. Rolot et al observed no increase in T_{VM} cells in the lungs in the context of *Schistosoma mansoni* infection, where the larvae are known to migrate to lungs before maturing in the liver [84]. Beyond a few studies, the tissue-specific role of T_{VM} cells during homeostatic and inflammatory conditions is vastly under-explored. T_{VM} cells are reported to preferentially localize to liver [97], therefore assessing the impact of pathogens like Salmonella [297] and hepatitis viruses [298,299] which are known preferentially induce liver inflammation, may be of interest.

Collectively, our study finds that that the age-associated defect in T_{VM} cells is not absolute and can be reversed. These findings may be exploited to retain functionality in old age and improve

antigen-specific responses in advanced age when there is increased incidence of infections and cancer.

Chapter VI

6. Impact of HIV infection on human T_{VM} cells

6.1. Introduction

Studies of T_{VM} cell phenotype and function have predominantly been performed using mouse models, with only a handful of studies examining the putative T_{VM} population in humans. Human T_{VM} cells share several features with mouse CD8 T_{VM} cells, including bystander cytotoxic potential, expression of NK receptors and high expression of Eomes and CD122[34,95–97]. Both mouse and human T_{VM} cells are also seen to have high expression of IFN-γ after IL-12 and IL-18 stimulation[96,143,169] along with increased basal expression of cytotoxic molecules like GrB and perforin[169]. Consistent with observations made in mouse studies, human T_{VM} cells also accumulate with age and lose TCR-mediated proliferative capacity[95].

HIV infection induces a chronic inflammatory state that can persist even after virological control is achieved[300,301]. This persistent state of inflammation is caused by numerous factors including increased microbial translocation[302] because of physical alterations to the gut barrier, like leaky junctions and formation of mucosal lesions[303,304] as well as an imbalance in immune cell subsets which leads to various comorbidities[305]. Due to these systemic effects of HIV infection, it exacerbates some aspects of inflammaging and accelerates the acquisition of age-related T cell phenotypes[292,293]. The chronic inflammation and immune dysfunction caused by HIV infection, results in similar alteration to immune cells[294,296] as observed during the normal

course of ageing with a reduction in naïve T cells and an accumulation of terminally differentiated CD8 T cells[184,306].

Given the sensitivity of T_{VM} cells to inflammation and ageing, and the fact that HIV infection induces premature immune ageing via induction of a profound inflammatory environment that persists even after virological control of disease, we are seeking to understand the impact of HIV infection on the prevalence and/or functionality of CD8 T cell subsets, and the T_{VM} subset in particular. T_{VM} cell function has recently been explored in the context of chronic conditions, such as cancer and chronic viral infections. Increased frequency of innate-like CD8 T cells has been associated with better outcomes in CML patients of that have discontinued tyrosine-kinase inhibitor treatment[172]. Consistent with these observations, an increase in tumor infiltrating lymphocytes (TIL) with a T_{VM} phenotype was observed in tumor models undergoing chemotherapeutic treatment[170]. T_{VM} cells have been reported to mediate cytotoxicity against tumor cells in an MHCI independent manner[170]. At the same time, there have been reports of T_{VM} cell accumulation during HIV in humans[169] and CMV infection in macaques[143] or increases in T_{VM} cell-like bystander memory CD8 T cell populations with several other viral infections[307]. Infection driven accumulation of T_{VM} cells during CMV and HIV infections has been attributed to occur in a IL-15-dependent manner[143,169]. Particularly in HIV infection, T_{VM} cells were increased even after virological control was achieved. T_{VM} cells have been shown to control HIV reactivation in a manner independent of HLA-I recognition, like the MHCI independent killing by T_{VM} cells in cancer models. During HIV infection, HLA-I blockade resulted in more efficient killing[169]. These results indicate that T_{VM} cells are efficient at adopting a TCR-independent, NK-like killing function in the context of human cancers and chronic infections

and therefore may play a vital role in protecting against disease states that induce chronic inflammation.

Given this emerging role of T_{VM} cells in human diseases, we undertook a preliminary analysis to understand how T_{VM} cell frequency and function changes with HIV infection and virological control. With a longitudinal analysis, we assessed whether differentiation state or senescent phenotype of CD8 T cells changes in people with HIV (PWH), with assessment at initiation of anti-retroviral therapy (ART) and then after 12 months of treatment. We also sought to determine whether HIV infection or virological control alters TCR-mediated or cytokine-driven T_{VM} cell function, alongside assessment of other CD8 T cell subsets. Understanding how CD8 T cells may be impacted by immune dysfunction in HIV could lead to strategies that can improve responses to curative interventions and reduce the likelihood of viral rebound in the absence of ART.

6.2. Results

6.2.1. T_{EMRA} cells increase with HIV infection, whereas T_N and T_{CM} subset is reduced

This study was conducted on samples collected from participants in the Melbourne HIV Cohort in collaboration with the Burnet Institute. The details of the samples included in this analysis are given in Table 6.1.

Table 6.1: Age, infection status, viral load and CD4 count at baseline (BL) and after 12 months of ART treatment (ART) of study participants

Replicate #	Age	Status	Viral load BL (copies/ml)	Viral load ART (copies/ml)	CD4 count BL (cells/µl)	CD4 count ART (cells/µl)
1	49	Uninfected	-	-	-	-
2	31	Uninfected	-	-	-	-
3	32	Uninfected	-	-	-	-
4	23	Uninfected	-	-	-	-
5	44	Uninfected	-	-	-	-
1	43	HIV+	91,091	20	718	1211
2	42	HIV+	15133	20	691	1184
3	47	HIV+	824081	20	536	380
4	44	HIV+	167777	20	380	515
5	40	HIV+	34900	20	641	616
6	34	HIV+	36,245	20	325	470
7	23	HIV+	232,000	21	835	675

We analysed PBMC samples taken longitudinally from both uninfected individuals and PWH, with the first sample acquired just before the initiation of ART (referred to as baseline (BL) sample) and the second taken 1 year post-ART treatment initiation (referred to as the ART sample), taken from the same individuals. The detail of the ART regimen is given in Table 6.2. All participants were male with the mean age of 39±8.1 and 35.8±4.7 years in PWH at baseline and the uninfected (U) cohort, respectively. There was no statistically significant difference observed in the ages between the two group (p=0.5 unpaired t-test, Welch's correction). All PWH were virologically suppressed at the 12 month-post ART timepoint and had viral load of 20 copies/ml of blood, which is the lower limit of detection (Table 6.1).

Table 6.2. Detail of ART regimes.

Replicate #	ART drugs			
1	Raltegravir	Tenofovir	Emtricitabine	
2	Efavirenz	Tenofovir	Emtricitabine	
3	Efavirenz	Tenofovir	Emtricitabine	
4	Atazanavir	Ritonavir	Tenofovir	Emtricitabine
5	Efavirenz	Tenofovir	Emtricitabine	
6	Efavirenz	Tenofovir	Emtricitabine	
7	Raltegravir	Tenofovir	Emtricitabine	

We undertook a broad analysis of phenotype, cytotoxic potential, senescence, and functionality of CD8 T cells in the PBMC samples. The gating strategy used to define each T cell subset is outlined in Figure 6.1A. We assessed differences in CD8 T cell subset from the uninfected to the infected state, and predictably observed a substantially lower proportion of

T_N cells ($CD45RA^{hi}CD27^{hi}PanKIR\text{-}NKG2A^-$) in pre-ART samples compared to uninfected controls which is a characteristic feature of HIV infection. With viral suppression there was marked increase in proportion of T_N cells in 6 out of 7 participants (Figure 6.1 B). Since we did not have the opportunity to access CD8 T cell numbers for this data, it is possible that this increase in T_N cell frequency is only due to a simultaneous reduction in frequency of T_{CM} cells which is discussed further.

The frequency of T_{VM} cells ($CD45RA^+PanKIR^+$ and/or $NKG2A^+$) did not differ markedly between PWH and uninfected individuals. We also did not observe an increase in T_{VM} cell frequency from pre-ART to after treatment (Figure 6.1 C), of note is that T_{VM} cell frequency was increased with treatment in only 2 out of 7 patients in the treated cohort. Our results contrast with a previous study which has shown an increase in T_{VM} cell proportion in treated individuals compared to uninfected and treatment-naïve groups. This could reflect our smaller sample size (n=5-7) in comparison to previous studies (n= 28-64)[169].

All antigen-experienced memory subsets, T_{CM} ($CD45RA^{lo}CD27^{hi}$), T_{EM} ($CD45RA^{lo}CD27^{lo}$) and T_{EMRA} ($CD45RA^{hi}CD27^{lo}PanKIR\text{-}NKG2A^-$) cells, trended higher in PWH compared to the uninfected controls (Figure 6.1 D, E), however this was significant only in case of T_{EMRA} cells (Figure 6.1 E).

With the commencement of ART, the T_{CM} cell subset was significantly reduced, by nearly 50%, (Figure 6.1 D), which is likely due to T_{CM} cells homing to lymph nodes once the viral load is reduced. There was no difference in T_{EM} cell frequency with treatment (Figure 6.1 E). Finally, ART treatment further increased T_{EMRA} cells by nearly 40% (Figure 6.2 F).

There have been numerous reports of rapid T_N cell attrition in HIV infection, alongside an increase in terminally differentiated memory subsets[195,294,295], where increased replication of memory CD8 T cells leads to acquisition of the terminally differentiated phenotype[306,308] . Collectively, these data demonstrate a familiar pattern, where HIV infection leads to a loss of undifferentiated naïve T cells and relative retention or accumulation of more differentiated memory phenotypes.

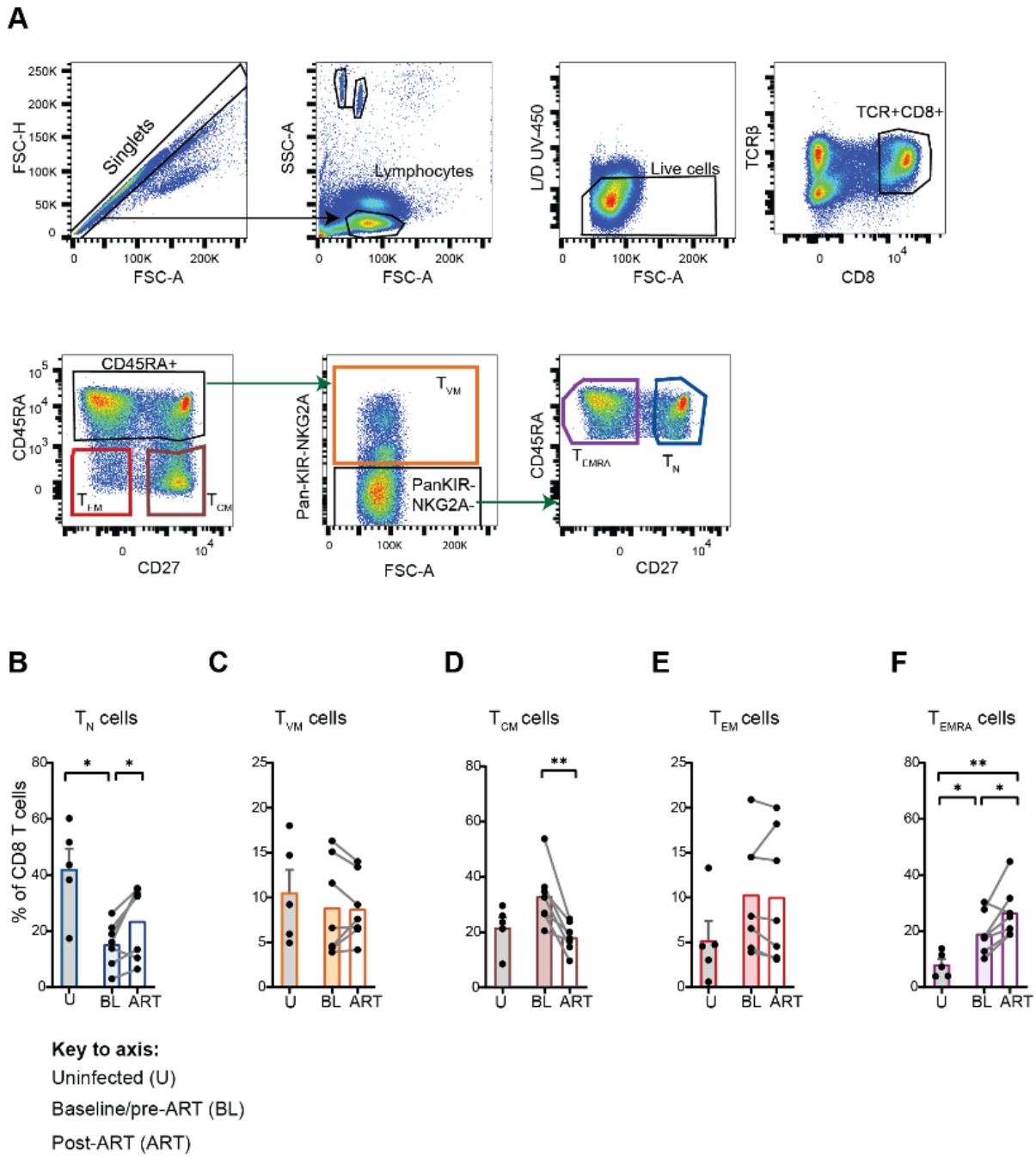

Figure 6.1. Frequency of CD8 T cell subsets in uninfected, HIV infected and ART treated samples. A) Gating strategy to define live CD8+TCRβ+ T_N, T_{VM}, T_{CM}, T_{EM} and T_{EMRA} subsets from human PBMC samples. Frequency of live CD8+TCRβ+ T cells that are B) T_N, C) T_{VM}, D) T_{CM}, E) T_{EM}, or F) T_{EMRA} cells in uninfected (U) donors or people with HIV pre-ART (BL) and post-ART (ART). Data is pooled from 2 experiments. * $p<0.05$, ** $p<0.01$ (Wilcoxon matched test for paired data and Mann-Whitney test for unpaired data). Bar graphs represent mean ± SEM.

6.2.2. T_{VM} cells become increasingly differentiated during HIV infection

To determine how the phenotype of CD8 T cell subsets changes with HIV infection and virological control, we tracked expression of markers associated with senescence and T cell dysfunction.

CD27 and CD28 are costimulatory molecules expressed on T cells. They are highly expressed on, and are indeed markers of, T_N cells[309] but the absence of both CD27 and CD28 is seen commonly in T cells from older individuals and has been correlated with a loss of TCR-associated functions[187,310,311]. CD27 and CD28 expression is known to be downregulated with progressive cell divisions and the double negative state could imply replicative senescence[312] but this population can also be enriched for cells with innate-like immune function[155]. Previous studies have attributed the CD27-CD28- state to cells derived from the effector memory pool. Indeed, we observed that ~90% and ~70% of T_{EM} and T_{EMRA} cells respectively were double negative in uninfected donors (Figure 6.2 B). However, we also observed that on average 60% of T_{VM} cells lacked expression of CD27 and CD28 which has not been described before (Figure 6.2A-B). With infection, frequencies of CD27-CD28- cells were not significantly different for all subsets but they did show an upward trend for T_{VM} and T_{EM} cells. With ART treatment, the frequency of T_{VM} cells that were CD27-CD28- showed a moderate but significant increase (Figure 6.2 A, B). This suggests that T_{VM} cells are more likely to acquire double negative state during infections which may indicate their potential to become senescent, and that this effect is not attenuated with suppression of viral load for T_{VM} and T_{EM} cells.

An increased proportion of T_{EM} and T_{EMRA} cells express CD57 compared to T_N and T_{CM} cells, and therefore it is considered as a marker for more differentiated cells[308]. Its expression is typically

associated with cells that have undergone excessive cell division due to chronic antigen stimulation and is therefore regarded as an indicator of replicative history[296,308] and indicates terminal differentiation and replicative senescence in T cells[128,313]. Fitting this characterisation, T_{VM} cells, along with T_{EM} and T_{EMRA} cells, had a substantial proportion (~40-65%) of CD57+ cells, while less differentiated T_N and T_{CM} cells showed minimal prevalence of CD57+ cells (<10%) (Figure 6.2 C, D). Similar frequency of CD57 expression has been previously described on human T_{VM} cells[96].

With infection, frequencies of CD57+ cells were unchanged for all subsets but, with ART treatment, the frequency of T_{VM} cells that were CD57+ increased significantly (Figure 6.2 C, D). Together, these results demonstrate that certain markers of differentiation and senescence continue to increase on T_{VM} cells in PWH even after virological control, which may indicate that persistent, low-grade, HIV-related inflammation can drive premature acquisition of age-related phenotypes in T_{VM} cells.

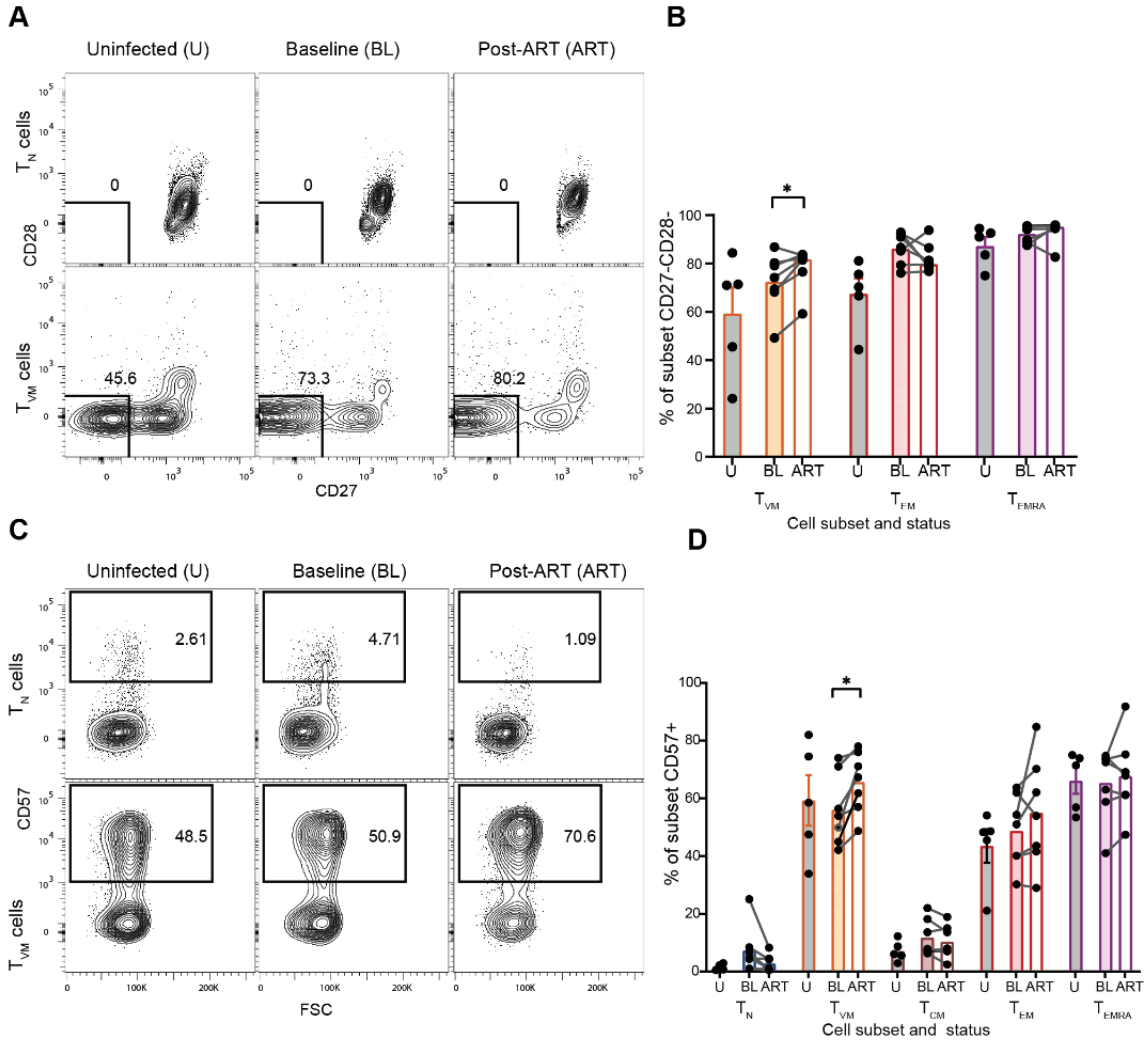

Figure 6.2. Senescent phenotype of CD8 T cells during HIV infection and in uninfected groups A) Representative contour plots showing the proportion of live CD8+TCRβ+ T_N and T_{VM} cells that are CD27-CD28-. B) Bar graphs of the frequency of T_{VM}, T_{EM}, T_{EMRA} cells that are CD27-CD28- in uninfected, pre-ART (BL) and post-ART (ART) samples. C) Representative contour plots showing the proportion of live CD8+TCRβ+ T_N and T_{VM} cells that are CD57+. D) Bar graphs of the frequency of T_N, T_{VM}, T_{CM}, T_{EM}, T_{EMRA} cells that are CD57+ in uninfected, pre-ART (BL) and post-ART (ART) samples. Data is pooled from 2 experiments. * $p<0.05$, (Wilcoxon matched t-test for paired data and Mann-Whitney test for unpaired data). Bar graphs represent mean ± SEM.

6.2.3. T_{VM} cells have increased cytotoxic potential which is maintained in infection and steady state

T_{VM} cells in the young environment are highly responsive to both TCR-mediated stimulation and cytokine stimulation and can be highly cytotoxic [34,85,96,100]. This shifts with biological ageing, leading to a loss of responsiveness to TCR-mediated stimulation[95]. Given the premature ageing phenotypes observed in people with HIV on ART above, we assessed whether T_{VM} cell cytotoxic capacity changed with HIV infection or ART treatment. Expression of perforin A and GrB was measured in CD8 T cells subsets directly *ex vivo* to assess cytotoxic capacity.

In uninfected individuals, expression of GrB was relatively variable across individuals with T_N cells appearing to have the least expression in uninfected state. While no significant differences were observed, the most consistent and highest frequency of GrB+ cells was seen in T_{EMRA} cells followed by T_{VM} cells (Figure 6.3 A, B), and this did not differ with HIV infection or ART treatment (Figure 6.3 A, B). In uninfected individuals, perforin was only expressed in less than 10% of T_N cells on average, whereas the highest frequency of perforin+ cells was seen in T_{EMRA} followed by T_{VM} and T_{EM} cells (Figure 6.3 C, D). Similar trends were observed in PWH for most of the CD8 T cells, except in T_{CM} cells where the frequency of perforin+ cells was substantially increased with infection and then significantly reduced with ART (Figure 6.3 C, D). These data indicate that production of some cytolytic molecules may reduce in some CD8 T cell subsets alongside viral load. However, more differentiated T_{VM} cells, T_{EM} cells, and T_{EMRA} cells exhibit a highly cytotoxic profile across all states, and this appears relatively unperturbed by infection

or treatment. This reaffirms that the differentiation state of these subsets appears ready to mediate cytotoxic function irrespective of infection status.

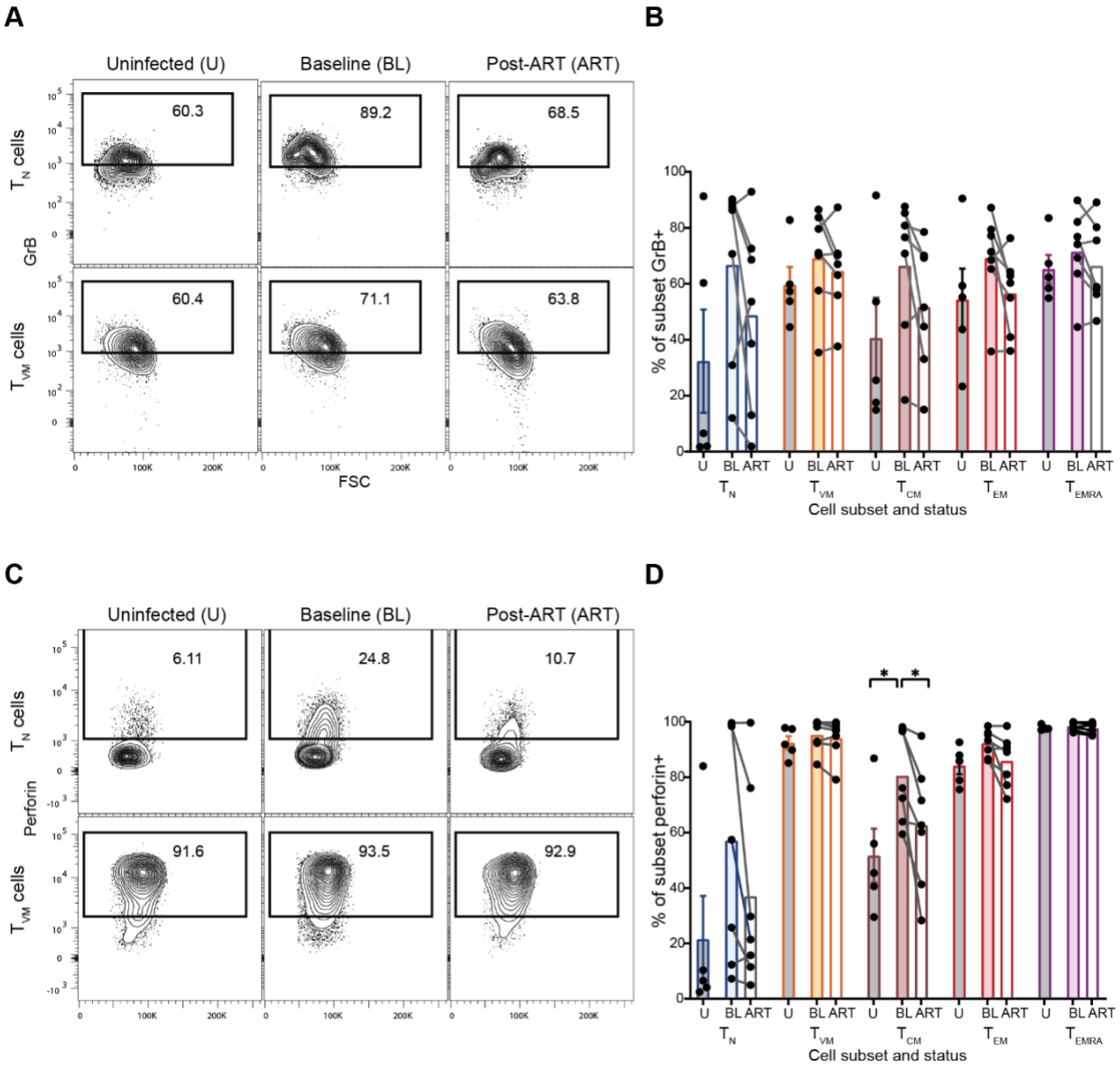

Figure 6.3. Cytotoxic potential of CD8 T cells in HIV infection and uninfected groups A) A) Representative contour plots showing the frequency of live CD8+TCRβ+ T_N and T_{VM} cells that are granzyme B (GrB)+. B) Bar graph depicting the frequencies of T_N, T_{VM}, T_{CM}, T_{EM}, T_{EMRA} cells that are GrB+ in uninfected, pre-ART (BL) and post-ART (ART) samples. C) Representative contour plots showing the frequency of live CD8+TCRβ+ T_N and T_{VM} cells that are perforin+. D) Bar graph depicting the frequencies of T_N, T_{VM}, T_{CM}, T_{EM}, T_{EMRA} cells that are perforin+ in uninfected, pre-ART (BL) and post-ART (ART) samples. Data is pooled from 2 experiments. * $p<0.05$, (Wilcoxon matched t-test for paired data and Mann-Whitney for unpaired data). Bars represent mean ± SEM.

6.2.4. T_{VM} cells have dampened TCR mediated but increased IL-15 mediated proliferation during active HIV infection

To assess whether the cytokine responsiveness of T_{VM} cells changed with HIV infection or level of viraemia, we sorted T_{VM}, T_{CM} and T_{EM} cells from pre- and post-ART samples, CTV labelled them (described in Section 2.8.1), stimulated them for 7 days with 200ng of IL-15c and then assessed proliferation (described in Section 2.8.4). Unfortunately, we were unable to evaluate T_{CM} and T_{EM} cells from uninfected samples given constraints with sorting capacity. There was no significant difference in T_{VM} cell proliferation with infection compared to uninfected controls, however there was a trend toward higher proliferation in the pre-ART group compared to uninfected T_{VM} cells (Figure 6.4 A, B, C). In PWH, T_{CM} and T_{EM} cells proliferated as well as, if not more than, T_{VM} cells in response to IL-15 (Figure 6.4 A, B). With ART treatment, T_{CM} and T_{EM} cells did not appear to show a reproducible difference in their proliferative capacity, while the mean divisions in T_{VM} cells trended downward for 5 out of 6 samples after treatment (Figure 6.4 A, B). This effect was significant when percentage of T_{VM} cells that had proliferated was compared between pre- and post-ART samples (Figure 6.4 C). This result would also support our earlier observation of a modest trend of increased proliferation with infection in T_{VM} cells compared to uninfected controls.

We previously observed a more differentiated phenotype in T_{VM} cells during HIV infection, with increased frequency of cells lacking CD27 and CD28 (Figure 6.2 A, B) or expressing CD57 (Figure 6.2 C, D), a phenotype often associated with proliferative defects in senescent CD8 T cells [296,314,315]. Therefore, we assessed whether this impacted TCR mediated proliferation. We sorted T_N, T_{VM}, T_{CM} and T_{EM} cells from pre- and post-ART samples, CTV labelled them,

stimulated them for 80-90 hours with anti-CD3/-11a and then assessed proliferation (described in section 2.8.2). There were no consistent or significant differences observed in proliferative capacity between pre- and post-ART samples for any T cell subset except T_{VM} cells, where a modest increase in proliferative capacity was observed after treatment in all 4 samples analysed ($p=0.06$) (Figure 6.4 D, E).

Our analysis of IL-15- and TCR-mediated proliferative capacity indicates that treatment may support minor recovery of TCR mediated functionality while dampening IL-15 induced proliferation in T_{VM} cells. However, despite the persistent inflammatory environment induced by HIV infection, we only observed modest changes in phenotype of CD8 T cells, and these changes generally trended across all subsets. This analysis would benefit from a larger sample size that can validate these findings.

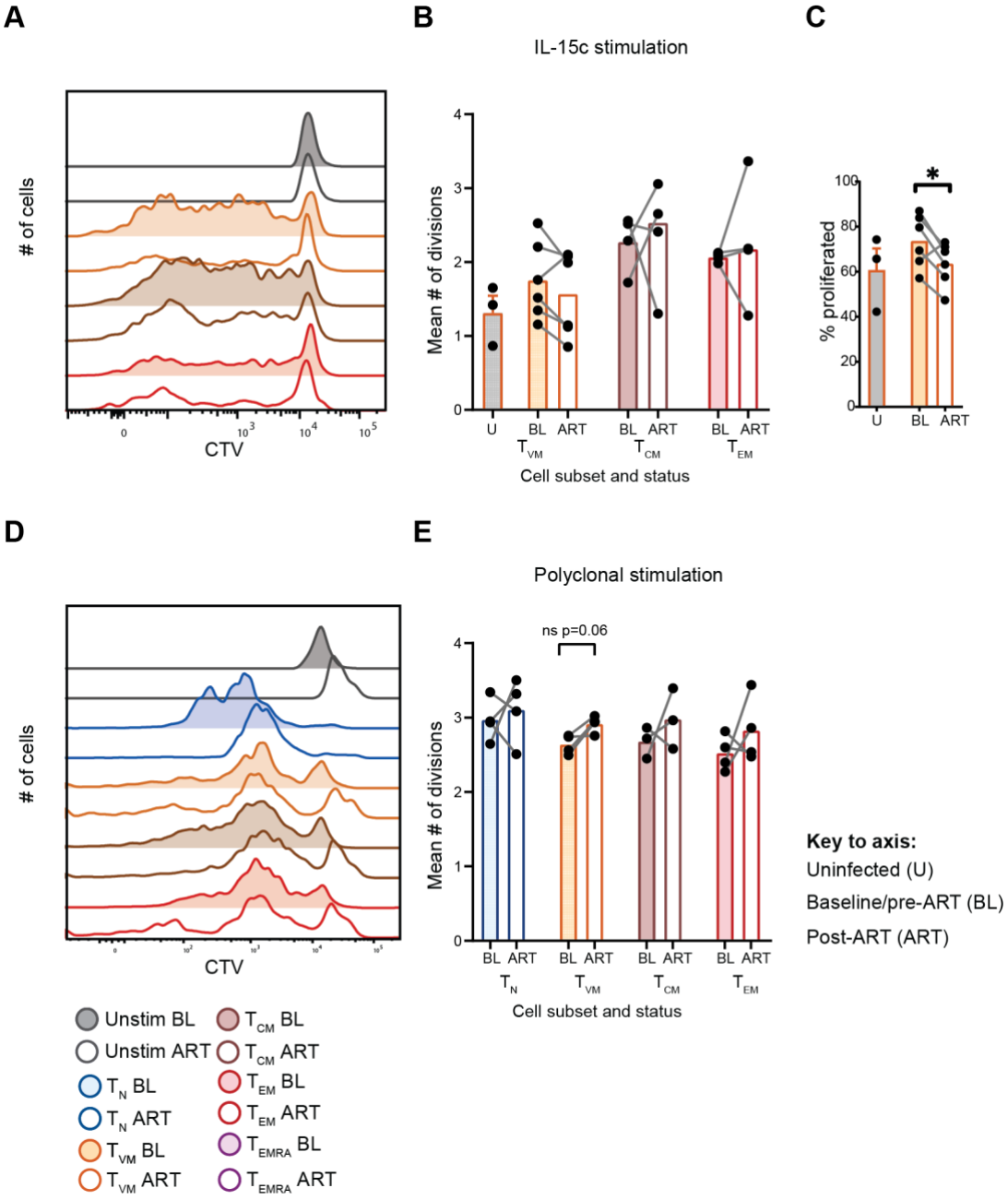

Figure 6.4. Proliferative capacity of CD8 T cell subsets in response to IL-15 or TCR stimulation in HIV infection. A) Representative histograms showing CTV staining in sorted T_{VM}, T_{CM}, T_{EM} cells after 7 days of IL-15c stimulation. B) Bar graph showing the mean number of divisions of each subset from uninfected (U), pre-ART (BL) and post-ART (ART) samples. C) Frequency of proliferated cells in stimulated T_{VM} cells from uninfected (U), BL, and ART samples. D) Representative histograms showing CTV dilution in T_N, T_{VM}, T_{CM}, T_{EM} cells after 80-90hrs of polyclonal stimulation (anti-CD3/11a), E) Bar graph showing the mean number of divisions of each subset from pre-ART (BL) and post-ART (ART) samples. Data is pooled from 2 experiments. * $p<0.05$ (Ratio t-test for paired data). Bars represent mean ± SEM.

6.3. Discussion

In this analysis we showed that T_{VM} cells exhibit a highly differentiated phenotype in the steady-state which can be further exaggerated in ART treated individuals. We also observed that T_{VM} cells, along with T_{EMRA} cells, have the highest cytotoxic potential among CD8 T cells, although did not differ substantially with HIV infection or treatment. Overall, the profile of T_{VM} cells aligns more closely with T_{EM} and T_{EMRA} cells, which may be an indication of their highly differentiated state in humans.

This dataset is helpful because most research on CD8 T cell function in HIV infection has been focused on HIV-specific CD8 T cells [316]. There is evidence that CD8 T cells that are not specific for HIV antigens also expand after infection [169,317,318], but the changes in function of bystander CD8 T cell with HIV infection or virological control and their role in an antiviral response is not clear. Our data begin to fill that knowledge gap for T_{VM} cells, which are a key CD8 T cell subset with high potential for bystander function.

HIV is known to accelerate immune ageing [292,293,296]. Thus, one may expect that T_{VM} cell functionality may be compromised due to their sensitivity to age related immune defects, marked by a loss of TCR mediated proliferative capacity in mice and humans[95,186]. However, we observed that T_{VM} cells have comparable TCR mediated proliferative capacity as T_{CM} and T_{EM} cells during active HIV infection. This is particularly interesting when we are observing a modest but significant increase in an expression profile, CD57+/CD27-CD28-, that is associated with terminal differentiation and loss of TCR function [295,314,315]. It is also possible that this T_{VM} cell phenotype is indicative of a highly activated state during HIV infection. This can be expected if T_{VM} cells are mediating viral clearance, as evidenced in a study where they

were shown to exert HIV inhibition via innate-like pathways[169]. The CD57+/CD27-CD28- phenotype of T_{VM} cells may be a better predictor of their activation state and replicative history, therefore does not correlate to TCR functionality in T_{VM} cells, as has been described in other terminally differentiated CD8 T cell subsets [295,308,310,314].

Given that a major subset of T_{VM} cells lacks CD28 expression, which is a major costimulatory molecule, it might be useful to explore if other costimulatory pathways are functioning efficiently in T_{VM} cells that maintain their response to TCR stimulus. In this regard, NKG2D has been reported as a costimulatory molecule in CD8 T cells[150,152], but NKG2D alone or without CD3 stimulation cannot induce co-stimulation [319]. One study has reported that NKG2D can enhance TCR-mediated cytokine production in $CD28^{lo}$ CD8 T cells but not in $CD28^{hi}$ CD8 T cells[154]. Considering that most T_{VM} cells do not express CD28, it might be useful to assess if co-stimulation with anti-NKG2D along with CD3 can enhance the proliferative response of T_{VM} cells, thus overcoming their reduced response compared to T_N and T_{CM} cells in the steady state[95].

We also observed a modest but significant reduction in T_{VM} cell response to IL-15 with treatment. These observations of dampened IL-15 response and increased TCR-driven proliferation align with the results from LCMV infection (Chapter 5). While we did not assess CD122 expression in this cohort, Wang and colleagues have reported downregulated CD122 expression on T_{VM} cells in ART treated individuals compared to treatment naïve and HIV-uninfected individuals[169]. Downregulation of IL-15 receptor could be responsible for the dampened IL-15 response in ART group, such a correlation would be supported by our observations made during LCMV infection. At the same time increased IL-15 levels during HIV

infection[320] or in individuals that achieve virological control and CD4 T cell recovery[233,234,321], may already be driving maximal T_{VM} cell proliferation, rendering them refractory to further stimulation. There are conflicting reports on whether IL-15 levels are reduced or increased during active HIV infection and ART. Therefore, a comprehensive analysis of this cytokine, which is critical for T_{VM} cells, may be a better predictor of T_{VM} cell response during HIV infection. It would also be of interest to explore other markers of inflammation, like IL-15 and chemokines like CXCL9/10/11, ligands for CXCR3, to better track how T_{VM} cells are responding during HIV infection.

The paucity of studies investigating the role of T_{VM} cells in humans is likely a consequence of lack of definite markers of T_{VM} cells in humans[322]. Most of the analysis of human T cell subsets does not differentiate between T_{VM} and T_{EMRA} cells[155,191,315]. T_{VM} cells present a highly differentiated and cytotoxic population in the steady-state like T_{EMRA} cells, however, in young individuals, T_{VM} cells are not a terminally differentiated population with severe TCR associated defects[95]. Moreover, the semi-differentiated profile of T_{VM} cells in humans is not a representation of an antigen-activated state but is imprinted from bystander activity during infections. These differences in functionality necessitate a much deeper analysis of what regulates T_{VM} cell function and proliferation and calls for better markers that differentiate T_{VM} cells from other terminally differentiated CD8 T cells subsets in humans.

One other study has observed a significant increase in T_{VM} cells in ART treated individuals with HIV compared to uninfected controls[169], but we did not make similar observations, this is especially striking when we consider that most of the Melbourne HIV cohort was CMV seropositive. In studies on macaques, CMV infection is known to increase T_{VM} cell subset[143].

This is likely due to having a much smaller dataset, which made it difficult to observe reproducible differences. Additionally, factors like how long the PWH were infected prior to ART initiation may also be different between our analysis and the previous study. Most patients in this cohort started ART at high CD4 counts and viral suppression was achieved within 1-2 months of therapy. Further analysis of T_{VM} cell function in context of other HIV treatment groups, such as the immunological non-responders or elite controllers might reveal new roles of T_{VM} cells during viral infections. Given that recent human studies have proposed a critical protective role of bystander memory populations during chronic inflammatory conditions like cancer[170,172] and infections[169,318], it is possible that the T_{VM} cell subset is reduced or dysfunctional in immunological non-responders. It is also worth exploring if the T_{VM} cell subset, given its highly differentiated state and sensitivity to age-related dysfunction, loses functionality after extended periods of virological control. In addition to this, to assess if T_{VM} cell population is maintained during the life course as observed in mouse studies, a more in-depth analysis of functional parameters like, cytokine production and senescence, is needed between young and aged human cohorts.

Chapter VII

7. Concluding Remarks

7.1. Discussion

In this thesis, I explored the impact of infection and inflammation on the generation, maintenance, and function of T_{VM} cells in the context of ageing. I was able to show that *de novo* generation of T_{VM} cells did not occur in the periphery during infections and cytokine exposure. The T_{VM} cell subset tended to maintain its developmental phenotype and only transient changes were observed after infections, except in case of LCMV infection. I described robust innate-like functions in young and aged T_{VM} cells but did not observe enhancement of these properties with age. This suggests that retention of cytokine sensitivity may be a mechanism to support survival of an ageing T_{VM} cell population without impacting proliferative capacity.

T_{VM} cells are a dynamic population of CD8 T cells that have been implicated as playing a key role not only during infectious episodes but also in mediating protection against cancer and autoimmune diseases (Figure 7.1). There is mounting evidence that infections, especially those that drive a robust cytokine response with high IFN and IL-15 production, can have a clear impact on T_{VM} cell homeostasis as evidenced by this work and various previous studies [84,143,167,169]. However, it remains unclear whether other environmental factors such as microbiome, diet or stress can affect the T_{VM} cell population.

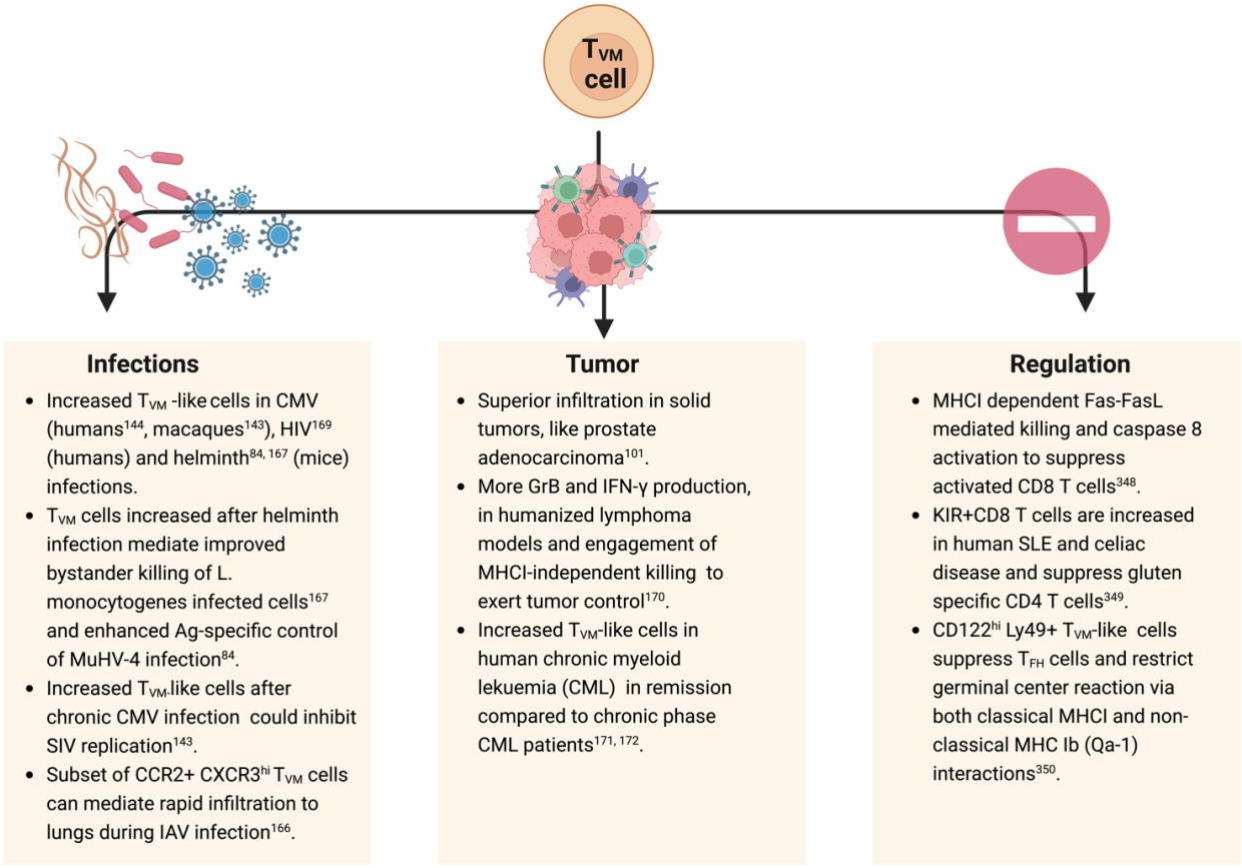

Figure 7.1: Role of T_{VM} cells during various infections, cancer and regulation of immune system.

There has been speculation that the generation and/or maintenance of T_{MP} cells might be impacted by cross-reactivity to commensal or environmental antigens, with observations of reduced numbers [193] and/or slower proliferation [323] of T_{MP} cells in germ-free mice. Indeed, there are instances where CD8 T cells cross-reactive to antigens derived from commensal microbes play a significant role in the antigen-specific response to different pathogens or in diseases like colitis and cancer [324–326]. With respect to the role of microbiota in generation of T_{VM} cells, it was observed that T_{VM} cells were unchanged in frequency and number in GF and SPF mice [94,101]. However, this observation did not discount the possibility that T_{VM} cells may have the ability to respond to commensal antigens. Stepanek and colleagues directly assessed

this by co-housing SPF mice with established T_{VM} cell populations with feral mice. Despite a significant shift in microbial colonisation in SPF co-housed mice, no differences in the T_{VM} cell compartment were observed compared to SPF mice that were not co-housed. It is possible that a more diverse microbiota can induce greater differences in T_{VM} cell population, however the fact that T_{VM} cells have limited access to the gut [97,203], the largest site of bacterial colonization [327,328], may also limit T_{VM} cell's exposure to the changing landscape of commensal bacteria. Stepanek and co also observed differences in microbial colonization in the salivary glands, but a comparison of T_{VM} cells populations specific to tissue sites was not made, which still leaves the possibility that T_{VM} cell compartment may be locally responding to commensal antigens in tissues, but these differences may not be reflected systemically.

A definite factor regulating T_{VM} cell development is host genetics. As discussed in previous chapters, T_{VM}-like cells follow different developmental trajectories in C57BL/6 mice compared to BALB/c mice, where they are also called T_{IM} cells. Owing to the presence of IL-4 producing PLZF+ NKT cells in the thymus of BALB/c mice, the T_{IM} cells arise in the thymus [92,93], whereas C57BL/6 mice lack thymic NKT cells and therefore T_{VM} cells are only detectable by surface expression of CD122 and CD44 in the periphery where IL-15 is present [35,101]. These different developmental pathways dictated by host genetics translate to alternate cytokine dependencies of T_{VM} cells during helminth infection, as we and others have shown. T_{VM} cells appear more dependent on IL-4 in the helminth infected BALB/c mice[84,167], whereas we have shown that IL-15 is critical for helminth-induced increase of T_{VM} cells in C57BL/6 mice (Chapter 4). Whether T_{IM} cells and T_{VM} cells are the same populations is still not clear. Similarly, while an increase in T_{VM} cells in feral mice compared to SPF mice has been observed, we and others [102]

found no difference in T_{VM} cell population when SPF mice were co-housed in dirty conditions. These observations also point to an influence of host genetics in the development and homeostasis of T_{VM} cells, as feral mice are an outbred population with a more diverse genetic makeup compared to inbred SPF lines.

Diet associated factors can also have an impact on CD8 T cell accumulation in various tissue sites. Obesity is associated with a state of chronic low-grade inflammation where adipocytes as well as macrophages, which are increasingly recruited to adipose tissue (AT), produce more pro-inflammatory cytokines, like TNF and IL-6 [329–331]. While the impact of diet and obesity has not been explored specifically in the context of T_{VM} cells, several studies have described an increase in CD8 T cells in the adipose tissue of obese mice compared to lean mice [332–336]. These CD8 T cells have been typically reported to have a T_{EM}/T_{RM} phenotype ($CD62L^{lo}$ $CD69^{hi}$ $CXCR3^{hi}$) where they contribute to macrophage recruitment and inflammation with increased innate-like production of IFN-γ [332–334,337]. Of note, T_{VM} cells are capable of undergoing activation and adopting T_{EFF} and T_{RM} cell fates [85,166] and thrive in inflammatory environment. It is very likely that the activated phenotype of CD8 T cells in AT is due to endogenous antigens, as suggested by multiple studies [332,337] and therefore the CD8 T cell population in AT can be potentially derived from T_{VM} cells. To understand the impact of diet and obesity on T_{VM} cells, adoptive transfer of T_{VM} cells would be necessary to track their activation in AT as well as in lymphoid organs.

A key feature regulating T_{VM} cell generation, homeostasis and activation, particularly with age, is their sensitivity to γc chain cytokines. Expression of CD122 and CD127 on T_{VM} cells increases with age, as shown in this work (Chapter 3), and suggests that $CD122^{hi}$ and $CD127^{hi}$ T_{VM} cells

may have a survival advantage with their improved sensitivity to homeostatic cytokines. Therefore preferential accumulation of T_{VM} cells with $CD122^{hi}CD127^{hi}$ expression with age may be linked to their enhanced sensitivity to homeostatic cytokines, IL-7 and IL-15, as suggested by previous studies [34,89,102]. IL-7 and IL-15 support survival by upregulating expression of anti-apoptotic protein, Bcl2 [23,31,222]. Recent evidence suggests that the role of IL-7 and IL-15 extends beyond suppression of apoptosis and may be shaping the metabolic programs in CD8 T cells to support long-term survival (Figure 7.1). In previous studies, reliance on oxidative phosphorylation (OXPHOS) and fatty acid oxidation (FAO) has been shown to support the metabolic requirements of T_{MEM} cells, thus aiding their survival in the quiescent state [338–340]. During OXPHOS, the difference between basal and maximal oxygen consumption rate (OCR) is referred to as spare respiratory capacity (SRC) and we recently demonstrated that high SRC was associated with survival of T_{VM} cells. We found that T_{VM} cells have the highest capacity for OXPHOS which translates to increased OCR and SRC compared to both T_N and T_{MEM} cells, and this capacity was further increased in T_{VM} cells with ageing and infection [34]. Additionally, the high SRC is supported by IL-15 signaling during infection and possibly through ageing as well, where neutralising IL-15 during IAV infection led to a marked decline and a reversal to baseline SRC in T_{VM} cells from infected mice [34]. Other evidence for these cytokines supporting T_{VM} cell survival is from observations of IL-7 playing a key role in regulating fatty acid synthesis in resting T_{MEM} cells. Kaech and colleagues described that T_{MEM} cell survival is supported by expression of Aquaporin 9 (Aqp9), a channel forming transmembrane protein upregulated with IL-7 stimulation, that shuttles glycerol inside the cell [341]. This allows T_{MEM} cells to esterify glycerol into triglycerides, which can then be catabolized to release fatty acids for FAO and

meet the metabolic requirements for long term survival. Our transcriptional data from a previous study shows that *Aqp9* transcript is increased by more than threefold in aged T_{VM} cells compared to young [95], which may indicate that T_{VM} cells are also using similar programs to support their survival. It is important to note that IL-15 can also induce Aqp9 expression in T_{MEM} cells, although to a lesser extent than IL-7 [341]. It is possible that the increased expression of *Aqp9* in T_{VM} cells is due to the cumulative effect of heightened sensitivity to both IL-7 and IL-15 in aged T_{VM} cells, and works to support prolonged survival of T_{VM} cells by supporting metabolic requirements (Figure 7.1).

Another way in which IL-15 can support survival of T_{VM} cells is by preserving telomere length. Dividing cells can undergo progressive loss in the terminal sections of their chromosomes, called telomeres. During DNA replication, telomere length is protected by an enzyme called telomerase [342,343]. Given that T_{VM} cells are poised for increased homeostatic turnover due to their reactivity to homeostatic cytokines and self-MHC, telomere shortening may be predicted to occur in these cells. While telomere length or telomerase activity has not been analyzed directly in T_{VM} cells, there is evidence that IL-15 signaling activates telomerase [344,345]. IL-15 has been shown to stimulate telomerase activity in memory CD8 T cells by Jak3 phosphorylation that then initiates P13K signaling [344]. Telomerase activity helps in evading apoptosis and therefore supports survival of cells [346,347] These studies imply that T_{VM} cells are predicted to have robust mechanisms in place to maintain telomerase activity via their heightened response to IL-15, which in turn also supports the survival of these cells. A model describing factors that regulate T_{VM} cell homeostasis and function through the lifespan is given in Figure 7.1.

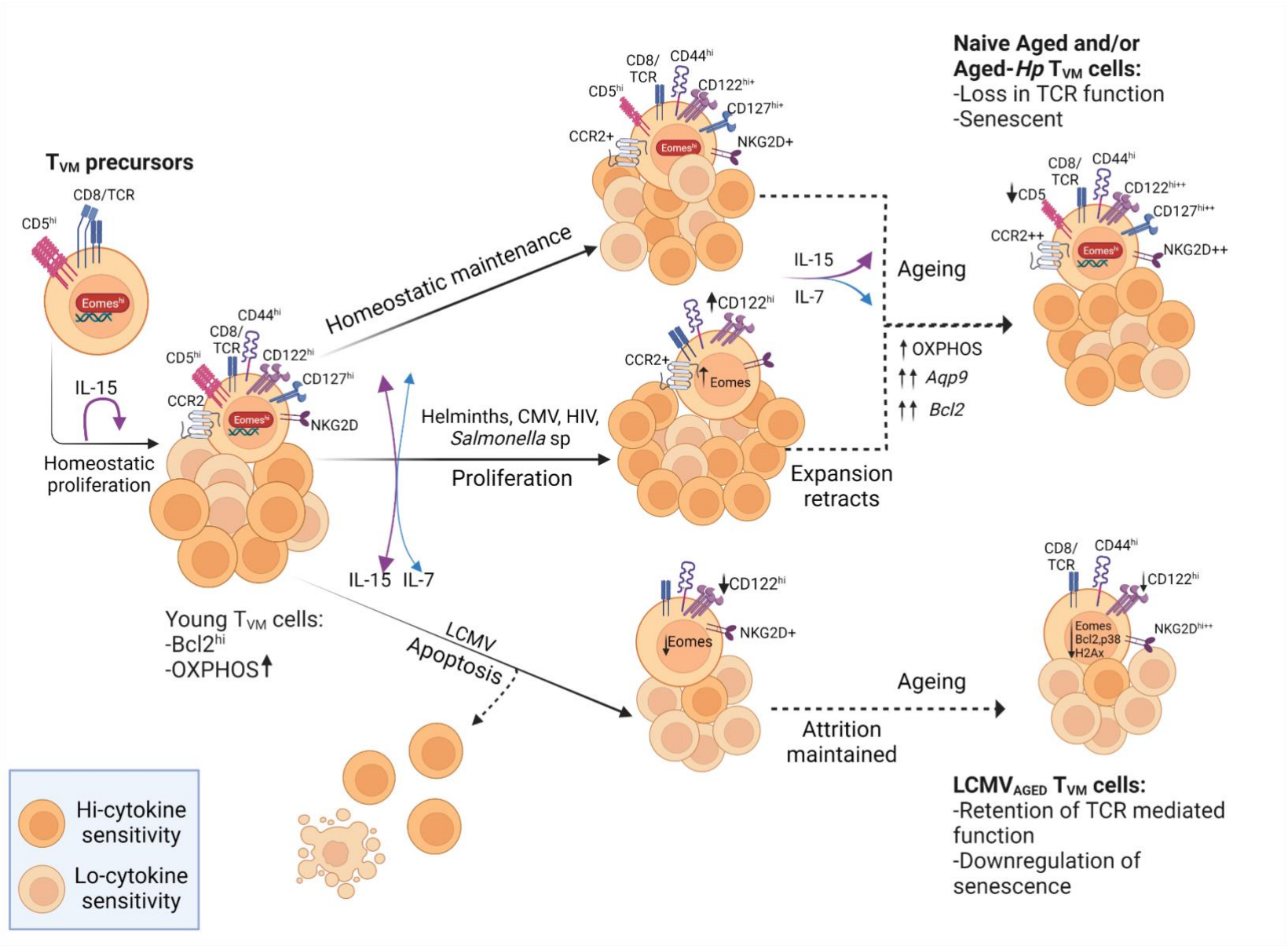
Naive Aged and/or
Aged-*Hp* T_{VM} cells:
-Loss in TCR function
-Senescent
T_{VM} precursors
CD8/TCR
$CD5^{hi}$
$Eomes^{hi}$
IL-15
Homeostatic proliferation
$CD44^{hi}$
CD8/ TCR
$CD5^{hi}$
$CD122^{hi}$
$CD127^{hi}$
CCR2
$Eomes^{hi}$
NKG2D
Young T_{VM} cells:
-$Bcl2^{hi}$
-OXPHOS↑
Homeostatic maintenance
$CD44^{hi}$
CD8/ TCR
$CD122^{hi+}$
$CD127^{hi+}$
$CD5^{hi}$
CCR2+
$Eomes^{hi}$
NKG2D+
IL-15
IL-7
Ageing
↑OXPHOS
↑↑ *Aqp9*
↑↑ *Bcl2*
$CD44^{hi}$
CD8/ TCR
↓CD5
$CD122^{hi++}$
$CD127^{hi++}$
CCR2++
$Eomes^{hi}$
NKG2D++
Helminths, CMV, HIV,
Salmonella sp
Proliferation
$CD122^{hi}$
CCR2+
↑Eomes
Expansion retracts
IL-15 IL-7
LCMV
Apoptosis
$CD122^{hi}$
↓Eomes
NKG2D+
Ageing
Attrition maintained
$CD44^{hi}$
CD8/ TCR
$CD122^{hi}$
Eomes
Bcl2,p38
↓H2Ax
$NKG2D^{hi++}$
$LCMV_{AGED}$ T_{VM} cells:
-Retention of TCR mediated function
-Downregulation of senescence
Hi-cytokine sensitivity
Lo-cytokine sensitivity

Figure 7.2. A model depicting peripheral survival of T_{VM} cells. Thymic T_{VM} cell precursors with distinct TCR repertoire and $Eomes^{hi}$ expression expand upon homeostatic IL-15 exposure in the periphery and acquire $CD44^{hi}CD122^{hi}$ expression. IL-15 signaling also results in expression of CCR2 and NKG2D on 30% and 5-10% of T_{VM} cells, respectively. Homeostatic exposure to IL-15 and to a lesser extent IL-7 supports T_{VM} cell survival throughout the lifespan by regulating Bcl2 expression, increased OXPHOS and triacylglyceride synthesis capacity and efficient telomerase activity compared to other CD8 T cells. Infectious stimuli can increase or decrease T_{VM} cell numbers; these changes are often transient, except with LCMV infection where depletion of more cytokine sensitive T_{VM} cells is maintained in advanced age leading to retention of better TCR function and suppression of senescence. Preferential survival of $CD122^{hi}CD127^{hi}$ T_{VM} cells leads to their accumulation with age, resulting in increased expression of these markers on aged T_{VM} cells compared to young, loss in TCR function and increase in senescent phenotype. Increasing sensitivity to homeostatic cytokines also results in increased proportion of T_{VM} cells expressing CCR2 (up to 75%) and NKG2D (up to 40%) and enhanced OXPHOS capacity compared to young T_{VM} cells.

The results from this thesis and previous studies have found that T_{VM} cells are not a homogenous population and, similar to T_N cells, individual T_{VM} cells may be destined to adopt different fates during antigen-specific responses. Previous studies have found differential ability of T_{VM} cells to differentiate in to T_{RM} cells in lungs based on CCR2 expression [166], and found intrinsic differences in T_{VM} cell capacity to mediate T_{EFF} and T_{MEM} cell responses in other systemic infections [85,291]. Our results from the LCMV infection model also imply that a distinct (and possibly fitter) T_{VM} cell subset survive the marked attrition, signifying that there is heterogeneity within the T_{VM} cell subset. The surviving cells were poised for better TCR-associated functions but had suppressed IL-15 responsiveness compared to T_{VM} cells from naïve mice (Chapter 5). Rudd and colleagues described that T_{VM} cells made early in life were more poised to mediate rapid effector responses [291]. These T_{VM} cells had open chromatin regions that allowed binding of Eomes, Tbx21 and Runx1 transcription factors, that are associated with generating effector responses. In contrast T_{VM} cells developed later in life were more destined for memory response and showed greater chromatin accessibility for Sp1, Egr2, nuclear factor kB (NF-kB) binding, which suppress differentiation to T_{EFF} cells during an immune

response [291]. Further evidence for heterogeneity was given by Qi and colleagues where they demonstrated differential capacity of T_{VM} cells in adopting varying differentiation status based on chemokine receptor expression. They showed that CCR2+ T_{VM} cells contributed more to the short-lived effector cells (SLECs) and displayed a $CD127^{lo}KLRG^{hi}$ phenotype 3 weeks after *L. monocytogenes* infection [166]. In line with this, CCR2+ T_{VM} cells also had enhanced expression of genes associated with a pro-inflammatory response, like *Ccl4* and *Ccl5* and increased cytotoxic potential, with increased expression of *GZMM* and the Ly49 family of receptors. When the potential of T_{VM} cells to recruit to sites of infection was tested during IAV infection, CCR2+ T_{VM} cells trafficked to the lungs more rapidly owing to their higher expression of CXCR3 compared to CCR2- T_{VM} cells. Overall T_{VM} cells outcompeted T_N cells in generating IAV-specific T_{RM} cells in the lungs, where CCR2- T_{VM} cells contributed more to development of resident-memory response during IAV infection compared to CCR2+ T_{VM} cells [166]. CCR2+ T_{VM} cells appear as early as 1-week after birth, and expression of CCR2 may be supported by both exposure to self-antigens as well as homeostatic IL-7 and IL-15 [166]. This inference is also supported by our observations of twofold higher expression of CD122 in CCR2+ T_{VM} cells compared to CCR2- T_{VM} cells in young mice (Chapter 3). Together these investigations indicate that the distinct transcriptional programs of T_{VM} cells defined early in life contributes to heterogenous functionality during infection and inflammation.

Further evidence of heterogeneity within T_{VM} cells comes from studies proposing that T_{VM}-like cells are a regulatory CD8 T (CD8 T_{REG}) cell population. With their self-reactive TCR repertoire [101,157], T_{VM} cells are predicted to follow a similar developmental trajectory in thymus as CD4 T_{REG} cells. It is therefore not surprising that T_{VM} cells have been implicated as a CD8 T_{REG}

population in multiple studies. Akane et al. described that CD122^{hi}CD49d^{lo} CD8 T cells regulate T cell homeostasis by Fas-FasL mediated killing and caspase 8 activation, and not by IL-10 production [348]. CD122^{hi}CD49d^{lo} CD8 T cells are *bona fide* T_{VM} cells in the periphery [86,94]. A regulatory role of T_{VM} cells was also proposed when CD122^{hi}Ly49+ CD8 T cells were seen to suppress activation of other T cell populations. These CD8 T_{REG} cells suppressed follicular helper T (T_{FH}) cell function and inhibited germinal centre reactions, thereby maintaining self-tolerance [349]. CD122^{hi}Ly49+ CD8 T cells are typically restricted to Qa-1 [350], a non-classical MHC Ib molecule [351], but can also be activated in a classical MHC-I restricted manner [352]. Strikingly, Qa-1b mutant mice develop lethal systemic lupus erythematosus (SLE)-like disease (Kim 2010). Similar KIR+CD8 T_{REG} cells have been described in humans, where they are found to be increased in people with autoimmune diseases like SLE and celiac disease and in people with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infections [353]. KIRs are the equivalent of Ly49 receptors in mice and similar to the role of mouse CD122^{hi}Ly49+ CD8 T cells in preventing T_{FH} cell activation during SLE [349], human KIR+ CD8 T_{REG} cells could also mediate suppression of CD4 T cells specific for gluten-derived antigens during celiac disease [353].

Critically, the potential regulatory role of T_{VM} cells is MHCI-dependent, whether it is a classical or non-classical interaction varies between studies. Multiple studies have reported that TCR activation is required for optimal suppressive activity of CD8 T_{REG} cells [348,349,352,353]. Given that T_{VM} cells are highly cytokine sensitive and mediate several effector functions without TCR engagement, it is possible that a particular subset of T_{VM} cells may have this regulatory function, which necessitates a deeper dissection of both T_{VM} cell heterogeneity and regulatory function.

T_{VM} cells may also play a role in development of autoimmune disease. In a recent study, T_{VM}-like cells were seen to increase during helminth infection and were are able to suppress drug-induced onset of type 1 diabetes (T1D). This study referred to all $CD122^{hi}$ CD8 T cells as CD8 T_{REG} cells [249]. Our own work has shown that T_N cells do not express CD122 and T_{MEM} cells express CD122, but do not expand during helminth infection (Chapter 4). Therefore, the $CD122^{hi}$ CD8 T cell subset that is expanding during helminth infection is likely to contain T_{VM} cells. *H. polygyrus* infection also induced increased colonization of some commensal species in the intestines whereas antibiotic treatment of *H. polygyrus* infected mice reversed this effect. A striking observation of a close link between microbiota and T_{VM} cell proliferation was observed in this study when antibiotic treatment also resulted in attenuation of helminth driven expansion of CD8 T_{REG} cells and therefore loss of protection against T1D offered by CD8 T_{REG} cells [249]. This loss in proliferation was attributed to decreased colonization of *Ruminococcus* species in the intestines with the antibiotic treatment without influencing *H. polygyrus* infection. When splenocytes were cultured in *Ruminococcus* culture supernatants, an increased number of CD8 T_{REG} cells were observed, suggesting that *Ruminococcus* species directly produced a factor that may expand T_{VM} cells. These observations are intriguing, especially in light of our results that propose a critical role of IL-15 in mediating T_{VM} cell proliferation during *H. polygyrus* infection. It is tempting to speculate that these bacterial species induce production of IL-15 from other immune cells which then stimulates T_{VM} cell proliferation. One study has proposed that gut bacteria can induce production of IL-15 from innate lymphoid cells (ILCs) in the gut mucosa [231]. These observations require further investigation to assess how intestinal commensal species can regulate the proliferation of T_{VM} cells and whether T_{VM} cells have access to the gut during *H. polygyrus* infection.

In recent years, many studies in mice have differentiated *bona fide* antigen-experienced memory CD8 T cells (i.e T_{MEM} cells) from other T_{MP} cells through the use of MHCI-tetramers or specific well-defined markers. in contrast, studies in humans still lack appropriate markers to differentiate *bona fide* antigen-experienced T_{MEM} cells from other T_{MP} cells, particularly definitive markers to identify T_{VM} cells. Currently, putative human T_{VM} cells are identified by their expression of NKG2A and KIRs within the CD45RA+ subset of CD8 T cells, with some studies also using high Eomes expression to identify T_{VM} cells [96,97]. Several recent studies have suggested that NKG2C may also be a marker of innate-like CD8 T cells. Expansion of NKG2C+ innate-like CD8 T cells with features very similar to T_{VM} cells was observed after CMV infection. These innate-like CD8 T cells produced IFN-γ with IL-12/IL-18 stimulation, used both TCR-dependent and -independent killing mechanisms, proliferated in response to IL-15, had restricted TCR usage and expressed high levels of CD122 and CXCR3 while lacking CD28 and PD-1 expression [143,144,146]. The lack of CD28 and CD27 expression is generally associated with replicative senescence [295,296,309], however our results from PWH and ART treated groups revealed that T_{VM} cells retained TCR mediated proliferation despite having increasing loss of CD27 and CD28 expression with ART (Chapter 6). These observations indicate that T_{VM} cells represent a functional CD8 T cell subset within the CD27-CD28- double negative population, largely comprised of T_{EMRA} cells [155]. This further underlines the need for specific markers to definitively distinguish the T_{VM} cell population, which retains TCR-associated proliferation in young individuals, from T_{EMRA} cells, which are non-proliferative even in young people [95].

It also appears that features that are distinctive for murine T_{VM} cells may not be a defining feature of putative human T_{VM} cells. For instance, CD122 expression profile of human T_{CM} and

T_{EM} cells compared to T_{VM} cells does not have a similar disparity of expression as observed in mouse T_{VM} and T_{MEM} cells, where mouse T_{VM} cells have more than twice the expression of CD122 compared to T_{MEM} cells. Similarly CD122 is undetectable in mouse T_N cells, but has considerable expression in human T_N cells and in turn human T_N cells have the ability to proliferate in response to IL-15 *in vitro* as shown by other studies [220] and our observations (data not shown). In mice, the rate of IL-15-driven proliferation is highest in T_{VM} cells compared to other subsets, however our work has demonstrated that human T_{EM} and T_{CM} cells also have similar, if not greater, capacity to proliferate in response to IL-15 stimulation as compared to T_{VM} cells (Chapter 6). Similar results have also been described in previous studies [42,354]. Another difference between mouse and human T_{VM} cells is the lower CD127 and CD5 expression in putative human T_{VM} cells compared to other CD8 T cell subsets [143,144], which contrasts with their increased expression in mouse T_{VM} cells as shown in our work (Chapter 3). This data suggests the survival and function of human and mouse T_{VM} cells may not be regulated the same way in the periphery, or the current markers being used to identify T_{VM} cells are not fully capturing the true T_{VM} cell population in human studies. A more in-depth analysis, such as single-cell genomics or analysis of chromatin accessibility in human T_{VM} cells and other CD8 T cell subsets may reveal better markers to identify human T_{VM} cells.

7.2. Limitations and future directions

The strength of our approach has been to test impact of individual inflammatory and infectious stimuli on the generation, homeostasis and function of T_{VM} cells. However, there were several key areas that we were unable to address and remain for future analyses.

While we tested the impact of several pathogens on T_{VM} cells in this analysis, there may still be other pathogens, commensals or co-incident infections that can have a role in regulating the peripheral maintenance of T_{VM} cells. Our models of infections mainly tested the response to acute infections that resolved within a few weeks. However, chronic and persistent life-long infections like CMV [355] and Epstein-Barr virus (EBV) in humans [356] could be playing a key role in modulating the T_{VM} cell population, as evidence by recent studies in humans [143,144].

In addition, our infection models used 8-12 week old adult mice, which corresponds to a 15-20 year old human [357]. However, exposure to pathogens in humans starts immediately after birth, which is a timeframe that we were not able to capture in our studies in mice. There are also many possible permutations of sequential and even co-incident infections that we were unable to model. These avenues can be dissected in future studies, which may provide a comparison of mouse and human T_{VM} cells. The recent observations of increased commensal species inducing an increase in T_{VM} cells during helminth infection [249] warrant further investigation of T_{VM} cells in context of diseases that are known to modulate the microbiome.

We observed robust expansion of T_{VM} cells after helminth infection and marked reduction coupled with retention of T_{VM} cells possessing increased TCR-associated function after LCMV infection. However, we were unable to assess if having an increased or decreased number of T_{VM} cells had any impact on subsequent infections or if the observed functional advantages after LCMV infection led to improved protection compared to T_{VM} cells from naïve mice. These directions can be explored in further studies to better assess if cytokine conditioning from infections results in improved antigen dependent and/or independent functions.

Heterogeneity within T_{VM} cells is an intriguing prospect, especially in light of regulatory roles proposed for T_{VM} cell-like populations where it would be useful to dissect if all T_{VM} cells are capable of suppressing immune cell activation. At the same time, clues from LCMV infection can guide which subset within T_{VM} cells is more prone to developing TCR-associated dysfunction with age.

7.3. Conclusions

In summary, we found that T_{VM} cells make use of selective inflammatory stimuli that may be generated during specific infections to undergo transient changes, however their long-term survival appears independent of exogenous inflammatory signals. Considering current literature together with findings from this study, it appears that homeostatic cytokine exposure is crucial for maintaining intrinsic programs in T_{VM} cells that favour survival of relatively cytokine sensitive T_{VM} cells, however this does not result in any proliferative advantage in response to IL-15 in aged T_{VM} cells compared to young (Figure 7.1). These results have advanced our understanding of innate-like function of CD8 T cells and its regulation by factors such as ageing and infection. This knowledge can inform strategies that harness unique T_{VM} cell functions for therapeutic use in infections and cancer.

Chapter VIII

8. Bibliography

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9. Appendices

9.1. Antibodies

Marker	Conjugate	Clone	Isotype	Manufacturer	Dilution
CD8α	BUV395	53-6.7	Rat IgG2a, k	BD Biosciences	400
CD44	PE-Cy7	IM7	Rat IgG2b, k	eBioscience	800
CD44	FITC	IM7	Rat IgG2b, k	BD Biosciences	400
CD45.1	APC-Cy7	A20	Mouse IgG2a, κ	Biolegend	200
CD45.2	PE	30-F11	Rat IgG2b, k	eBioscience	200
TCRβ	AF700	H57-597	Ar Ham IgG	Biolegend	400
TCRβ	BV421	H57-597	Ar Ham IgG	Biolegend	400
CD49d	AF647	R1-2	Rat IgG2b, k	Biolegend	400
CD49d	PE-Cy7	R1-2	Rat IgG2b, k	Biolegend	400
CD122	BV421	TM-b1	Rat IgG2b, k	BD Biosciences	200
CD122	FITC	TM-b1	Rat IgG2b, k	eBioscience	200
CD122	PE	TM-b1	Rat IgG2b, k	Biolegend	200
CD124	PE	mIL4R-M1	Rat IgG2b, k	BD Biosciences	100
CD127	PE	A7R34	Rat IgG2a, k	eBioscience	200
CD5	PerCP-Vio® 700	53-7.3	Rat IgG2a, k	Miltenyi Biotec	100
NKG2D	BV421	CX5	Rat IgG1	BD Biosciences	200
NKG2D	BV711	CX5	Rat IgG1	BD Biosciences	200
NKG2D	Purified	CX5	Rat IgG1, κ	Biolegend	25
NKG2A/C/E	Biotin	20D5	Rat IgG2a, k	eBioscience	200
CXCR3	APC	220803	Rat IgG2a	In Vitro Technologies	20
CCR2	unconjugated	EPR20844-15	Rabbit IgG	Biolegend	200
CXCR5	BV786	L138D7	Rat IgG2b, k	Biolegend	50

Marker	Conjugate	Clone	Isotype	Manufacturer	Dilution
CD172α (SIRPα)	BUV496	P84	Rat IgG1	BD Biosciences	400
CD11c	FITC	HL3	Ar Ham IgG_1, l2	BD Biosciences	400
Siglec H	AF647	551	Rat IgG1, k	Biolegend	1000
I-A (b/d/q), I-E(d/k)MHC Class II	APC-Cy7	M5/114.15.2	Rat IgG2b, k	Biolegend	800
NK1.1	PacBlue	PK137	Mouse IgG2a, k	Biolegend	400
CD19	PacBlue	6D5	Rat IgG2a, k	Biolegend	400
IL-15	Biotin		Polyclonal rabbit	Peprotech	100
CD8	BV605	H1T8α	Mouse IgG1, κ	BD Biosciences	100
TCR α/β	AF700	IP26	Mouse IgG1, κ	Biolegend	100
KIR2DL1/S1/S3/S5	APC	HP-MA4	Mouse IgG2b, κ	Biolegend	100
KIR3DL1/DL2	APC	REA970	rhuman IgG1	Miltenyi Biotec	100
NKG2A	APC	REA110	recombinant human IgG1	Miltenyi Biotec	100
CD45RA	BV786	HI100	Mouse IgG2b, κ	BD Biosciences	200
CD27	BUV395	L128	Mouse IgG1	BD Biosciences	400
CD28	APC-H7	CD28.2	Mouse IgG1, κ	BD Biosciences	100
CD57	Pacific Blue	HCD57		Biolegend	800
Intracellular markers					
Ki-67	PE	SolA15	Rat IgG2a, κ	eBioscience	400
Bcl2	FITC	10C4	Rat IgG1, κ	eBioscience	200
Eomes	PE-Cy7	Dan11Mag	Rat IgG2a, κ	eBioscience	200
Phospho-p38 MAPK (Thr180/Tyr182)	unconjugated	D3F9	Rabbit	Cell Signaling Technology	400
Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204)	unconjugated	D13.14.4E	Rabbit	Cell Signaling Technology	400

Marker	Conjugate	Clone	Isotype	Manufacturer	Dilution
H2AX (pS139)	PE	N1-431	Mouse IgG1, κ	BD Biosciences	200
H2AX (pS139)	BV421	N1-431	Mouse IgG1, κ	BD Biosciences	200
TNF	PE	MP6-XT22	Rat IgG1, k	Biolegend	400
IFN-γ	FITC	XMG1.2	Rat IgG1, k	BD Biosciences	400
Granzyme B	PacBlue	GB11	Mouse IgG2b, k	Biolegend	200
Granzyme B	BV510	GB11	Mouse IgG2b, k	BD Biosciences	100
Perforin	FITC	dG9	Mouse IgG2b, k		200
Streptavidin	PE			Invitrogen	500
Streptavidin	APC			Invitrogen	500
Anti-Rabbit IgG F(ab')2 Fragment	PE		Goat	Cell Signaling Technology	500
Fixable dyes, Viability stains					
Propidium Iodide				BD Biosciences	800
AquaBlue				Invitrogen	400
Near Infra Red				Invitrogen	800
Fixable viability stain 700				BD Biosciences	800
Cell Trace Violet				Invitrogen	1000

9.2. Media and Buffers

Hanks buffered salt solution (HBSS): HBSS was prepared by Media Preparation Unit of the Biomedicine Discovery Institute at Monash University, Clayton campus.

Lung Digestion mix:

Lung digestion mix was prepared in RPMI-1640 media with 2.5 mg/mL of Type I collagenase (Gibco) and 105 U/mL of recombinant DNase I (Sigma Aldrich, Missouri, USA-Aldrich).

Complete Roswell Park Memorial Institute media (cRPMI):

cRPMI was prepared by supplementing the following nutrients and antibiotics in RPMI 1640 media (Gibco):

-10% FBS (Sigma Aldrich, Missouri, USA-Aldrich)

-2 mM L-glutamine

-1 mM MEM sodium pyruvate

-100 µM MEM non-essential amino acids

-5 mM HEPES buffer

-55 µM 2-mercaptoethanol

-100 U/mL penicillin (Gibco)

-100 µg/mL streptomycin (Invitrogen, Thermo Fisher Scientific, USA).

Ammonium-Chloride-Potassium (ACK) lysis buffer: 8.02 g ammonium-chloride (NH_4Cl), 0.84 g sodium bicarbonate ($NaHCO_3$), 0.37 g EDTA dissolved in 100ml of Milli-Q water.

Fc Block: Mouse Fc blocking solution was prepared by adding 1% normal mouse serum and 1% normal rat serum (Stem Cell Technologies, Vancouver, Canada) in 2.4G2 supernatant (Section 2.5.1). Fc block was diluted 1:2 in FACS or MACS buffer before use.

FACS Buffer: 1X PBS with 1% bovine serum albumin (BSA) (Bovogen Biologicals, Victoria, Australia) and 0.02% Na-azide (Sigma Aldrich, Missouri, USA-Aldrich).

MACS buffer: 1X PBS with 0.5% BSA (Scientifix) and 2mM EDTA.

Sort Buffer: 1X PBS with 2% FBS.

Percoll® density gradient media: Standard isotonic Percoll® solution was made with 63% Percoll® (Sigma Aldrich, Missouri, USA-Aldrich), 7% 10x PBS, 29.5% 1x PBS and 0.5% 0.5 mM EDTA.

NycoPrep™ 1.077/EDTA: 1mM EDTA was added to NycoPrep™ 1.077 (AXIS Shield, Soctland) and stored in single use aliquots.

DC Spleen Digestion mix: 7mg Type II Collagenase (Worthington Biochemical Corporation, New Jersey, USA) and 0.01% Grade II DNAse 1 (Roche Diagnostics) was dissolved in RPMI (Gibco) with 2% FCS and stored in single use aliquots at -20°C.

9.3. Supporting Data

JCU Aged dirty mice health report:

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Cerberus Sciences

Submitter: Monash University - Biochemistry
19 Innovation Way
Clayton, Victoria Australia
Contact: Claerwen Jones

Submission No: 18/M012
Your ref: 180109
Date received: 1 February 2018
Date of issue: 14 February 2018

Location: From JCU / Strain ID: C57BL/6 **Species: Mouse**

Bacteria and Fungi	**Pos / Tested**	**Laboratory**	**Method**
Bordetella bronchiseptica	0 / 1	Cerberus	Culture
Bordetella spp	0 / 1	Cerberus	Culture
CAR bacillus	0 / 1	Cerberus	EIA / IFA
Citrobacter rodentium	0 / 1	Cerberus	Culture
Clostridium piliforme	0 / 1	Cerberus	EIA / IFA
Corynebacterium kutscheri	0 / 1	Cerberus	Culture
Helicobacter spp	1 / 1	Cerberus	PCR
Klebsiella oxytoca	0 / 1	Cerberus	Culture
Klebsiella pneumoniae	0 / 1	Cerberus	Culture
Mycoplasma pulmonis	0 / 1	Cerberus	EIA / IFA
Other significant organisms	0 / 1	Cerberus	Culture
Pasteurella pneumotropica	1 / 1	Cerberus	Culture
Pasteurella pneumotropica	1 / 1	Cerberus	PCR
Pasteurellaceae group	1 / 1	Cerberus	Culture
Pasteurellaceae group	1 / 1	Cerberus	PCR
Proteus spp	0 / 1	Cerberus	Culture
Pseudomonas aeruginosa	0 / 1	Cerberus	Culture
Salmonella spp	0 / 1	Cerberus	Culture
Staphylococcus aureus	0 / 1	Cerberus	Culture
Streptococcus pneumoniae (alpha haem)	0 / 1	Cerberus	Culture
Streptococcus spp (beta haem)	0 / 1	Cerberus	Culture
Ectoparasite	**Pos / Tested**	**Laboratory**	**Method**
Demodex musculi	0 / 1	Cerberus	Exam
Mycoptes musculinus	0 / 1	Cerberus	Exam
Myobia musculi	0 / 1	Cerberus	Exam
Polyplax serrata	0 / 1	Cerberus	Exam
Radfordia spp	0 / 1	Cerberus	Exam
Endoparasite	**Pos / Tested**	**Laboratory**	**Method**
Encephalitozoon cuniculi	0 / 1	Cerberus	EIA / IFA
Giardia muris	0 / 1	Cerberus	Wet prep/Motility
Pinworm - Aspiculuris spp	0 / 1	Cerberus	Wet prep/Motility
Pinworm - Aspiculuris tetraptera	0 / 1	Cerberus	PCR
Pinworm - Syphacia obvelata	1 / 1	Cerberus	PCR
Pinworm - Syphacia spp	0 / 1	Cerberus	Wet prep/Motility
Spironucleus muris	0 / 1	Cerberus	Wet prep/Motility
Histopath	**Pos / Tested**	**Laboratory**	**Method**
Mouse Histology	0 / 1	Cerberus	Histo
Non-Pathogenic Protozoa	**Pos / Tested**	**Laboratory**	**Method**
Chilomastix bettencourti	0 / 1	Cerberus	Wet prep/Motility
Entamoeba muris	0 / 1	Cerberus	Wet prep/Motility
Tritrichomonas muris	0 / 1	Cerberus	Wet prep/Motility

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Virus	Pos	/	Tested	Laboratory	Method
Adenovirus Type 1	0	/	1	Cerberus	EIA / IFA
Adenovirus Type 2	0	/	1	Cerberus	EIA / IFA
Ectromelia Virus	0	/	1	Cerberus	EIA / IFA
Hantaan Virus	0	/	1	Cerberus	EIA / IFA
K Virus	0	/	1	Cerberus	EIA / IFA
Lymphocytic Choriomeningitis Virus	0	/	1	Cerberus	EIA / IFA
Minute Virus of Mice	0	/	1	Cerberus	EIA / IFA
Mouse Hepatitis Virus	0	/	1	Cerberus	EIA / IFA
Mouse Norovirus	1	/	1	Cerberus	EIA / IFA
Mouse Parvovirus	0	/	1	Cerberus	EIA / IFA
Mouse Thymic Virus	0	/	1	Cerberus	IFA
Murine Cytomegalovirus	0	/	1	Cerberus	EIA / IFA
Pneumonia Virus of Mice	0	/	1	Cerberus	EIA / IFA
Polyoma Virus	0	/	1	Cerberus	EIA / IFA
Reovirus Type 3	0	/	1	Cerberus	EIA / IFA
Rotavirus	0	/	1	Cerberus	EIA / IFA
Sendai Virus	0	/	1	Cerberus	EIA / IFA
Theiler`s Encephalomyelitis virus	0	/	1	Cerberus	EIA / IFA

Comment:

Samples Refs. were:

Sample Male EM#28.

The following samples were POSITIVE for antibody directed against Mouse Norovirus by EIA / IFA: Sample Male EM#28 (From JCU).

The following samples were POSITIVE for Helicobacter spp by PCR: Sample Male EM#28 (From JCU).

The following samples were POSITIVE for Pasteurella pneumotropica by Culture: Sample Male EM#28 (From JCU).

The following samples were POSITIVE for Pasteurella pneumotropica by PCR: Sample Male EM#28 (From JCU).

The following samples were POSITIVE for Pasteurellaceae group by PCR: Sample Male EM#28 (From JCU).

The following samples were POSITIVE for Pasteurellaceae group by Culture: Sample Male EM#28 (From JCU).

The following samples were POSITIVE for Pinworm - Syphacia obvelata by PCR: Sample Male EM#28 (From JCU).

The Pasteurellaceae group of organisms are a large family of Gram-negative bacteria. Most of these organisms are commensals on mucosal surfaces of mammals and birds and are not regarded as pathogenic organisms. Included in this family are several pathogens of relevance to laboratory animal species: Pasteurella pneumotropica (mice and rats), and Pasteurella multocida (rabbits).

If an initial positive result is obtained for the Pasteurellaceae group, then further tests are run to determine if P. pneumotropica and/or P. multocida are present.

For interpretation of these results: If there is a positive result for Pasteurellaceae group, with negative, or no P.pneumotropica or P.multocida result - this reflects commensal bacteria in the sample and no pathogenic organisms of concern.
If there is a positive Pasteurellaceae group result, and positive P.pneumotropica and/ or P.multocida then these are of potential pathogenic relevance.

The following samples were POSITIVE for Pinworm - Syphacia obvelata by PCR: Sample Male EM#28 (From JCU).

Sample Male EM#28 (From JCU - Mouse Norovirus):

Elevated OD by ELISA. Confirmed POSITIVE by IFA. DK

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Sample Male EM#28 (From JCU - Mouse Histology):

Macropathology:
A male C57B/6 mouse earmarked #28 is received, worn approximately October 2016, ageing experiment, that high-fat diet from November 16 to September 17. Ethics A 2500.
No significant findings on gross post-mortem.

Histopathology:
Liver:
Moderate diffuse hepatic lipidosis.

Caecum:
A cross-section through one adult nematode, consistent with the oxyurid Syphacia species.

Kidney:
Moderate lymphoplasmacytic infiltrations into the interstitial tissues, rare basophilic tubules.

Salivary glands:
Moderate lymphocytic infiltrate into the interstitial tissues.

Spleen:
Mild diffuse extramedullary haematopoiesis.

Lung, heart, gastrointestinal system (stomach, small intestine, colon), pancreas, brain:
No significant findings.

Diagnosis:
Moderate hepatic lipidosis.
Syphacia species pinworm infestation.
Moderate lymphoplasmacytic interstitial nephritis.
Moderate lymphocytic interstitial infiltrates of the salivary gland.
Mild diffuse splenic extramedullary haematopoiesis.

Comments:
The hepatic lipidosis is probably secondary to the high-fat diet.

There are two species of pinworm in mice Syphacia obvelata and Aspiculuris tetraptera. Syphacia obvelata occurs in the Caecum with eggs deposited externally around the anus. Aspiculuris tetraptera occurs within the colon. Both pinworm have been linked to alterations parameters used in research.

The renal, salivary gland, and splenic changes are all common incidental background findings in many strains of mice.

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