The Role of NF-κB Transcription Factors in Virtual Memory (T_{VM}) $CD8^+$ T cells

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Abstract

Virtual memory (T_{VM}) cells are a subset of memory phenotype (MP) $CD8^+$ T cells that arise naturally in naïve, lympho-replete mice. In contrast to conventional, antigen-primed memory (T_{AM}) $CD8^+$ T cells that are generated following an antigen-dependent T cell effector immune response, T_{VM} cells develop from naïve $CD8^+$ T cells in response to cytokine-dependent homeostatic proliferative signals, in particular interleukin (IL)-15.

Utilizing two murine models, we show that the NF-κB transcription factor RelA plays a T cell-intrinsic role in the homeostatic maintenance of T_{VM} cells. Lethally irradiated mice reconstituted with $RelA^{-/-}$ foetal liver-derived hematopoietic stem cells (HSCs) fail to maintain a $RelA^{-/-}$ T_{VM} cell population. This phenotype, which appears in part due to an inability to compete with residual ($RelA^{+/+}$) T_{VM} cells for survival/proliferation signals coincides with reduced expression by $RelA^{-/-}$ T_{VM} cells of the IL-2/15 receptor β-chain (CD122). Conditional inactivation of RelA in T cells ($Lck^{cre}Rela^{fl/fl}$ mice) reveals an issue in maintenance with these mice exhibiting a decline in the T_{VM} cell population from 6 weeks of age to ~50% of normal numbers by 12 weeks, after which this reduced level of T_{VM} cells is maintained throughout adult life. In addition to a reduced expression of CD122, IL-7 receptor α-chain (CD127) expression is lower on $Lck^{cre}Rela^{fl/fl}$ T_{VM} cells when compared to age-matched littermate controls ($Lck^{cre}RelA^{wt/wt}$ mice), a finding suggestive of an IL-7 survival defect.

Further investigation of T_{VM} cells lacking RelA confirmed that these changes in CD122 and CD127 cell surface expression levels coincided with reduced viability when cultured in the presence of IL-15 or IL-7. Moreover, the cytokine-induced proliferation of RelA-deficient T_{VM} cells was reduced in higher concentrations of IL-15. Whilst these findings establish that RelA contributes to IL-15 concentration-dependent T_{VM} cell survival and division, the mechanism(s) by which RelA regulates these functions are likely to be complex, given that T_{VM} cells lacking RelA were also found to be susceptible to activation-induced cell death when stimulated with higher concentrations of IL-15.

Collectively, the data presented in this thesis indicated that the canonical NF-κB transcription factor RelA plays an important role in controlling the homeostatic maintenance of T_{VM} $CD8^+$ T cells, in part, by regulating the expression of cytokine receptors important for T_{VM} cell survival and division.

Declaration

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.

Darcy P Ellis

1st July, 2019

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Table of Contents

Abbreviations

Abbreviation	Abbreviation Definition
Ab	Antibody
AICD	Activation induced cell death
AIRE	Autoimmune regulator
ANOVA	Analysis of variance
APC	Antigen presenting cell
APC	Allophycocyanin (fluorophore)
ASC	apoptosis-associated speck-like protein containing CARD
Bcl	B cell lymphoma proteins
BSA	Bovine serum albumin
c-Rel	V-rel avian reticuloendotheliosis viral oncogene homolog
CARD	C-terminal caspase recruitment domain
CBP	CREB binding protein
CCL	CC chemokine ligand
CCR	CC chemokine receptor
CD	Cluster of differentiation
CD103	Intergrin αE
CD122	IL2R β-chain
CD127	IL7R α-chain
CD132	Common γ-chain
CD25	IL2R α-chain
CD45	Leukocyte common antigen, expressed on all white blood cells.
CD62L	L-selectin, *Sell* gene product
cIAP	cellular Inhibitor of apoptosis proteins
CLP	Common lymphocyte progenitor
CMP	Common myeloid progenitor
CNS	Conserved non-coding sequence
Cre	Cre recombinase
CREB	cAMP response element-binding protein
cTEC	cortical Thymic epithelial cell
CTLA-4	Cytotoxic T lymphocyte associated protein 4
CTV	Cell trace violet
Cy	Cyanine fluoroscent dye
DC	Dendritic cell
ddH2O	double distilled water
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DN	Double negative
DNA	Deoxyribonucleic acid
DP	Double positive
DTT	Dithiothreitol
E	Embryonic day
EDTA	Ethylene diamine tetraacetic acid
EGTA	Ethylene glycol tetraacetic acid

ELISA	Enzyme linked immunosorbent assay
EMSA	Electrophoretic mobility shift assay
Eomes	Eomesodermin
FACS	Fluorescent activating cell sorter
Fc	Fragment crystallizable region of antibodies
FCS	Foetal calf serum
FITC	Fluoroscene fluorophore
Foxp3	Forkhead box P3
FSC	Forward scatter
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFP	Green fluorescent protein
Gy	Gray, SI unit of absorbed radiation
HBSS	Hank's balanced salt solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HP	Homeostatic proliferation
HRP	Horseradish peroxidase
HSC	Haematopoetic stem cell
ICOS	Inducible T cell costimulator
Id3	DNA-binding protein inhibitor id-3
IFN	Interferon
Ig	Immunoglobulin
IκB	Inhibitor of kappa-B
IKK	IκB kinase
IL	Interleukin
Irf9	Interferon regulatory factor 9
ITAM	Immunoreceptor tyrosine-based activation motif
Jak	Janus kinase
kDa	kilo Dalton
KLF2	Krüppel-like factor 2
LAG3	Lymphocyte-activation gene 3
Lck	Lymphocyte-specific protein tyrosine kinase
LIP	Lymphopenia-induced proliferation
LN	Lymph node
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MFI	Median fluorescence intensity
MHC	Major histocompatability complex
MP	Memory phenotype
mTEC	medullary Thymic epithelial cells
NADPH	Nicotinamide adenine dinucleotide phosphate
NEMO	NF-κB essential modulator, IKKγ
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer cell
NKT cell	Natural killer T cell
NLS	Nuclear localisation signal
p100	Full length NF-κB2 protein

p105	Full length NF-κB1 protein
p50	Processed form of NF-κB1 protein
p52	Processed form of NF-κB2 protein
p65	RelA
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PD-1	Programmed cell death protein 1
PE	Phycoerythrin
PerCP	Peridinin chlorophyll fluorescent protein
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PLCγ	Phospholipase C gamma
pLN	peripheral Lymph node
PLZF	Promyelocytic Leukaemia Zinc Finger protein
Rag1	Recombination activating gene 1
RelA	V-rel avian reticuloendotheliosis viral oncogene homolog A
RelB	V-rel avian reticuloendotheliosis viral oncogene homolog B
RFP	Red fluorescent protein
RHD	Rel homology domain
RNA	Ribonucleic acid
RO	Reverse osmosis
RPMI	Roswell Park Memorial Institute medium
SEM	Standard error of the mean
Ser	Serine
SP	Single positive
Src	Proto-oncogene tyrosine-protein kinase Src
SSC	Side scatter
STAT	Signal transducer and activator of transcription
T-bet	T-box transcription factor TBX21
TAD	Transactivating domain
TAE	Tris/acitic acid/EDTA
T_{AM}	conventional antigen-primed memory, CD8+
TBE	Tris/boric acid/EDTA
TBS	Tris-buffered saline
TCR	T cell receptor
Tg	Transgenic
T_{IM}	Innate memory cell, CD8+
TNF	Tumour necrosis factor
TNFR	Tumour necrosis factor receptor
TNFRSF	Tumour necrosis factor receptor super family
Treg	Regulatory T cell
T_{VM}	Virtual Memory cell, CD8+
Ub	Ubiquitin
V(D)J	Variable, diversity, joining gene segments of T and B antigen receptors
WT	Wild type
YFP	Yellow fluorescent protein
β-ME	Beta-mercaptoethanol

Chapter 1:

Literature Review

1.1 CD8$^+$ T cells

CD8$^+$ T cells are an essential part of cell-mediated immunity required for the clearance of compromised cells, including those cells that have become cancerous, damaged or infected with intracellular pathogens, such as viruses. During an immune response to an intracellular pathogen, naïve CD8$^+$ T cells recruited to the site of infection become activated, proliferating and differentiating into short-lived effector cells that utilize cytotoxins such as perforin, granzymes and granulysin to lyse and eliminate infected cells (Pearce et al, 2003). Upon successful clearance of infected cells, approximately 90 to 95% of these pathogen-specific effector CD8$^+$ T cells die in the so- called 'contraction phase' of the CD8$^+$ T cell response via a process of apoptotic programmed cell death. The remaining antigen-specific effector CD8$^+$ T cells differentiate into long-lived T cells that can facilitate a rapid recall response in the event of a subsequent infection by the same pathogen (Joshi and Kaesh, 2008). This population of CD8$^+$ T cells represents the basis of CD8$^+$ T cell-mediated immunity.

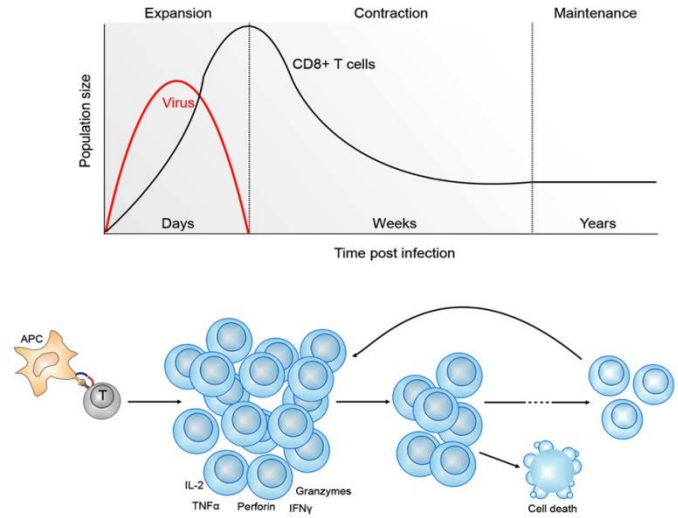


Figure 1.1 CD8+ T cell effector response to viral infection. Naïve CD8$^+$ T cells are activated upon recognition of intracellular foreign antigen, leading to rapid expansion and differentiation into short-lived effector cells. Successful elimination of virally infected cells is followed by the contraction phase, where most effector CD8$^+$ T cells die via apoptosis. A small population of effector cells differentiate into memory CD8$^+$ T cells which are maintained long-term to facilitate a faster immune response to a secondary infection. (Russ et al, 2013)

1.1.1 T cell development

All T cells develop from bone marrow-derived multipotential lymphoid precursors that undergo a series of differentiation steps in the thymus. The sequential process of T cell development in the thymus is guided by signals received by the T cell antigen receptor (TCR) and co-stimulatory receptors expressed on developing thymocytes (Godfrey and Zlotnik et al, 1993). This process generates a population of mature T cells with a diverse repertoire of receptors that recognize a broad range of foreign peptides, but not self-antigens (Singer et al, 2008). $CD4^-CD8^-$, double negative (DN) T cell precursors transition through a series of differentiation steps (DN1 to DN4) classified by the differential expression of CD44 and CD25 (Godfrey et al, 1993). During this developmental transition, T cells initially undergo a commitment to either the αβ or γδ T cell lineages as a result of the rearrangement and assembly of specific TCR genes (Gerondakis et al, 2014). Precursors committed to becoming T cells that express αβ TCRs, first undergo β-chain TCR rearrangement prior to DN3 (von Boehmer, 2005). Following the successful selection of DN thymocytes expressing a correctly rearranged TCR β chain, developing T cells reach DN4 and undergo TCRα gene rearrangement to become $CD4^+CD8^+$ (DP) $TCR\alpha\beta^+$ thymocytes. DP thymocytes are then screened by a dual overlapping process of positive and negative selection as they move from the thymic cortex to the medulla, while at the same time becoming either $CD4^+$ or $CD8^+$ single positive (SP) thymocytes. This process is under the control of the autoimmune regulator (AIRE) protein, a transcription factor expressed by medullary thymic epithelial cells (mTECs), which controls the expression of various tissue-specific self-antigens to be presented to DP thymocytes via MHC molecules (von Boehmer and Melcher, 2010). Cells that interact with self-peptide presented by MHC class Ia molecules become $CD8^+$ T cells, while interaction with MHC class II molecules promotes $CD4^+$ T cell development. The strength and duration of TCR signals received by developing thymocytes is a crucial step in determining developmental outcomes as only cells that interact with self-peptide-MHC complexes with low to moderate affinity undergo positive selection and receive survival signals, while

cells that interact with high affinity undergo negative selection and die by apoptosis. Cells that fail to interact with self-peptide-MHC complexes do not receive a survival signal and die by neglect. With death by neglect and negative selection resulting in the elimination of more than 95% of DP thymocytes, the remaining cells that survive this rigorous screening process exit the thymus as naïve conventional T cells.

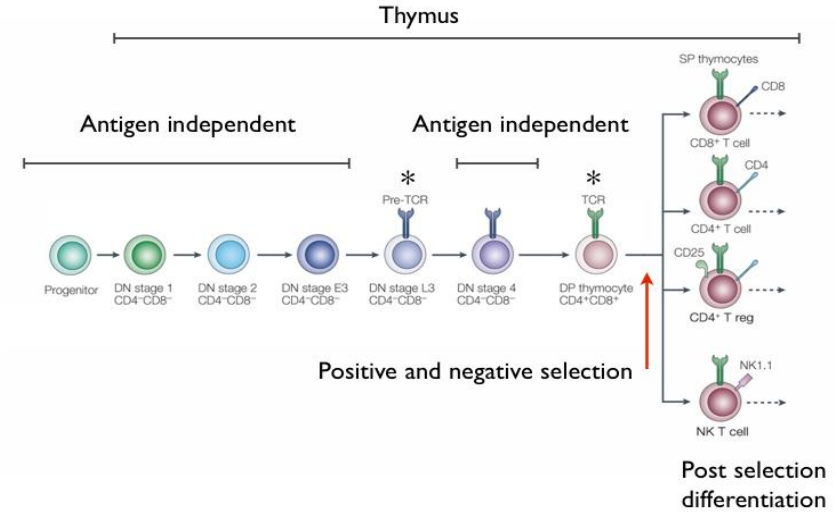


Figure 1.2. Thymic development of T cell subsets. The development of T cells follows a series of differentiation steps in the thymus leading to the selection of T cells with antigen receptors that recognise a broad range of foreign peptides, but not self antigens. Post-selection differentiation gives rise to the various thymic T cell populations. (Siebenlist et al, 2005)

1.1.2 Non-conventional CD8$^+$ T cells

Non-conventional CD8$^+$ T cells represent a subset of so-called 'innate-like T cells' that also develop from DP thymocytes. Unlike conventional CD8$^+$ T cells which only acquire effector-like properties following antigen-dependent activation, non-conventional CD8$^+$ T cells acquire their effector function during development, typically expressing surface markers associated with an activated or memory phenotype, such as CD44, CD122 (IL-2 receptor β-chain) and NK1.1 (Gordon et al, 2011). These innate-like T cells

include CD1d restricted natural killer T (NKT) cells and $CD8^+$ T cells expressing TCRs specific for non-classical MHC class Ib molecules, including H2-M3-specific T cells, $CD8^+\alpha\alpha$ T cells and mucosal-invariant T cells (Berg, 2007). These innate-like $CD8^+$ T cells have been shown to participate in host defense against bacteria and viruses and despite having not encountered foreign antigen are able to produce effector cytokines including IFNγ following activation (Berg, 2007).

1.2 Memory-phenotype (MP) $CD8^+$ T cells

Conventional memory $CD8^+$ T cells are generated following the production and contraction of the effector response upon recognition of foreign antigen (Akue et al, 2012). The generation and long-term maintenance of memory $CD8^+$ T cells provides a more rapid and robust immune response upon successive exposure to antigens. These memory $CD8^+$ T cells show diverse and pliable populations that possess distinct functional properties and are broadly characterized as effector-memory (T_{EM}) or central memory (T_{CM}) populations based on expression of the adhesion molecule L-selection (CD62L) and the chemokine receptor type 7 (CCR7) . T_{EM} cells ($CD62L^{lo}CCR7^{lo}$) circulate through inflamed peripheral tissue and display immediate effector functions, whereas T_{CM} cells ($CD62L^{hi}CCR7^{hi}$) home to T cell zones of secondary lymphoid organs and possess greater proliferative potential (Jameson and Masopust, 2009). Memory-like $CD8^+$ T cells can also be generated without encountering foreign antigen, but rather in response to homeostatic signals (Akue et al, 2012). A population of these memory-phenotype (MP) $CD8^+$ T cells, known as innate $CD8^+$ T cells, arises in the thymus in response to certain cytokine signals (Cho et al, 2000).

1.2.1 Innate (T_{IM}) $CD8^+$ T cells

Innate $CD8^+$ T cells typically display phenotypic and functional properties reminiscent of conventional memory $CD8^+$ T cells. While small numbers of these $CD8^+$ T cells normally develop in the thymus, the frequency of different innate $CD8^+$ T cells can be much higher in various strains of mutant mice (Lee et al, 2011). The development of these innate $CD8^+$ T cells is dependent on peptides presented by MHC class Ib molecules expressed on hematopoietic cells rather than MHC class Ia molecules present on TECs. Similarities between innate $CD8^+$ T cells and conventional memory $CD8^+$ T cells include their high levels of CD44 and CD122 expression, plus the ability to quickly produce effector cytokines such as IFNγ following activation. Originally identified in mice lacking the Tec family of non-receptor tyrosine kinases Itk and Rlk, subsequent studies also found that these MP $CD8^+$ T cells were expanded in mice deficient for Kruppel-like factor 2 (KLF2), CREB binding protein (CBP) and Inhibitor of DNA binding 3 (Id3) when compared to wild type (WT) control mice (Lee et al, 2011). Innate $CD8^+$ T cells also express the T-box transcription factor Eomesodermin (Eomes), which is associated with the up-regulation of CD122 and increased responsiveness to IL-15. It was subsequently shown that the expansion of this innate $CD8^+$ T cell population in certain mutant mouse strains was due to an increased number of $PLZF^+$ cells such as NK cells residing in the thymus, which promoted a memory phenotype via a 'by-stander effect' through increased IL-4 production (Raberger et al, 2008). IL-4 promotes the generation of these innate MP $CD8^+$ T cells in the thymus by inducing Eomes expression via the Akt and STAT6 signaling pathways (Carty et al, 2014). These findings also helped explain the variation in the size of innate $CD8^+$ T cell populations in different mouse strains. For example, higher numbers of innate $CD8^+$ T cells in BALB/C compared with C57BL/6 mice are due to three to five times more IL-4-producing $PLZF^+$ cells present in the thymus (Weinrich et al, 2010). This shows that variation in the generation of these innate MP $CD8^+$ T cells occurs naturally in response to changes in cytokine signaling during T cell development. The finding that altered MP $CD8^+$ T cell development in the thymus is controlled in part by cytokine signaling is akin to the

cytokine-dependent generation of antigen-inexperienced, MP $CD8^+$ T cells that arise from conventional naïve $CD8^+$ T cells in the periphery. These cells which typically develop under conditions of natural or induced lymphopenia have been termed lymphopenia induced proliferation (LIP) or homeostatic proliferation (HP) memory $CD8^+$ T cells.

1.2.2 Lymphopenia Induced Proliferation (LIP)/Homeostatic Proliferation (HP) $CD8^+$ T cells

Besides conventional memory development that occurs as a result of antigen exposure, T cells are able to develop a memory-like phenotype in response to homeostatic processes. During conditions of lymphopenia, naïve $CD8^+$ T cells can undergo extensive proliferation in order to fill a vacant or depleted immune compartment. This phenomenon occurs when small numbers of naïve $CD8^+$ T cells are adoptively transferred into a chronically lymphopenic host such as a syngeneic Nude and Rag-1-deficient mice, during acute lymphopenia induced by sub-lethal irradiation (Ichii et al, 2002), or naturally in neonates during the period when the peripheral immune system is being established (Akue et al, 2012). This lymphopenia-induced expansion is accompanied by an up-regulation of the cell surface markers CD44, CD122 and Ly6C, and the ability to rapidly produce effector cytokines such as IFNy upon stimulation (Goldrath et al, 2004). Unlike conventional antigen-primed memory (T_{AM}) $CD8^+$ T cells, LIP/HP $CD8^+$ T cells arise in the absence of overt antigen stimulation and are not dependent on IL-2 or CD28 costimulation (Cho et al, 2000). Instead, these cells arise in response to signals that promote the maintenance of naïve T cells in the periphery, including endogenous peptide/MHC-class-I (pMHC-I) interactions and exposure to IL-7 (Kieper and Jameson, 1999). Originally, it was proposed that the memory phenotype acquired by LIP/HP $CD8^+$ T cells was transient, and upon reestablishment of the T cell compartment, LIP/HP generated MP $CD8^+$ T cells would revert back to a naïve state. This hypothesis offered a thymus-independent mechanism for the restoration of the naïve compartment after a loss of $CD8^+$ T cells (Goldrath et al, 2000). However, the donor-derived naïve $CD8^+$ T cells that are generated in

sub-lethally irradiated recipients receiving naïve OT-I $CD8^+$ T cells ($CD44^{lo}Ly6C^{lo}$) were subsequently shown to be due to small numbers of hematopoietic stem cells contaminating the transferred donor cell population and require recipients to have an intact thymus (Ge et al, 2002). Similarly, naturally occurring MP $CD8^+$ T cells that arise in neonates during lymphopenia-induced expansion retain an activated or memory phenotype (Akue et al, 2012).

1.2.3 Virtual Memory (T_{VM}) $CD8^+$ T cells

Natural lymphopenia, such as that which exist in neonates before the peripheral T cell compartments is established, leads to the generation of $CD8^+$ T cells bearing phenotypic and functional traits of conventional memory cells (Lee et al, 2013). These neonatal MP $CD8^+$ T cells, coined 'virtual memory' (T_{VM}) cells, were shown to emerge in the periphery of mice at 2 weeks of age and gradually accumulate up to 4 weeks of age, after which this population plateaus at between 5-20% of the total $CD8^+$ T cell population (Tian et al, 2007; Akue et al 2012; Lee et al, 2013). This frequency of T_{VM} cells remains fairly constant throughout adult life, but has been shown to increase dramatically to >40% of $CD8^+$ T cells in aged (20 month old) mice (Chiu et al, 2013). The accumulation of MP $CD8^+$ T cells that occurs in aged mice, previously believed to occur as a result of lifelong antigenic stimulation (Nikolich-Zugich, 2008), could be explained by the increase in $CD8^+$ T_{VM} cells that dominate the central memory compartment (>90%) in aged WT mice (Chiu et al, 2013). A similar trend is seen in humans where a decline in the naïve $CD8^+$ T cell compartment is accompanied by a dramatic increase in the number of MP $CD8^+$ T cells (Wertheimer et al, 2014). Whether this increase in MP $CD8^+$ T cells in aging humans occurs as a result of T_{VM} cell accumulation is yet to be determined as human counterparts of murine T_{VM} cells have not been conclusively identified (Nikolich-Zugich, 2014). The finding that the T_{VM} cell population exhibits diverse TCR specificities that are retained during bystander immune responses reinforces the concept that these

cells are generated and maintained by homeostatic processes rather than foreign antigen-driven stimulation (Akue et al, 2012).

Although sharing many characteristic with antigen-induced or 'true memory' (T_{AM}) $CD8^+$ T cells, T_{VM} cells are phenotypically and functionally distinct. Similarities between T_{VM} and T_{AM} cells include similar cell cycle regulation and elevated expression of Eomes and T-bet, T-box transcription factors associated with the acquisition of an activated or MP in $CD8^+$ T cells (Lee et al, 2013). However, although T_{VM} cells up-regulate expression of Eomes and T-bet, both of which are required for cytokine expression by memory $CD8^+$ T cells, T_{VM} cells are much less efficient at rapidly producing IFNy following TCR stimulation than T_{AM} cells (Lee et al, 2013). T_{VM} cells also differ from T_{AM} $CD8^+$ T cells in regards to the expression of CD49d, the alpha subunit of the α4β1 integrin, which serves as a lymphocyte homing receptor (Christensen et al, 1995). CD49d is upregulated on T_{AM} cells following antigen-induced T cell activation, but is expressed at low levels on T_{VM} cells (Haliszczak et al, 2009). The generation of a large number of functional MP $CD8^+$ T_{VM} cells in very young mice with a capacity to rapidly respond to pathogens is thought to represent a stop-gap mechanism that affords neonates protection prior to the establishment of conventional memory networks (Ichii et al, 2002). However, the maintenance of a T_{VM} population throughout adult life indicates that this $CD8^+$ T cell population must also serve other roles.

A number of cytokines have been shown to influence T_{VM} cell homeostasis. IL-7, which is crucial for maintaining naïve T cells numbers under normal non-lymphopenic conditions, is elevated following T cell depletion. This increased availability of IL-7 together with signals generated by self-pMHC-I complexes drives the proliferation and differentiation of naïve $CD8^+$ T cells into MP cells (Kieper et al, 2002). While MP $CD8^+$ T cells employ IL-7 for their survival, unlike naïve $CD8^+$ T cells, they do not require self-pMHC-I interactions for proliferation (Sandau et al, 2007; Surh and Sprent, 2008). Instead, MP $CD8^+$ T cells depend on IL-15 to maintain basal proliferation (Sandau et al, 2007). T_{VM} cells, like all MP $CD8^+$ T cells up

regulate expression of the IL-15 receptor (IL-15R) comprising the IL-15α chain plus CD122 and CD132, the respective IL-2Rβ chain and common γ chain (Colpitts et al, 2012). Consistent with the strong dependence on IL-15, T_{VM} cell numbers are severely reduced in IL-15- and IL-15Rα-deficient mice (Kennedy et al, 2000). In addition to IL-7 and IL-15, other cytokines have either been shown to play a role or implicated in T_{VM} cell generation and maintenance. For example, the size of the T_{VM} population appears to have some dependency on IL-4 based on the finding that the frequency of MP $CD8^+$ cells is reduced in mice lacking the IL-4R (Akue et al, 2012). An expanded population of T_{VM} cells was also observed in the periphery in mice lacking Ndfip1, a regulator of the E3 ubiquitin ligases Nedd4 and Itch, due to an over-production of IL-4 (Kurzweil et al, 2014). More recently, type I interferons (IFNs) have been shown to play direct and indirect roles in the generation and maintenance of MP $CD8^+$ T cells (Martinet et al, 2015). Type I IFN signaling is required for the production and trans-presentation of IL-15 by dendritic cells and inflammatory monocytes to $CD8^+$ T cells (Colpitts et al, 2012). Furthermore, type I IFN signaling may directly regulate Eomes expression in $CD8^+$ T cells, leading to the induction of CD122 and increased responsiveness to IL-15. The induction of Eomes in response to type I IFNs occurs via activation of the ISGF3 complex, consisting of STAT1, STAT2 and IFN regulatory factor (IRF) 9 (Martinet et al, 2015). Aside from the role of Eomes, the transcriptional network responsible for the differentiation of T_{VM} cells is largely unknown. However, previous studies investigating the transcriptional regulators of $CD8^+$ T cell development and function have identified Nuclear Factor of kappa B (NF-κB), a small family of related transcription factors as participants in the development, differentiation and survival of MP $CD8^+$ T cells (Boothby et al, 1997; Voll et al, 2000; Hettmann et al, 2003; Schmidt-Supprian et al, 2004; Dutta et al, 2006; Jimi et al, 2008; Gugasyan et al, 2012).

1.3 NF-kappa B (NF-κB)

NF-κB is a family of transcription factors expressed in almost all cell types and plays crucial roles in mediating various responses to a remarkable diversity of external stimuli. Initially discovered and characterized as a transcription factor required for the expression of the immunoglobulin kappa light chain in B cells, subsequent studies have demonstrated that NF-κB proteins are ubiquitously expressed and serve as critical regulators of the inducible expression of many genes (May and Ghosh, 1998). The NF-κB family of transcription factors functions as homodimers and heterodimers and regulates numerous physiological processes, in particular immune and stress responses. In the immune system, NF-κB signaling pathways have been shown to play crucial roles in inflammation and the survival, differentiation and proliferation of various types of immune cells (Gerondakis and Siebenlist, 2010).

1.3.1 The NF-κB family of transcription factors

The Rel/NF-κB family of transcription factors is comprised of dimers made up of five related proteins that can be sub-divided into two distinct sub-families (Gilmore, 2006). The Rel proteins consist of cRel, RelB and RelA (p65), whereas the NF-κB proteins are represented by NF-κB1 and NF-κB2. All members of the Rel/NF-κB family of proteins contain a highly conserved DNA binding domain called the Rel Homology Domain (RHD) located within the amino-terminal half of each protein (Hayden and Ghosh et al, 2012). The RDH encompasses sub-domains required for site-specific DNA binding, dimerization with other Rel/NF-κB proteins and a nuclear localization signal (NLS). Only Rel proteins contain intrinsic transactivation domains (TAD), which are able to activate transcription. By contrast, the p50 and p52 forms of NF-κB1 and NF-κB2 respectively, are derived from the amino-terminal halves of larger precursors (p105 NF-κB1 and p100 NF-κB2). The mature p50 and p52 forms of NF-κB1 and NF-κB2 are generated by proteolysis of the precursor proteins. Because p50 and p52 lack a TAD, these family members are unable to promote transcription unless they form dimers with members of the Rel sub-

family (Gerondakis and Siebenlist, 2010). While DNA bound p50 and p52 homodimers typically repress gene expression due to the absence of intrinsic TADs, these NF-κB transcription factors can induce transcription when coupled with co-activators such as Bcl-3 (Gilmore, 2006). Due to the broad array of physiological processes mediated by this family of transcription factors to a diverse array of external signals, activation of these proteins is tightly regulated at a cellular level (Oeckinghaus and Ghosh, 2009).

1.3.2 Regulation of NF-κB

The NF-κB pathway operates as two distinct, yet interactive halves, termed the canonical and non-canonical NF-κB pathways. The NFκ-B1, RelA and c-Rel transcription factors mediate canonical NF-κB pathway functions, while NF-κB2 and RelB are responsible for the effector functions of the non-canonical pathway. In most cells, NF-κB proteins exist in an inactive state in the cytoplasm as a result of interaction with Inhibitor of κB (IκB) proteins, a family of structurally related proteins (Oeckinghaus and Ghosh, 2009).These IκBs, either encoded by unique genes (IκBα, IκBß, IκBε) or as precursor proteins, p105 and p100 that also encode the mature NF-κB1 and NF-κB2 proteins respectively, serve to bind to the nuclear localization sequences of the NF-κB transcription factors, preventing their nuclear translocation (Siebenlist et al, 2005). All IκB proteins contain between five and seven ankyrin repeats within their C-terminal, which mediate the interaction with the RHD of Rel/NF-κB proteins, thereby masking the NLS and DNA binding domain (Oeckinghaus and Ghosh, 2009). The canonical and non-canonical pathways are activated in response to a wide variety of signals, including antigen receptors and TNF superfamily members. Upon receiving a stimulatory signal, various signaling pathways downstream of these receptors engage an IκB kinase (IKK) complex comprising IKKα, IKKß and NEMO. When the IKK complex is activated, it phosphorylates IκBs, targeting them for proteasome-mediated degradation. Signals that specifically activate the IKKß kinase target IκBα, IκBß and IκBε bound to

canonical NF-κB complexes, while activated IKKα targets the NF-κB2 precursor protein partnered with RelB, resulting in the degradation of the C-terminal IκB sequence in the p100 NF-κB2 precursor, thereby generating the active RelB/NF-κB2 dimer. These canonical and non-canonical dimers then enter the nucleus and bind to target kappa B (κB) sequences present in the regulatory regions of thousands of genes. The different dimers, which have related yet distinct functions serve to control transcription in both a positive and negative manner via a variety of mechanisms.

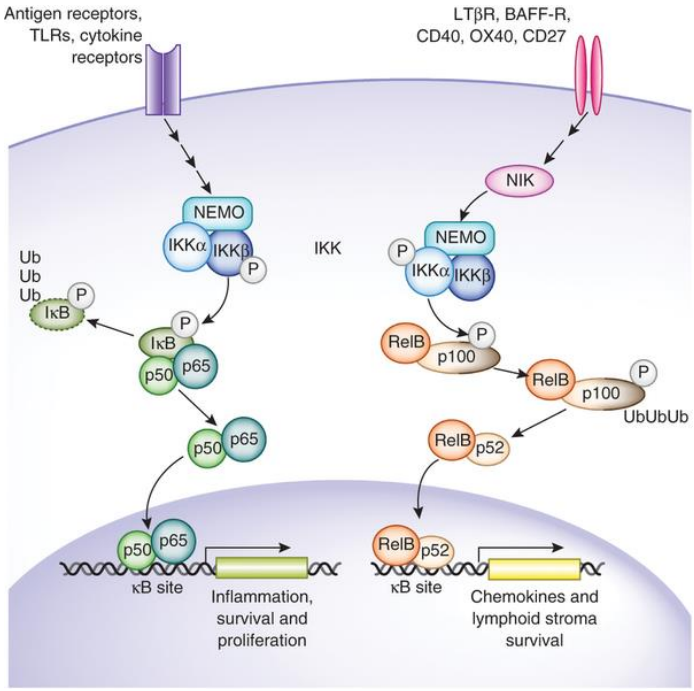


Figure 1.3. Activation of NF-κB transcription factors.In a resting cell, the NF-κB dimer is retained in the cytoplasm by inhibitor of κB, or IκB proteins. Activation by receptor signaling, including antigen receptors, activates the IκB kinase, or IKK complex, which phosphorylates IκB leading to its ubiquitination and degradation, releasing the NF-κB dimer, which translocates to the nucleus to regulate transcription by binding to a consensus sequence or kB site of a variety of genes, including those that control inflammation, survival and proliferation amongst others. (Gerondakis et al, 2014)

1.4 NF-κB in CD8$^+$ T cells

Since its discovery almost 30 years ago, extensive work has been carried out investigating the numerous roles of NF-κB transcription factors in the development and proper function of the immune system. Studies looking specifically at T cells have established that the NF-κB signaling pathway plays key roles in various stages of CD8$^+$ T cell development, differentiation, survival and maintenance.

1.4.1 NF-κB in thymocyte development

NF-κB has been shown to play crucial roles at multiple stages of T cell development (Gerondkis et al, 2014). Firstly, NF-κB provides survival signals to developing T cells during the DN3 stage of thymocyte differentiation. Low levels of nuclear NF-κB during DN1 and DN2 stages of thymocyte development is followed by an up-regulation at the DN3 stage, which is again down-regulated at the DN4 stage with transition to DP thymocytes. Most likely through the activity of p50-RelA heterodimers, activation of the pre-TCR at the DN3 stage promotes, at least in part, NF-κB-mediated survival of developing thymocytes (Aifantis et al, 2001). This finding was supported by subsequent studies using mice that express a dominant-negative variant of IκB-α, which is resistant to phosphorylation and proteolysis (mIκB-α mice). This variant of IκB-α is under control of a T cell-specific transcriptional regulatory element and contains mutations in the phosphorylation sites of this regulatory protein (Ser32 to Ala and Ser36 to Ala). When over expressed in cells, this mutation results in complete inhibition of TCR-mediated nuclear localization and activation of NF-κB in T cells (Boothby et al, 1997). mIκB-α mice display a significant reduction in DN4 stage thymocytes, indicating a role for NF-κB in mediating survival of developing thymocytes from pre-TCR signals.

Downstream of DN thymocyte differentiation, NF-κB controls both positive and negative selection in CD8$^+$ T cells. It appears that NF-κB contributes to both modes of selection in CD8$^+$ T cells by determining the signaling threshold that determines positive versus negative selection (Jimi et al, 2008). Weak to

moderate NF- κB activity in $CD8^+$ thymocytes promotes MHC-I-dependent positive selection by providing survival signals necessary to overcome low levels of the pro-survival factor Bcl2 (Jimi et al, 2008). High NF-κB activity in $CD8^+$ thymocytes however appears necessary to promote negative selection. While this finding is surprising, given the many pro-survival roles NF-κB serves in numerous cell types (Hayden and Ghosh, 2012), there are other examples where NF-κB activity appears to mediate pro-apoptotic responses (Dutta et al, 2006). Aside from roles in T cell development, NF-κB has also been implicated in the generation and maintenance of MP $CD8^+$ T cells (Hettmann et al, 2003; Schmidt-Supprian et al, 2004).

1.4.2 NF-κB in memory-phenotype (MP) $CD8^+$ T cells

The generation of memory cells following an immune response is crucial for providing long-term protection from pathogens (Obar et al, 2011). Although an understanding of the mechanisms of memory $CD8^+$ T cell differentiation have been significantly enhanced in recent years, considerably more remains to be determined about the various roles of different signaling pathways in this process. One of the transcriptional networks shown to be required for memory $CD8^+$ T cell generation and maintenance is NF-κB (Hettmann et al, 2003). mIκB-α mice, as described previously, exhibit a 67-fold reduction in the spleen and a 171-fold reduction in lymph nodes of MP $CD8^+$ T cells (Hettmann et al, 2003). Furthermore, when B6.2.16 mIκB-α double transgenic $CD8^+$ T cells were activated with HY peptide and adoptively transferred into *Rag1*$^{-/-}$ recipient mice, mIκB-α expressing $CD8^+$ T cells failed to expand and displayed a 2-fold decline in number 10 days post-transfer. Comparatively, the number of HY peptide-activated B6.2.16 control $CD8^+$ T cells increased 2-fold by 10 days post-transfer, resulting in a 12-fold difference in cell recovery accredited to impaired expansion and survival of B6.2.16 mIκB-α MP $CD8^+$ T cells. Another study reported a similar finding in mice that lack IKKβ in T cells, where *Ikkβ*$^{-/-}$ naïve $CD8^+$ T cells failed to proliferate when injected into *Rag1*$^{-/-}$ hosts (Schmidt-Supprian et al, 2004). While these studies identify a

critical role for NF-κB in the generation of MP $CD8^+$ T cells from activated $CD8^+$ T cells, it cannot identify the influence of individual NF-κB family member as the mouse strains in these studies contain a defect in the activation of multiple NF-κB transcription factors (Boothby et al, 1997). Therefore, it remains to be determined what role individual NF-κB family members play in the differentiation, proliferation and survival of MP $CD8^+$ T cells.

As a first step towards understanding the roles played by specific NF-κB proteins in memory $CD8^+$ T cells, NF-κB1 has been shown to prevent the development of MP $CD8^+$ T cells in the thymus (Gugasyan et al, 2012). A large increase in a MP $CD8^+$ T cell population was found to develop in the thymus of *Nfkb1*$^{-/-}$ mice. These MP $CD8^+$ T cells, which comprise ~40% of CD8 SP thymocytes in *Nfkb1*$^{-/-}$ mice are able to leave the thymus and contribute to the peripheral $CD8^+$ T cell pool, leading to a marked expansion of the peripheral MP $CD8^+$ T cell population in these mice. The MP $CD8^+$ T cells in the thymus of *Nfkb1*$^{-/-}$ mice differ from other innate $CD8^+$ memory T cells in that their generation is not due to an increased number of $PLZF^+$ T cells that over-produce IL-4 as seen in other mutant mouse strains, does not require IL-15 and is dependent on MHC class Ia, not Ib for antigen presentation (Gugasyan et al, 2012). Instead, radiation chimeras indicate an absence of NF-κB1 in both hemopoietic and non-hemopoietic cells contributes to this enhanced MP $CD8^+$ T cell phenotype. This in part involves reduced negative selection, whereby $CD8^+$ T cells with higher affinity TCRs are not eliminated as efficiently. Recent unpublished results have shown that the large MP $CD8^+$ thymic population in *Nfkb1*$^{-/-}$ mice appears to be very similar to T_{VM} cells (S. Gerondakis, unpublished results). *Nfkb1*$^{-/-}$ mice were also found to promote the enhanced division of T_{VM} $CD8^+$ cells, irrespective of whether these cells express NF-κB1 (S. Gerondakis, unpublished results). In fact, *Nfkb1*$^{-/-}$ MP $CD8^+$ T cells divide less efficiently than their WT counterparts when adoptively transferred into *Nfkb1*$^{-/-}$ hosts. Collectively this indicates that opposing NF-κB1 dependent mechanisms influence the homeostasis of T_{VM} cells via $CD8^+$ T cell intrinsic and extrinsic mechanisms. Many questions remain to be determined about how NF-κB1 regulates the generation and homeostasis of T_{VM} $CD8^+$ T

cells, including identifying the signal(s) that promote T_{VM} cell expansion in *Nfkb1*$^{-/-}$ mice, the types of cells that produce these signals and how $CD8^+$ intrinsic NF-κB1 expression controls T_{VM} cell division.

Aside from NF-κB1, virtually nothing is known about the roles the other canonical and non-canonical NF-κB proteins play in T_{VM} development and homeostasis. Given that mIκB-α transgene expression (Hettmann et al, 2003) and conditional targeting of IKKβ in T cells (Schmidt-Supprian et al, 2004) markedly reduces the MP $CD8^+$ T cell population, which contrasts with the loss of NF-κB1, other NF-κB family members must be required for the generation of MP $CD8^+$ T_{VM} cells. Herein evidence is provided to support the hypothesis that the canonical NF-κB family members NF-κB1 and RelA play opposing regulatory roles in T_{VM} cell generation and maintenance. The loss of NF-κB1 was found to promote T_{VM} cell expansion, in part via cell-extrinsic mechanisms involving the suppression of inflammation. In contrast, we show that the loss of RelA leads to impaired maintenance of the T_{VM} cell population, inferring a crucial role for RelA in the survival and/or expansion of T_{VM} cells, possibly by regulating homeostatic responses mediated via γc cytokine signals.

* Further review of the literature, pertaining to the findings described in each of the results chapters, is provided in the introduction section of each chapter.

Chapter 2:

Methods

2.1 Reagents

2.1.1 Chemicals and buffers

Buffers for cell culture were obtained from Gibco Invitrogen, unless otherwise stated. H_2O refers to reverse-osmosis (RO) treated water, unless otherwise stated. Double Distilled (dd) and MilliQ water were used for most applications. Ultra-pure H2O (#10977-023) is DNAse and RNAse free and sterile filtered through a 0.1µm membrane.

Acidified MilliQ H2O

MilliQ H2O was adjusted to pH 2.5 with concentrated (~6M) HCl (1.00317; Merck), using a Thermo Orin Star (A211) pH meter.

1000x β-mercaptoethanol (β-ME) for cell culture

A 1000x stock (50mM) of β-ME was made by diluting 14M β-ME (M7522; Sigma-Aldrich) in DMEM. 100µL aliquots were stored at -20°C.

Complete Advanced RPMI Tissue Culture Medium

Advanced RPMI medium (12633-012; Invitrogen) was supplemented with 10% (v/v) heat inactivated FCS, 2mM Glutamax (#35050-061), 100U/mL penicillin and streptomycin (#15140-122), 50µM β-ME and 50mM HEPES (#15630-080).

Complete DMEM Tissue Culture Medium

1x DMEM containing GlutaMAX (10569-044) was supplemented with 10% (v/v) heat inactivated FCS and 100U/mL penicillin and streptomycin (15140-122).

Dithiothreitol (DTT)

A 100mM stock of DTT was prepared by dissolving 154.25mg of DTT (D5545; SigmaAldrich) in 10mL ultra-pure H2O. Aliquots were stored at -20°C.

FACS Buffer

FACS buffer consisted of 1mL heat inactivated FCS added to 49mL sterile 1x HBSS.

1x Foxp3 Fix/Permeabilisation Solution (00-5523; eBioscience) for FACS

1x Foxp3 Fix/Permeabilisation solution was made by diluting 1 part Foxp3 Fix/Permeabilisation Solution with 3 parts Foxp3 Fix/Permeabilisation Diluent as per manufacturer's instructions.

1x Hank's Balanced Salt Solution (HBSS)

1x HBSS was made by diluting 1 part sterile 10x HBSS pH 7.4 (#14065-056) with 9 parts autoclaved MilliQ H2O.

Heat Inactivated Foetal Calf Serum

Foetal Calf Serum (26140-079; Gibco) was heat inactivated at 56°C for 30 min, then divided into 50mL aliquots under sterile conditions and stored at 4°C.

HSC Cryopreservation Solution

The cryopreservation solution for haematopoietic stem cells consisted of 90% (v/v) heat inactivated FSC and 10% (v/v) DMSO.

Hypotonic Lysis Buffer for EMSA

10mM HEPES pH 7.9 (11344-041; Gibco), 10mM KCl, 0.1mM EDTA and 0.1mM EGTA were diluted in ddH2O. 0.5mM DTT, 0.5mM PMSF, 4pg/mL aprotinin and 4pg/mL leupeptin were added directly before use.

Leupeptin

A 2mg/mL stock solution of Leupeptin was prepared by dissolving 5mg Leupeptin (Cat. #11 017101 001; Roche) in 2.5mL ultra-pure H2O. Stocks were stored at -20°C.

Neomycin Drinking Water

A 100x stock of Neomycin was prepared by dissolving 16.7g of Neomycin trisulphate (N1876-100G; Sigma-Aldrich) in 100mL of autoclaved MilliQ water. This was stored at 4°C in a light protected container. 10mL of 100x Neomycin stock was then diluted in 990mL of acidified MilliQ water and provided to mice in light excluding drinking bottles.

5x NF-κB Binding Buffer

250µL of 2M Tris-Cl pH7.5, 1mL of 5M NaCl, 100µL of 0.5M EDTA pH8.0, 2.5mL of Glycerol and 500µL of 10% NP-40 were made up to 10mL with ddH2O and stored at room temperature. 50µL of 0.1M DTT was added to 1mL binding buffer directly before use.

Onyx Buffer

2x Onyx Buffer was made with 40mM of Tris-Cl pH7.4, 270mM of NaCl, 3mM of MgCl2, 2mM of EGTA, 2% (v/v) Triton X-100 and 20% (v/v) glycerol, and stored at 4°C. 1x Onyx Buffer was made fresh up to 1mL with 500µL 2x Onyx Buffer and 5µL 2mg/mL Aprotinin, 5µL 2mg/mL Leupeptin, 5µL 100mM PMSF, 5µL 100mM DTT added directly before use.

1x Permeabilisation Buffer (00-5523; eBioscience) for FACS

1 part Permeabilisation buffer was diluted with 9 parts ddH2O as per manufacturer's instructions.

Protein Extraction Buffer for EMSA

420mM NaCl, 20mM HEPES pH 7.9 (#11344041), 1.5mM MgCl2, 0.2mM EDTA and 25% glycerol were dissolved in ddH2O. 0.5mM DTT, 0.5mM PMSF, 4pg/mL aprotinin and 4pg/mL leupeptin were added directly before use.

Running Buffer for Western blots

Running buffer was made by diluting 50mL of 20x MOPS Running Buffer (NP0001) with 950mL ddH2O, as per manufacturer's instructions and inverted to mix.

Transfer Buffer for Western blots

Transfer buffer was made by diluting 50mL of 20x Transfer Buffer (NP0006) with 850mL ddH2O and 100mL of methanol (1.06018; Merck), as per manufacturer's instructions and inverted to mix

1x Tris/Acetic Acid/EDTA (TAE) for Agarose Gels

A 1x solution of TAE was prepared by diluting 200mL of 50x TAE (#1610743; BioRad) with 10L H2O, as per manufacturer's instructions and inverting to mix.

0.5x Tris/Boric Acid/EDTA (TBE) for EMSA

A 0.5x TBE solution was made by diluting 100mL of 5x Novex TBE (LC6675; Invitrogen) with 900mL ddH2O, and inverting to mix.

2.1.2 Antibodies

Table 2.1. Antibodies used for flow cytometry

Antigen	Fluorophore	Clone	Company	Catalogue No.	Dilution
CD103	APC	2E7	eBioscience	17-1031-82	1:200
CD103	PerCP-Cy5.5	2E7	BioLegend	121415	1:50
CD122	FITC	TM-β1	BioLegend	123207	1:400
CD127	BV421	A7R34	BioLegend	135027	1:400
CD132	PE	TUGm2	BioLegend	132305	1:400
CD215	PerCP-eFluor710	DNT15Ra	eBiosciences	46-7149-80	1:200
CD24	BV421	M1/69	BioLegend	101825	1:200
CD25	APC-Cy7	PC61.5	BD Pharmingen	561038	1:200
CD3ε	PE-Cy7	145-2C11	eBioscience	25-0031-81	1:400
CD4	PE-Cy7	RM4-5	BD Pharmingen	552775	1:400
CD4	BV650	RM4-5	BD Biosciences	563747	1:400
CD44	PE	IM7	BD Pharmingen	561860	1:400
CD44	Alexa700	IM7	BD Pharmingen	560567	1:400
CD45.1	Alexa700	A20	BD Biosciences	561235	1:250
CD45.2	Alexa488	104	BioLegend	109816	1:400
CD45.2	PerCP-Cy5.5	104	BD Pharmingen	552950	1:100
CD49d	Alexa647	R1-2	BioLegend	103614	1:400
CD5	APC	53-7.3	eBioscience	17-0051-82	1:400
CD5	FITC	53-7.3	BioLegend	100605	1:400
CD62L	APC	MEL-14	BD Biosciences	553152	1:400
CD62L	PE-CF594	MEL-14	BD Pharmingen	562404	1:400
CD69	PE	H1.2F3	BD Pharmingen	562455	1:400
CD69	PerCP-Cy5.5	H1.2F3	BD Pharmingen	561931	1:100
CD8α	Pacific Blue	53-6.7	BD Pharmingen	558106	1:200

CD8α	BV650	53-6.7	BioLegend	100742	1:400
CTLA-4	APC	UC10-4B9	eBioscience	17-1522-80	1:400
Eomes	eFluor 660	WD1928	Invitrogen	50-4877-42	1:200
IFN-γ	FITC	XMG1.2	BD Pharmingen	554411	1:200
Ki-67	PE	B56	BD Pharmingen	556027	1:20
Live/Dead	Aqua	-	Invitrogen	L34957	1:200
NK1.1	PE-Cy7	PK136	eBioscience	25-5941-82	1:400
NKG2D	BV421	9C11G4	BD Bioscience	744866	1:200
PD-1	PE	J43	BD Pharmingen	561788	1:100
p-STAT5 (Tyr694)	Not conjugated	D47E7	Cell Signalling	4322S	1:200
Rabbit IgG	Alexa488	-	Cell Signalling	4412	1:1000
TNF-α	PE	MP6-XT22	BioLegend	506306	1:200

Table 2.2. Antibodies used for Western blotting

Antigen	Fluorophore	Clone	Company	Catalogue No.
Rabbit anti-mouse RelA	Not conjugated	C22B4	Cell Signalling	4764S
Rabbit anti-mouse GAPDH	Not conjugated	D16H11	Cell Signalling	5174P
Goat anti-rabbit IgG (H+L)	Horseradish Peroxidase (HRP)	-	Southern Biotech	4050-05

Table 2.3. Antibodies used for Electrophoretic Mobility Shift Assay (EMSA) super shifts

Antibody	Company	Catalogue No.
Goat anti-human RelA	Santa Cruz	SC-109 XG
Rabbit anti-mouse cRel	Santa Cruz	SC-71 X
Rabbit anti-mouse NF-κB1	Santa Cruz	SC-1114 X
Mouse anti-human NF-κB2	Santa Cruz	SC-7386 X

2.2 Mice

All mouse studies were performed with approval from the relevant Animal Ethics Committees at Monash University. All mice were bred and housed in Monash Animal Research Platform (MARP) facilities on the AMREP or Clayton campuses. All experimental strains have been backcrossed on an inbred C57BL/6 background that expresses the *Ptprcb* (CD45.2) allele. Congenic B6.SJL-*Ptprca Pepcb*/BoyJ (CD45.1) mice, as well as B6.129S7-*Rag1tm1Mom*/J (*Rag1*$^{-/-}$) mice, also on a C57BL/6 background were obtained from the WEHI animal facility at Kew (Melbourne, Australia). All adult mice were humanely euthanized by carbon dioxide asphyxiation, or cervical dislocation.

2.2.1 Nfkb1$^{-/-}$ *mice*

Nfkb1$^{+/-}$ mice that are heterozygous for the loss of NF-κB1 were mated to generate litter-matched *Nfkb1*$^{+/+}$ and *Nfkb1*$^{-/-}$ mice.

2.2.2 Nur77^{GFP} *reporter mice*

Nur77^{GFP} reporter mice (Purchased from The Jackson Laboratory) express a transgene encoding green fluorescent protein (GFP) under the transcriptional control of the *Nr4a1* (Nur77) locus (Moran et al, 2011).

2.2.3 Nfkb1$^{+/-}$*Asc*$^{-/-}$ mice

Nfkb1$^{+/-}$*Asc*$^{-/-}$ mice were generated by introducing a null allele for ASC onto the *Nfkb1*$^{-/-}$ genetic background. *Nfkb1*$^{+/-}$*Asc*$^{-/-}$ mice that lack ASC and are heterozygous for the loss of NF-κB1 were mated to generate litter-matched *Nfkb1*$^{+/+}$*Asc*$^{-/-}$ and *Nfkb1*$^{-/-}$*Asc*$^{-/-}$ mice.

2.2.4 Rela$^{+/-}$ *mice*

Rela$^{+/-}$ (p65B6 strain) mice are heterozygous for a null allele of *Rela* (Beg *et al.*, 1995) (kindly provided by Professor David Baltimore). Due to embryonic lethality of *Rela*$^{-/-}$ mice at embryonic day (E)14.5, timed matings were performed with *Rela*$^{+/-}$ male and female mice to generate *Rela*$^{+/+}$, *Rela*$^{+/-}$ and *Rela*$^{-/-}$ E13-13.5 embryos.

2.2.5 Timed mating

The death of *Rela*$^{-/-}$ mice at E14.5 due to liver toxicity precludes studying the immune system in these mice (Beg *et al.*, 1995). To overcome this limitation, *Rela*$^{+/-}$ male and female mice were time mated so

that Haematopoietic Stem Cells (HSCs) in the foetal liver of E13-13.5 *Rela*$^{-/-}$ embryos could be used to reconstitute the immune system of lethally irradiated mice. Male and female *Rela*$^{+/-}$ mice were time mated such that gestation could be accurately timed. Pregnant females were sacrificed at E13-13.5, the embryos were killed by decapitation and single cell suspensions from foetal livers resuspended in HSC cryopreservation buffer. Foetal liver suspensions were first chilled on ice for 20 min, then placed at -80°C overnight, before being placed long-term in vapour-phase liquid nitrogen storage (MVE). Connective tissue from each embryo was digested and used as a source of DNA for genotyping (2.4.1).

2.2.6 Haematopoietic stem cell transplants

Frozen vials of *Rela*$^{+/+}$ and *Rela*$^{-/-}$ E13-13.5 foetal liver suspensions were thawed quickly at 37°C and immediately washed twice in 9mL neat DMEM to remove DMSO. Thawed foetal liver cells resuspended in 220µL DMEM or 1x mtPBS were then injected into the caudal tail vein of lethally irradiated congenic CD45.1 mice, which had received two doses of 5.5Gy separated by three hours (Gammacell 1000 Elite or Gammacell 40 Exactor irradiators). Irradiated mice were maintained on acidified MilliQ H2O containing 1.67mg/mL neomycin in darkened bottles for six weeks following HSC transplantation to prevent opportunistic infection.

2.2.7 Lck^{cre}*Rela*$^{fl/fl}$ *mice*

Mice heterozygous for a floxed allele of *Rela* (*Rela*$^{fl/wt}$) were intercrossed with the *Lck*^{cre} deleter strain that expresses a Cre recombinase transgene under the transcriptional control of the T cell specific *Lck* proximal promoter (Algul et al, 2007). *Lck*^{cre} is expressed early in thymocyte development, thereby ensuring the efficient targeting of genes in all conventional T-lineage cells (Bolen et al, 1991). In all experiments conducted using *Lck*^{cre} targeting of floxed RelA, *Lck*^{cre}*Rela*$^{wt/fl}$ mice were mated to generate litter-matched mice homozygous for the wild-type (*Lck*^{cre}*Rela*$^{wt/wt}$) or floxed (*Lck*^{cre}*Rela*$^{fl/fl}$) *Rela* gene

2.2.8 Lck^{cre}*Rela*$^{fl/fl}$*Bcl2-Tg*$^{+/-}$ *mice*

Lck^{cre}*Rela*$^{wt/fl}$*Bcl2-Tg*$^{+/-}$ mice were generated by crossing *Lck*^{cre}*Rela*$^{fl/fl}$ mice with the vav-bcl-2 strain that expresses high levels of a human *Bcl2* transgene under the transcriptional control of the pan hematopoietic cell restricted mouse *vav2* promoter (Ogilvy et al, 1998; 1999). In all studies using an introduced Bcl-2 transgene, *Lck*^{cre}*Rela*$^{wt/fl}$*Bcl2-Tg*$^{+/-}$ and *Lck*^{cre}*Rela*$^{wt/fl}$ mice were mated to generate litter-matched mice heterozygous for the *Bcl2* transgene (*Bcl2-Tg*$^{+/-}$) and homozygous for the WT (*Rela*$^{wt/wt}$) or floxed (*Rela*$^{fl/fl}$) *Rela* gene.

2.3 Isolation of cells

2.3.1 Isolation of Lymphocytes

Spleen, pooled peripheral lymph nodes (pLNs) and bone marrow (BM) samples isolated from mice following humane euthanasia, were placed in DMEM media or FACS buffer on ice. Secondary lymphoid organs (spleen and pLNs) were mechanically disrupted by gently teasing with the frosted ends of microscope slides (#7107-PPN; Livingstone); femur and tibia were flushed with FACS buffer using a 25 gauge (G) needle to collect BM sample. Lymphocytes from the liver were isolated by mechanical disruption by gently teasing with the frosted ends of microscope slides (#7107-PPN; Livingstone) and pushed through a 70µm filter, followed by density gradient enrichment. Spleen and liver samples were specifically depleted of red blood cells (RBCs) using hypotonic lysis by resuspending cells in RBC Lysing Buffer (R7757 ; SigmaAldrich) for 5 min at room temperature. Single cell suspensions from lymphoid organs were then passed through a pre-moistened 70µm strainer (#352350; Falcon) to remove tissue aggregates and the flow through cells washed with FACS buffer.

2.3.2 Cell counting

Cell numbers were determined manually by counting cell suspensions stained with 0.4% Trypan Blue (T8154; SigmaAldrich) using a Neubauer Improved Bright Line Haemocytometer (La Fontaine). Viability was assessed by Trypan Blue exclusion. Cells were resuspended at a concentration of 1x108 cells per mL for antibody stains unless otherwise stated.

2.3.3 Enrichment of $CD8^+$ T cells using eBioscience Mouse CD8 T cell MagniSort kit (#8804-6822-74)

Single suspensions of splenocytes and lymph node cells in FACS buffer were incubated with 200µL of MagniSort Mouse CD8 T cell Enrichment Antibody Cocktail (#8804-6822-74; eBioscience) per mL of cells for 10 min at room temperature. Cells were then washed with 2 to 3 volumes of FACS buffer, centrifuged at 1500rpm for 5 min at 4°C and resuspended in FACS buffer. Cells were next incubated with 200µL/mL of MagniSort Negative Selection Beads for 5 min at room temperature and made up to a final volume of 2.5mL with FACS buffer. Cells were then placed in either a MagniSort magnet (MAG-4902; eBioscience) or EasySep magnet (#18000; StemCell Tech) and incubated for 5 min at room temperature. The supernatant, which is enriched for $CD8^+$ T cells, was decanted into a 15mL tube, while the beads retained by the magnet were washed with 2.5mL FACS buffer, to collect any additional unbound cells.

2.3.4 Fluorescence Activated Cell Sorting (FACS)

FACS was used to isolate populations of cells to a high degree of purity for use in a number of experiments. Single cell suspensions in FACS buffer of magnetic bead enriched $CD8^+$ T cells (2.3.3) were stained with pre-titrated fluorescently labelled monoclonal antibodies identify viable $CD8^+$ T cell subsets. Live/Dead Aqua (L34957; Invitrogen) was used to exclude dead cells, while anti-CD8α BV650 conjugated antibodies (Clone: 53-6.7; #100742; BioLegend) were used to identify $CD8^+$ T cells and anti-CD44 PE (Clone: PC61.5; #561038; BD Pharmingen) and anti-CD49d AF647 (Clone:R1-2 ; #103614 ; BioLegend) were used to identify distinct $CD8^+$ T cell subsets (naïve: $CD44^{lo}CD49d^{int}$; T_{VM}:$CD44^{hi}CD49d^{lo}$; T_{AM}: $CD44^{hi}CD49d^{hi}$). Labelled cells were resuspended at a concentration of 1-2x10^7 cells per mL, filtered through a 35µm nylon filter (#352235; Falcon) into polypropylene FACS tubes (#352063; Falcon) and run on BD FACSAria or BD Influx FACS machines using a 70µm nozzle. Viable $CD8^+$ T cell subsets were sorted into 15mL tubes or 5mL FACS tubes containing at least 1mL of RPMI/20% FCS. Purity of the T_{VM} cell populations was assessed following each sort and found to be >95% for all experiments (Fig.2.1).

2.4 Molecular techniques

2.4.1 Tail digests

To isolate genomic DNA for genotyping, a 2-3mm sample from the tip of the tail was taken from mice at neonatal day 10-14 by staff at Monash Animal Services. These tail samples, or connective tissue from embryos when foetal liver HSC transplants were performed (*2.2.6*), were digested overnight in 100µL or 200µL of Digest Buffer (#102-T: Viagen) containing 8mg/mL Proteinase K (03 115852 001; Roche) respectively. The following day the Proteinase K was inactivated by heat killing at 85°C for 1 hr, and samples stored at 4°C.

2.4.2 Genotyping

DNA isolated from mouse tail samples or embryos were amplified by Polymerase Chain Reaction (PCR), using primers that would specifically identify wild-type and mutant alleles for the genes concerned. Briefly, PCR reactions were made up to a final volume of 20µL with 10µL of 2x GoTaq Green (M7123; Promega), forward and reverse primers at the correct concentrations, ultra pure H_20 and 2µL of the DNA sample. PCR amplification reactions were performed using an Applied Biosystems Thermal Cycler 2720 with appropriate cycle conditions.

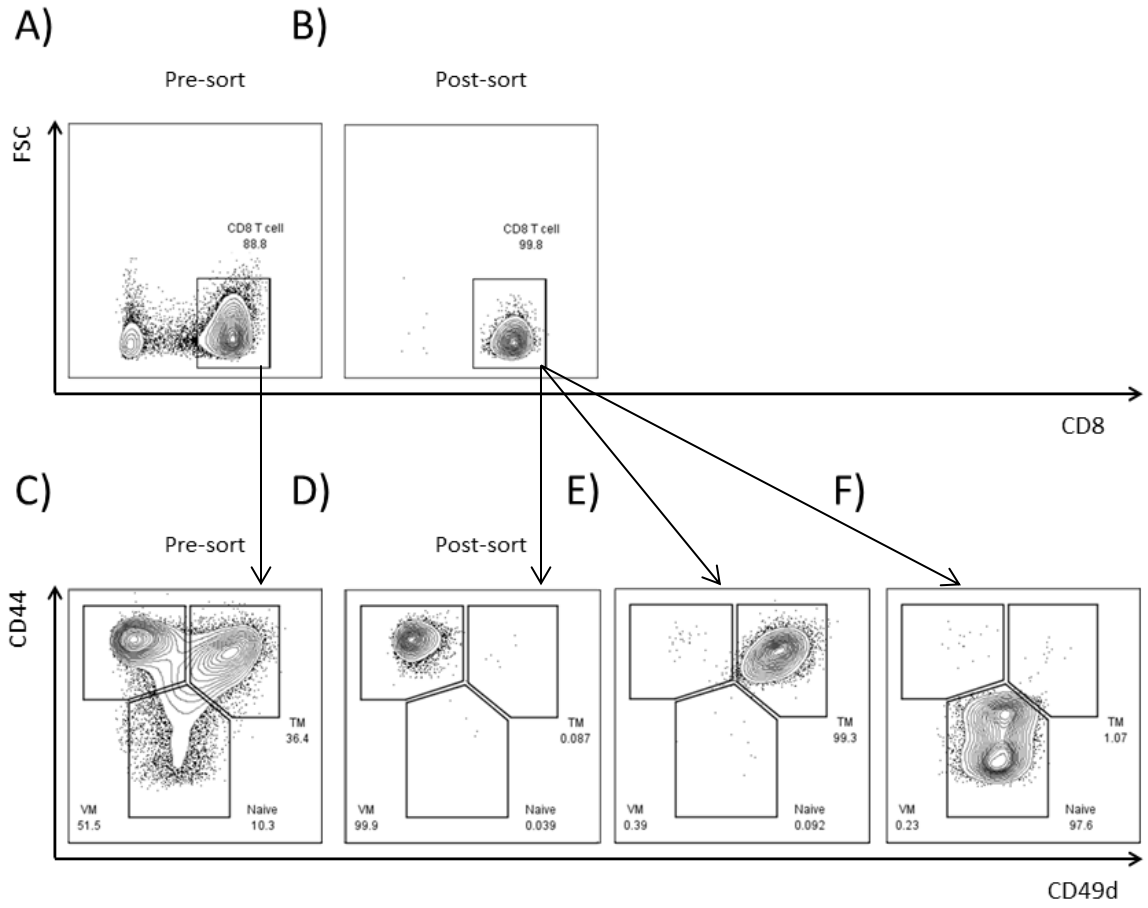


Figure 2.1 Representative flow cytometry plots showing purity of FACS sorted CD8$^+$ T cells. Enriched CD8$^+$ T cells were sorted using FACS utilizing anti-CD8α BV650 conjugated antibodies (Clone: 53-6.7; #100742; BioLegend) to identify CD8$^+$ T cells and anti-CD44 PE (Clone: PC61.5; #561038; BD Pharmingen) and anti-CD49d AF647 (Clone:R1-2 ; #103614 ; BioLegend) to identify distinct CD8$^+$ T cell subsets (**A**) Analysis of CD8$^+$ T cell purity in pre-sort sample. (**B**) Analysis of CD8$^+$ T cell purity in post-sort sample. (**C**) Analysis of proportions of distinct CD8$^+$ T cell subsets in pre-sort sample. (**D**) Analysis of VM cell purity in post sort sample. (**E**) Analysis of TM cell purity in post-sort sample. (**F**) Analysis of naïve CD8$^+$ T cell purity in post sort sample. (naïve: CD44^{lo}CD49d^{int}; VM; Virtual Memory:CD44^{hi}CD49d^{lo}; TM; True Memory: CD44^{hi}CD49d^{hi}).

2.4.3 Gel electrophoresis

The PCR amplified products were separated according to molecular weight by agarose gel electrophoresis. Samples were loaded on a 1.8-2% agarose gels containing ethidium bromide at a concentration of 0.5µg/mL. Electrophoresis was undertaken using 1x TAE buffered gels. The gels were illuminated under UV light and photographed in a Cambridge UVITEC Gel Dock, and printed using a Mitsubishi P95DW thermal printer.

2.4.4 RNA extraction

All pipetting was performed with RNase-free filtered tips. T_{VM} cells were isolated by FACS from 12-week-old *Lck*^{cre}*Rela*$^{wt/wt}$ and *Lck*^{cre}*Rela*$^{fl/fl}$ mice, washed in 1x mtPBS and snap frozen in 500µL Trizol (#15596-026; Invitrogen). Samples were thawed at 37°C for 5min and homogenised by repeated passage (7-8 times) through a 20G needle. Samples were then vortexed briefly and left at room temperature for 5 mins. 200µL of chloroform (C2432-25ML; SigmaAldrich) was added to samples, which were then mixed thoroughly to by vigorous inversion for 30 sec and left at room temperature for 3 mins. Samples were centrifuged at 12,000 xg for 15 min at 4°C and the aqueous phase collected into an RNAsefree microcentrifuge tube. 1x volume (~270µL) of 70% (v/v) ethanol (E7023-500ML; SigmaAldrich) was added to samples that were then vortexed for 1 min, then transferred to RNeasy columns (74104; QIAgen) and incubated for 1 min at room temperature. Samples were centrifuged at 8,000 xg for 15 sec and 350µL of RW1 buffer added. The centrifugation step was repeated and the column flow through discarded. 500µL of RPE buffer (200µL RPE concentrate, 800µL Ethanol) was added to samples, which were then centrifuged at 8,000 xg for 15 sec. Another 500µL of RPE buffer was added and centrifugation step repeated, this time for 2 min. Excess liquid was removed from columns, which were then placed in new microcentrifuge tubes, at which point 14µL of RNAse free water added was directly to column and incubated for 1 min at room temperature. Columns were then centrifuged for 5 min at 15,500 xg to elute the RNA.

2.4.5 RNA sequencing (RNAseq) analysis

RNA samples were shipped on dry ice to the Micromon Genomics facility located in the Biomedical precinct at Monash University for whole genome RNA profiling.

2.5 Cellular and protein analysis

2.5.1 Flow cytometry studies

2.5.1.1 Staining cells using antibodies for cell surface markers

Single cell suspensions (5-10x10^6 cells) were aliquoted into 96-well round bottom plates (CLS3799; Costar Corning) and pelleted by centrifugation in a Hereaus Megafuge 2.0R centrifuge at 1,500rpm for 5 mins at 4°C. Stains were performed in 50-100µL of FACS buffer for 30 min on ice with light excluded, using pre-titrated fluorescently labelled monoclonal antibodies. To identify different populations of $CD8^+$ T cells, Live/Dead Aqua, plus anti-CD8α, anti-CD4, anti-CD44 and anti-CD49d antibodies were used in combination with antibodies specific for cell surface markers that help to further characterize naïve and MP $CD8^+$ T cells. Stained cells were washed twice with 100-150µLFACS buffer. After staining and washing, cells were resuspended in FACS buffer and filtered through a pre-washed 35µm nylon strainer into a 5mL polystyrene FACS tubes (#352235; Falcon) for analysis on flow cytometer.

2.5.1.2 Intracellular staining of Eomes, phospho STAT-5 and Ki-67

Antibody stains for the transcription factors Eomes and STAT-5, plus the nuclear localized protein Ki-67, require fixation and permeablisation of cells to allow fluorescently labelled antibodies to access these intracellular antigens. After cell surface stains were complete, intracellular staining was performed using 5-10x10^6 cells resuspended in 200µL 1x Foxp3 Fix/Permeabilisation solution (Foxp3 Staining Kit (#00-5523; eBioscience)) and incubated overnight at 4°C protected from light. Samples were centrifuged, washed twice in 200µL of 1x Permeabilisation buffer and resuspended in 100µL 1x Permeabilisation buffer with titrated fluorescently labelled antibody for 30 min at room temperature. Samples were then washed twice with 150µL of 1x Permeabilisation buffer and once with FACS buffer, resuspended at a concentration of 1-2x10^7 cells per mL in FACS buffer and filtered through a pre-moistened 35µm nylon strainer into a 5mL polystyrene FACS tube (#352235; Falcon) for analysis.

2.5.1.3 Flow cytometry analysis

An analysis of cells stained with up to 12 different fluorophores was conducted on BD LDRII and BD LSR Fortessa X-20 flow cytometers at Monash FlowCore using BD FACSDiva software. In addition to the experimental samples, a set of compensation controls were analysed prior to every experiment. Compensation controls consisted of an unstained sample and single stained samples for all the fluorophores used in an experiment. Single stained controls were made using fluorescently labelled

antibodies that gave a signal as bright or brighter than the respective signal from the experimental samples. Final compensation was applied in FlowJo software (Tree Star Inc.). Samples were run through the cytometer at a rate of ~9,000 events per second.

2.5.1.4 General gating and analysis strategies

A general strategy was employed for analysing all flow cytometry data for live and fixed samples in FlowJo. A leukocyte gate was applied to remove small debris, followed by Forward Scatter Width (FSC-W) versus (vs.) Forward Scatter Height (FSC-H) and Side Scatter Width (SSC-W) vs. Side Scatter Height (SSC-H) gates to exclude doublets. Dead cells were excluded by gating for Live/Dead Aqua negative cells. In the spleen and pLNs, a CD4 vs. CD8 gate was used to identify $CD8^+$ T cells, after which a CD44 vs. CD49d gate was employed to identify naïve, T_{VM} and T_{AM} $CD8^+$ T cell subsets. A CD45.1 vs. CD45.2 gate in HSC chimeras used to distinguish host and donor cells prior to identification of T cell populations.

2.5.2 Western blotting

T_{VM} cells were isolated from *Lck*^{cre}*Rela*$^{wt/wt}$ and *Lck*^{cre}*Rela*$^{fl/fl}$ mice by FACS and washed in 1x mtPBS to remove FCS proteins present in collection buffer. Samples (4x105 cells per 10µL volume) were lysed in 1x Onyx buffer containing protease inhibitors for 1 hr on ice, after which supernatants containing the proteins of interest were separated from cell debris by centrifugation (10,000 xg for 5 min at 4°C). 10µL samples of cell lysate were mixed with 3.85µL of 4x NuPage Sample Buffer (NP0007; Invitrogen) supplemented with 10% β-ME, vortexed and denatured at 95°C for 10 min in Eppendorf Safe-Lock microcentrifuge tubes (0030 120.086). Samples were then loaded onto a Novex Bis-Tris 10% 10-well gel (NP0301BOX; Invitrogen) along with 10µL SeeBlue2Plus Protein marker (LC5925; Invitrogen) and the samples fractionated for ~1.5hr at 150V in 1x Running Buffer, using a Novex XCell SureLock Mini-Cell electrophoresis tank. After the gel had been run, the fractionated protein samples were transferred onto Hyperfilm-C nitrocellulose membranes (RPN303E; Amersham) by electrophoresis (30V for 1 hr) in 1x Transfer buffer. After the protein transfer was complete, membranes were first incubated in 10mL of Blocking Solution overnight at 4°C, then incubated with antip65 (clone C22B4, #4764; Cell Signalling) or anti-GAPDH (clone D16H11, #5174; Cell Signalling) antibodies in 5mL Blocking Solution for 1.5-2hr at room temperature. Membranes were then washed five times with Blocking Solution and incubated with Goat anti-Rabbit IgG Horseradish peroxidase (HRP) conjugated secondary antibody (#4050-05; SouthernBiotech) for 1 hr. Membranes were washed a further five times with Blocking Solution and twice with 1x mtPBS, then 2.5mL each of ECL Western Blotting Detection Reagents 1 and 2 (W94883333;

Amersham) were mixed and incubated on the membranes for 1 min. Amersham Hyperfilm ECL (28906837) exposed to the membranes in dark room was developed using Agfa Developer Solution (G153) and Agfa Fixative (G354).

2.5.3 Electrophoretic Mobility Shift Assay (EMSA)

Nuclear extracts were prepared from FACS sorted T_{VM} cell populations as described (Grumont and Gerondakis, 1994). Approximately 10^6 cells were harvested, washed in 1x mtPBS, then gently resuspended in 200µL of Hypotonic lysis buffer and allowed to swell on ice for 15 mm. After the addition of 25µL of 10% NP4O, samples were vortexed for 10 sec and then centrifuged at 12,000 for 30 sec. The resultant supernatant, containing the cytoplasmic fraction, was snap frozen on dry ice and stored at -80°C. 5µL of Protein Extraction buffer was then added to the pellet; the sample placed on ice and agitated every 5 min for a period of 20 mins. The sample extract was centrifuged at 12,500rpm and the supernatant containing the nuclear fraction snap frozen and stored at - 80°C. 1µL from each sample was diluted with 3µL of 5x NF-κB binding buffer, 1µL of 1mg/mL poly dI:dC, 1µL 100µg/mL BSA (#9998S; Cell Signalling), 1µL of radiolabeled κB3 probe (Grumont and Gerondakis, 1994), made up to 15µL with ddH2O and then incubated for 30 min at room temperature. Samples were then loaded on to a 6% non-denaturing polyacrylamide gel (EC6365BOX; Invitrogen) using 3µL of 5x Ficoll loading buffer. Samples electrophoresed at 100V for 90 min in 0.5x TBE running buffer, and then the gel was transferred onto 3mm Whatman filter paper and dried under vacuum at 85°C for ~90 min (Model 543; BioRad). The dried gel was exposed to Kodak Carestream Biomax MS film (#829 4985) typically between 24 hr and 48 hr at 80°C and developed using Agfa Developer Solution (G153) and Fixative (G354). Antibody supershifts were performed by pre-incubating individual samples with antibodies specific for particular canonical NF-κB proteins (Table 2.3) for 30 min at room temperature, before adding the radiolabelled κB3 probe as outlined above.

2.6 Cell Culture

All sterile Falcon cell culture plasticware was obtained from BD and Corning.

2.6.1 Cytokine stimulation assays

T_{VM} cells were purified from the spleens and lymph nodes of $Lck^{cre}Rela^{wt/wt}$, $Lck^{cre}Rela^{fl/fl}$, $Lck^{cre}Rela^{wt/wt}$ $Bcl2\text{-}Tg^{+/-}$ and $Lck^{cre}Rela^{fl/fl}Bcl2\text{-}Tg^{+/-}$ mice by FACS. Cells were then stained in 2.5µM Cell Trace Violet (CTV) (C34557; Invitrogen) for 10 min at 37°C, washed with 5x volumes complete advanced RPMI and

resuspended at a concentration of $1x10^6$ cells per mL. Aliquots of cells ($1x10^5$ cells in 100µL) were placed into a 96-well flat bottom tissue culture plate (#355072; Falcon). 100µL of 2ng/mL recombinant mouse IL-2 (14-8021-64; eBiosciences), recombinant mouse IL-7 (14-8071-62; eBiosciences), recombinant mouse IL-15 (14-8153-62; eBiosciences), recombinant mouse IL-12 () or recombinant IL-18 (), alone or in combination, were then added to the cells, and the cultures were incubated at 37°C for 1, 3 or 5-days. Following incubation, cells were stained with Live/Dead Aqua and analysed by flow cytometry to determine survival and proliferation. For effector cytokine production assays, cells were stained intracellularly with anti-IFN-γ and anti-TNF-α using the appropriate staining protocol (*Chapter 2.5.1.4*).

2.6.2 TCR stimulation assays

T_{VM} cells were purified from the spleens and lymph nodes of $Lck^{cre}Rela^{wt/wt}$ and $Lck^{cre}Rela^{fl/fl}$ mice by FACS. Cells were then stained in 2.5µM Cell Trace Violet (CTV) (C34557; Invitrogen) for 10 min at 37°C, washed with 5x volumes complete advanced RPMI and resuspended at a concentration of $1x10^6$ cells per mL. Aliquots of cells ($1x10^5$ cells in 100µL) were placed into a 96-well flat bottom tissue culture plate (#355072; Falcon) pre-coated with Rat anti-mouse CD3 antibodies (Clone 145-2C11) at 1µg/mL. 100µL of 2ng/mL recombinant mouse IL-2 (14-8021-64; eBiosciences), recombinant mouse IL-7 (14-8071-62; eBiosciences) or recombinant mouse IL-15 (14-8153-62; eBiosciences) were then added to the cells, and the cultures were incubated at 37°C for 1, 3 or 5-days. Following incubation, cells were stained with Live/Dead Aqua and analysed by flow cytometry to determine survival and proliferation. For effector cytokine production assays, cells were stained intracellularly with anti-IFN-γ and anti-TNF-α using the appropriate staining protocol (*Chapter 2.5.1.4*).

2.7 Statistical Analysis

Where shown, all graphs display mean ± Standard Error of the Mean (SEM). Significance was assessed by way of Student's t-test for bivariate experiments or two-way ANOVA with Bonferonni's post-test for multiple variable experiments.

Chapter 3:

The influence of canonical NF-κB family members in T_{VM} cell development

3.1 Introduction

The generation and functional capacity of memory $CD8^+$ T cells has been shown to require two members of the T-box transcription factor family, T-bet and Eomesodermin (Eomes) (Intlekofer et al, 2005; Banerjee et al, 2010). These transcription factors are highly expressed in activated $CD8^+$ T cells and play critical roles determining the differentiation states of effector and memory $CD8^+$ T cells following exposure to antigen (Szabo et al, 2002; Sullivan et al, 2003; Pearce et al, 2003; Intlekofer et al, 2005). Eomes in particular is associated with the functional characteristics that typify a central memory (T_{CM}) phenotype, such as long-term persistence and increased secondary expansion following antigen re-encounter (Banerjee et al, 2010). T_{VM} cells, unlike T_{AM} cells, preferentially display features reminiscent of a T_{CM} phenotype and have recently been shown to express high levels of T-bet and Eomes (Lee et al, 2013). Upregulation of Eomes expression in memory $CD8^+$ T cells has been linked to signals provided by type I IFNs and IL-15, two cytokines closely associated with T_{VM} cell generation and maintenance (Boyman et al, 2007; Martinet et al, 2015). Besides T-bet and Eomes, not much is known about other transcription factors that make significant contributions to the regulation of T_{VM} development and function. However, based on insights from previous studies that implicate the NF-κB pathway in memory-phenotype (MP) $CD8^+$ T cell biology (Hettmann et al, 2003; Schmidt-Supprian et al 2004; Gugasyan et al, 2012), the possibility emerged that NF-κB may be involved in the development and function of T_{VM} cells. The inhibition of NF-κB nuclear localization and activation in the T cells of mice expressing a mIκB-α transgene resulted in a significant reduction of MP $CD8^+$ T cells in the spleen and peripheral lymph nodes (pLNs) (Hettmann et al, 2003). A similar finding was reported in mice that lack IKKβ (*Ikkβ*$^{-/-}$) in T cells (Schmidt-Supprian et al, 2004). In contrast, NFκB1-deficient mice (*Nfkb1*$^{-/-}$) exhibit a large increase in a MP $CD8^+$ T cell population in both the thymus and peripheral lymphoid organs (Gugasyan et al, 2012). Although further characterization of this expanded MP $CD8^+$ T cell population in *Nfkb1*$^{-/-}$ mice is required, high cell surface expression of CD122 and upregulation of Eomes in these cells

is suggestive of a T_{VM} cell phenotype. Given IKKβ specifically activates canonical NF-κB complexes, collectively these studies indicate that one or both of the other canonical NF-κB family members cRel and RelA play an opposing regulatory role to NF-κB1 in T_{VM} cell generation and maintenance.

Over the past 30 years, the various functions of the NF-κB signaling pathway have been elucidated largely through the use of knockout and transgenic mouse models (Gerondakis et al, 2005). Using systemic or conditional gene targeting, together with transgenic models that overexpress wild-type or null alleles encoding a particular NF-κB, IκB or IKK protein, the complexities of the NF-κB signaling pathway continue to be unraveled. Albeit useful in deciphering the diverse array of cellular processes regulated by NF-κB, including but not limited to survival, proliferation, differentiation and inflammation, the use of these mouse models is not without problems. One such example is the systemic ablation of RelA (p65) or IKKβ, both of which result in embryonic lethality due to hepatocyte apoptosis at embryonic days (E) 14.5 and 12.5 for mice that lack RelA and IKKβ respectively (Beg et al, 1995; Tanaka et al, 1999). This occurs through apoptotic signals mediated by TNF that are transduced through the type I TNF receptor (TNFR1) (Hsu et al, 1995). The study of these two NF-κB pathway components within the context of the immune system therefore relies either on the use of hematopoietic stem cell (HSC) chimeric mice, or cell type-specific gene targeting. Previous studies have shown that by E10.5 the HSCs present in the foetal liver are able to repopulate the entire hematopoietic system when transplanted into irradiated adult recipient mice (Johnson and Moore, 1975). The generation of mice with immune systems that lack either IKKβ or RelA is routinely achieved by engrafting foetal liver HSCs from E13-13.5 *Rela*$^{-/-}$ and E12-12.5 *Ikkβ*$^{-/-}$ embryos into radio-ablated adult recipient mice (Doi et al, 1997; Alcomo et al, 2001; Senftleben et al, 2001). This strategy by-passes the issue of hepatocyte-associated embryonic lethality, with the hematopoietic cells that arise in these chimeric mice being of a different genotype to the non-hematopoietic cells of the recipient mouse, thereby allowing an analysis of the impact a selective loss of either RelA or IKKβ has on the hematopoietic compartment. Whilst *Ikkβ*$^{-/-}$ HSC chimeric

mice exhibit defects in the generation of the T cell compartment (Senftleben et al, 2001), to date, a basic analysis of lymphoid cell development in *Rela*$^{-/-}$ HSC chimeric mice found that the T cell compartment appeared ostensibly normal (Gerondakis et al, 2006). Importantly, because RelA-deficient HSCs are able to reconstitute the hematopoietic compartment of irradiated recipient mice and generate all hematopoietic cell lineages (Doi et al, 1997; Alcomo et al, 2001), using this strategy should allow an initial investigation of the role RelA plays in the development of T_{VM} cells. The outcome of this investigation, presented in the findings below, provides strong evidence that the canonical NF-κB family member RelA plays an opposing regulatory role to NF-κB1 in T_{VM} cell generation and maintenance.

3.2 Results

3.2.1 The canonical NF-κB family members NF-κB1 and RelA are present in the nucleus of ex-vivo T_{VM} cells

Contrasting findings have emerged from studies using *Nfkb1*$^{-/-}$ mice and T cell-specific mIκB-α transgenic or *Ikkβ*$^{-/-}$ mice when investigating the role the canonical NF-κB pathway plays in MP CD8$^+$ T cells (Hettmann et al, 2003; Schmidt-Supprian et al, 2004; Gugasyan et al, 2012). Whereas MP CD8$^+$ T cell numbers are elevated in *Nfkb1*$^{-/-}$ mice (Gugasyan et al, 2012), these cells are markedly reduced in the mIκB-α and *Ikkβ*$^{-/-}$ mouse models (Hettmann et al, 2003; Schmidt-Supprian et al, 2004). These differences most likely reflect the impact of blocking the activity of individual versus multiple NF-κB family members, with T cells from mIκB-α transgenics and *Ikkβ*$^{-/-}$ mice, unable to activate the gamut of canonical NF-κB homodimers or heterodimers. These different MP CD8$^+$ T cell phenotypes, which provide strong evidence for the involvement of multiple NF-κB family members in the generation and/or homeostasis of MP CD8$^+$ T cells, prompted an investigation of NF-κB activity in the nucleus of T_{VM} cells as a starting point in this study. NF-κB DNA binding activity was examined in the nucleus of immediate *ex-vivo* naïve, T_{VM} and T_{AM} CD8$^+$ T cells co-purified by FACS sorting from the pooled spleen and pLNs of 8-12

week-old C57BL/6 mice using Electrophoretic Mobility Shift Assay (EMSA) as described previously (Grumont and Gerondakis, 1994). These EMSAs revealed that naïve ($CD44^{lo}CD49d^{lo}$), T_{AM} ($CD44^{hi}CD49d^{hi}$) and T_{VM} ($CD44^{hi}CD49d^{lo}$) $CD8^+$ T cells all expressed a common nuclear NF-κB complex (C1) of varying abundance with a mobility characteristic of NF-κB1 homodimers, whereas T_{VM} cells expressed a unique NF-κB nuclear complex (C2) (Fig. 3.1A). Antibody super-shift analysis utilizing antibodies that recognize the specific NF-κB proteins NF-κB1, cRel, RelA and NF-κB2 were used to determine the composition of the NF-κB complexes present in the nucleus of these $CD8^+$ T cell subsets (Fig. 3.1B). As predicted, the common C1 complex was found to comprise NF-κB1 homodimers, while the C2 complex expressed exclusively in the nucleus of T_{VM} cells was a NF-κB heterodimer comprised of NF-κB1 and RelA.

3.2.2 The constitutive expression of RelA-containing NF-κB dimers in the T_{VM} cell population is not induced by tonic TCR signals

The presence of RelA heterodimers in the nucleus of immediate *ex-vivo* T_{VM} cells was unexpected, given RelA nuclear localization in conventional T cells is normally induced by stimuli that trigger T cell activation, such as TCR and co-stimulatory signals (Kingeter et al, 2010; Gerondakis et al, 2014). Given T_{VM} cells, as indicated by their low expression of CD49d, have not encountered antigen signals that are sufficiently strong to activate these cells, it seemed unlikely that the nuclear expression of RelA is induced by signaling through the TCR. Naïve T cells do however maintain their survival in the periphery, in part, through tonic TCR signaling (Takada and Jameson, 2009). Based on the finding that T_{VM} cells express higher levels of CD5, a surrogate marker of TCR signal strength (Azzam et al, 1998), than naïve $CD8^+$ T cells, it has been proposed that T_{VM} cells generally have a greater affinity for peptide/MHC class-I complexes than their naïve counterparts (Quinn et al, 2016). Furthermore, a recent study undertaken by White and colleagues (2016) found that those naïve $CD8^+$ T cells expressing a TCR with greater affinity for peptide/MHC Class-I complexes, identified by heightened CD5 expression, preferentially converted

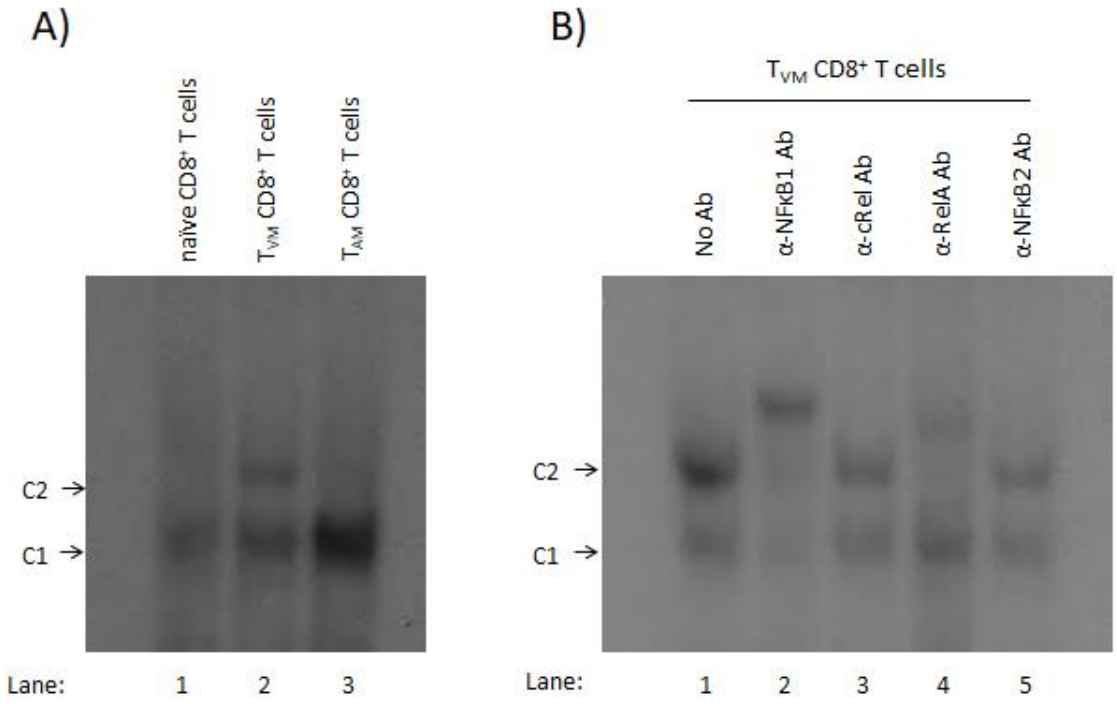


Figure 3.1. T_{VM} cells exclusively express RelA-containing NF-κB complexes. NF-κB activity was analysed in naïve, T_{VM} and T_{AM} $CD8^+$ T cell subsets by EMSA. (**A**) Naïve (lane 1), T_{VM} (lane 2) and T_{AM} (lane 3) $CD8^+$ T cells all expressed a common nuclear NF-κB complex (C1), whereas T_{VM} cells also expressed a unique nuclear NF-κB complex (C2). (**B**) Antibody (Ab) supershift analysis for NF-κB family members active in the nucleus of splenic T_{VM} cells. Compared to T_{VM} cells not treated with Ab (lane 1), a shift in the mobility of C1 and C2 with the addition of the anti(α)-NF-κB1 Ab (lane2) suggest NF-κB1 homodimers and heterodimers are active within the nucleus of these cells. The addition of α-RelA Ab (lane 4) shifts the mobility of C2, suggesting that RelA heterodimers are active in the nucleus of T_{VM} cells. Splenic T_{VM} cells display a supershift demonstrating the presence of NF-κB1 homodimers and NF-κB1:RelA heterodimers. C1 & C2=NF-κB/DNA complexes; naïve=$CD8^+CD44^{lo}CD49d^{int}$; T_{VM}=$CD8^+CD44^{hi}CD49d^{lo}$ Virtual Memory cells; T_{AM}=$CD8^+CD44^{hi}CD49d^{hi}$ conventional antigen-primed memory cells. Representative of three experiments.

to T_{VM} cells when transferred into lympho-replete congenic hosts. This raised the possibility that the increased affinity for peptide/MHC class-I (pMHC-I) complexes displayed by TCRs expressed on T_{VM} cells may be sufficient to promote RelA activation in response to tonic TCR signals, without driving T_{VM} cells into a fully activated state. To test this hypothesis, we utilized $Nur77^{GFP}$ reporter mice which express a transgene encoding green fluorescent protein (GFP) under the transcriptional control of the *Nr4a1* (Nur77) locus (Moran et al, 2011). Nur77 is normally up-regulated in developing thymocytes and mature T cells following TCR stimulation, where its level of expression correlates closely with the strength of antigen receptor signaling (Osborne et al, 1994; Moran et al, 2011). To determine if T_{VM} cells experience a level of tonic TCR signaling different from other peripheral $CD8^+$ T cell subsets that could be sufficient to promote RelA activation, GFP levels were examined by flow cytometry in naïve and MP (T_{AM} and T_{VM}) $CD8^+$ T cell subsets taken from 12-week old $Nur77^{GFP}$ reporter mice (Fig.3.2A). A comparison of $Nur77^{GFP}$ levels in the different $CD8^+$ T cell subsets revealed that the Nur77 reporter was expressed in T_{VM}s at levels comparable with that of naïve $CD8^+$ T cells, but lower than T_{AM} cells (Fig.3.2B). So whilst T_{VM} cells may express TCRs with a higher affinity for self-antigens than naïve $CD8^+$ T cells, the levels of tonic TCR signalling was equivalent in these T cell subsets. Based on these findings, the select nuclear expression of RelA in T_{VM} cells is unlikely to reflect these cells being subjected to stronger tonic TCR signals.

3.2.3 The T_{VM} cell population is markedly expanded in mice that lack NF-κB1

The initial findings of this study have shown that T_{VM} cells express multiple nuclear NF-κB complexes, namely NF-κB1/RelA heterodimers and NF-κB1 homodimers. Whilst NF-κB1 (p50) coupled with coactivators such as Bcl-3, or heterodimerized with other canonical trans-activating NF-κB family members can induce transcription, p50 homodimers typically repress gene transcription due to this protein lacking an intrinsic transactivation domain (TAD) (Gerondakis and Siebenlist, 2010). Mice lacking NF-κB1 (*$Nfkb1^{-/-}$*) have previously been shown to develop a unique population of MP $CD8^+$ T cells in the

thymus (Gugasyan et al, 2012). These MP CD8$^+$ T cells (CD44^{hi}CD25^{lo}) present in the thymi of adult *Nfkb1*$^{-/-}$ mice comprise ~40% of the CD8 SP thymocyte population, a much higher frequency than the ~15% of CD8 SP with this phenotype in age-matched WT littermate controls. These *Nfkb1*$^{-/-}$ MP CD8$^+$ T cells also exit the thymus and contribute to the peripheral CD8 T cell pool, leading to higher than normal numbers of peripheral MP CD8$^+$ T cells in these mice. These collective findings prompted an investigation into the role NF-κB1 might play in the generation and maintenance of T_{VM} cells.

To determine if T_{VM} cells contribute to the elevated number of peripheral MP CD8$^+$ T cells in *Nfkb1*$^{-/-}$ mice, immune organs from 6-month-old *Nfkb1*$^{+/+}$ and *Nfkb1*$^{-/-}$ mice were analyzed by flow cytometry. Mice lacking NF-κB1 have been reported to display certain age-related pathologies including systemic tissue inflammation, increased cellular senescence, autoimmune disease and gastric cancer (Bernal et al, 2014; deValle et al, 2016; O'Reilly et al, 2018). Some of these pathologies have also been reported to occur in *Nfkb1*$^{+/-}$ mice, albeit with less severity (O'Reilly et al, 2018), indicating these defects are subject to haplo-sufficiency. Taking this into consideration, age-matched *Nfkb1*$^{+/-}$ littermates were also included in the analysis to determine if changes in the MP CD8$^+$ T cell population were also NF-κB1 dose-dependent (Supplementary Fig.1). This analysis revealed a significant increase in the proportion of antigen-inexperienced MP (CD44^{hi}CD49d^{lo}) CD8$^+$ T cells (Fig.3.3A), but not conventional antigen-primed T_{AM} (CD44^{hi}CD49d^{hi}) CD8$^+$ T cells (Fig.3.3B), in the spleen, pLNs and BM of *Nfkb1*$^{-/-}$ mice when compared to age-matched WT controls (*Nfkb1*$^{+/+}$ mice). In direct contrast to this finding, the frequency of naïve CD8$^+$ T cells was found to be significantly reduced in all of the lymphoid organs tested in *Nfkb1*$^{-/-}$ mice (Fig.3.3C). Further investigation of the splenic CD8$^+$ T cell compartment revealed that whilst the total number of antigen-inexperienced MP CD8$^+$ T cells was significantly increased in the spleen of *Nfkb1*$^{-/-}$ mice when compared to WT controls (Fig.3.3.E), the total number of splenic naïve CD8$^+$ T cell was statistically comparable between the two groups (Fig.3.3.E). These data show that the expanded MP CD8$^+$ T cell population, previously reported in *Nfkb1*$^{-/-}$ mice (Guyasyan et al, 2012), show phenotypic

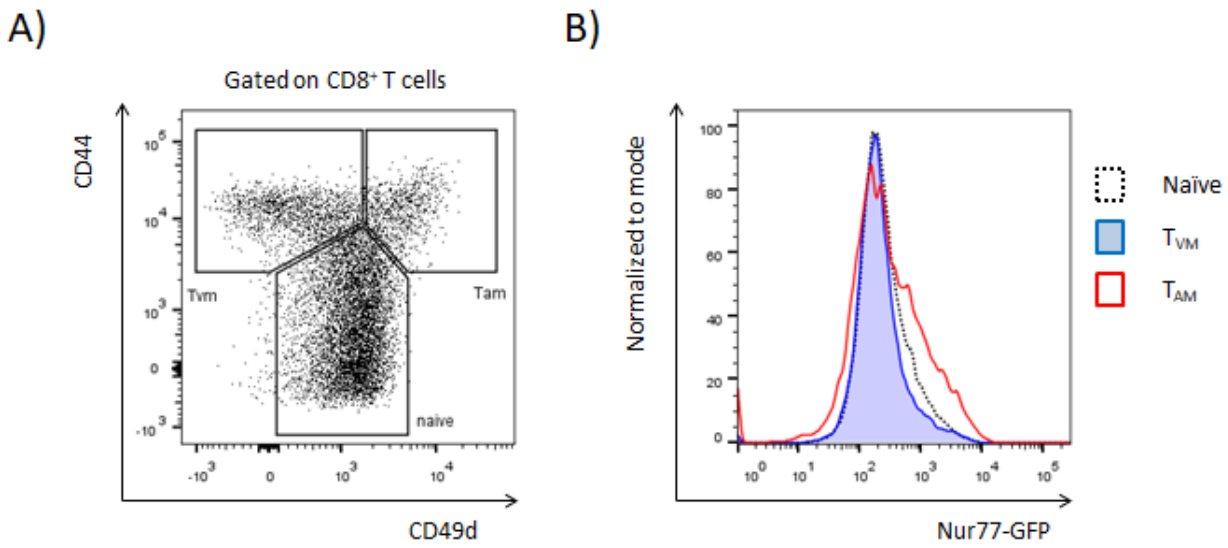
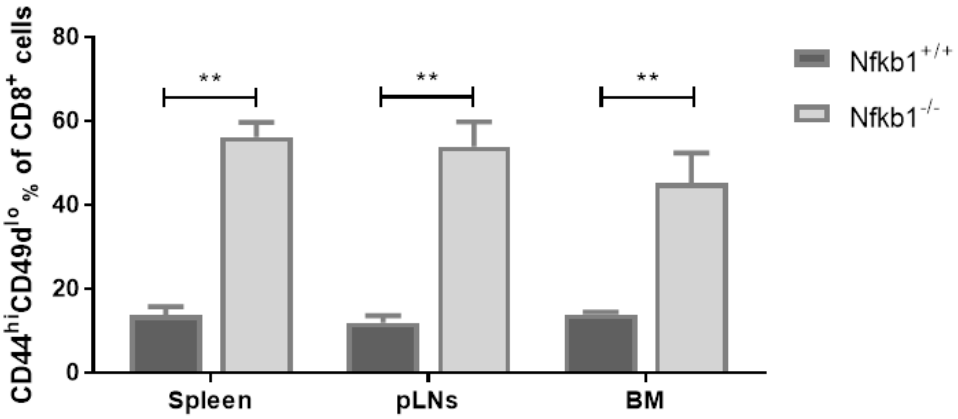


Figure 3.2. Naïve and T_{VM} CD8 T cells encounter similar levels of tonic TCR signaling in the periphery. Nur77 expression levels were measured in naïve, T_{VM} and T_{AM} $CD8^+$ T cell subsets in the spleen of 3-month-old $Nur77^{GFP}$ reporter mice using flow cytometry. (**A**) Representative plot of naïve, T_{VM} and T_{AM} gating strategy on $CD8^+$ gated cells. (**B**) Expression levels of $Nur77^{GFP}$ in naïve, T_{VM} and T_{AM} $CD8^+$ T cell subsets. Nur77 reporter was expressed in T_{VM}s at levels comparable with that of naïve $CD8^+$ T cells, but lower than T_{AM} cells. Naïve=$CD8^+CD44^{lo}CD49d^{int}$ T_{VM}=$CD8^+CD44^{hi}CD49d^{lo}$ Virtual Memory cells; T_{AM}=$CD8^+CD44^{hi}CD49d^{hi}$ conventional antigen-primed memory cells; GFP=green fluorescent protein. Representative of two independent experiments.

A)


CD44hiCD49dlo % of CD8+ cells
80
60
40
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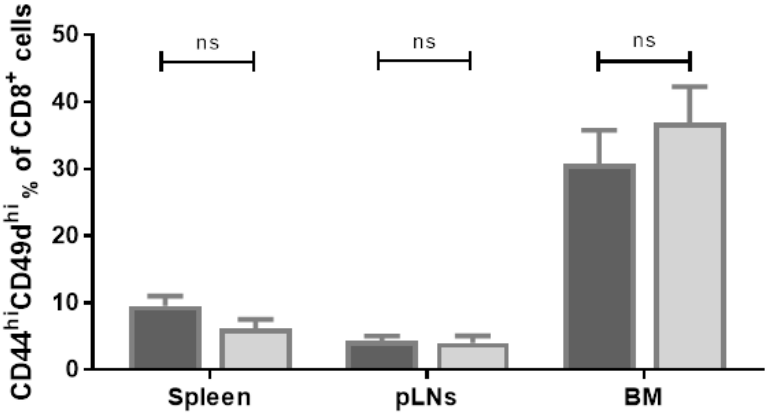
0
**
**
**
Spleen
pLNs
BM
Nfkb1+/+
Nfkb1-/-

B)


CD44hiCD49dhi % of CD8+ cells
50
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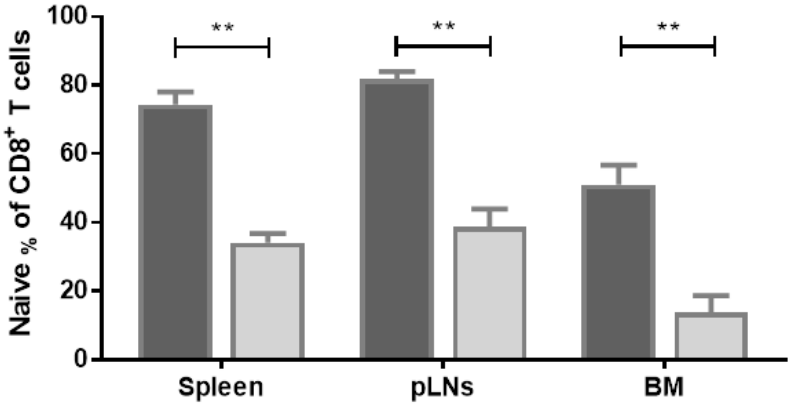
ns
Spleen
pLNs
BM

C)


Naive % of CD8+ T cells
100
80
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Spleen
pLNs
BM

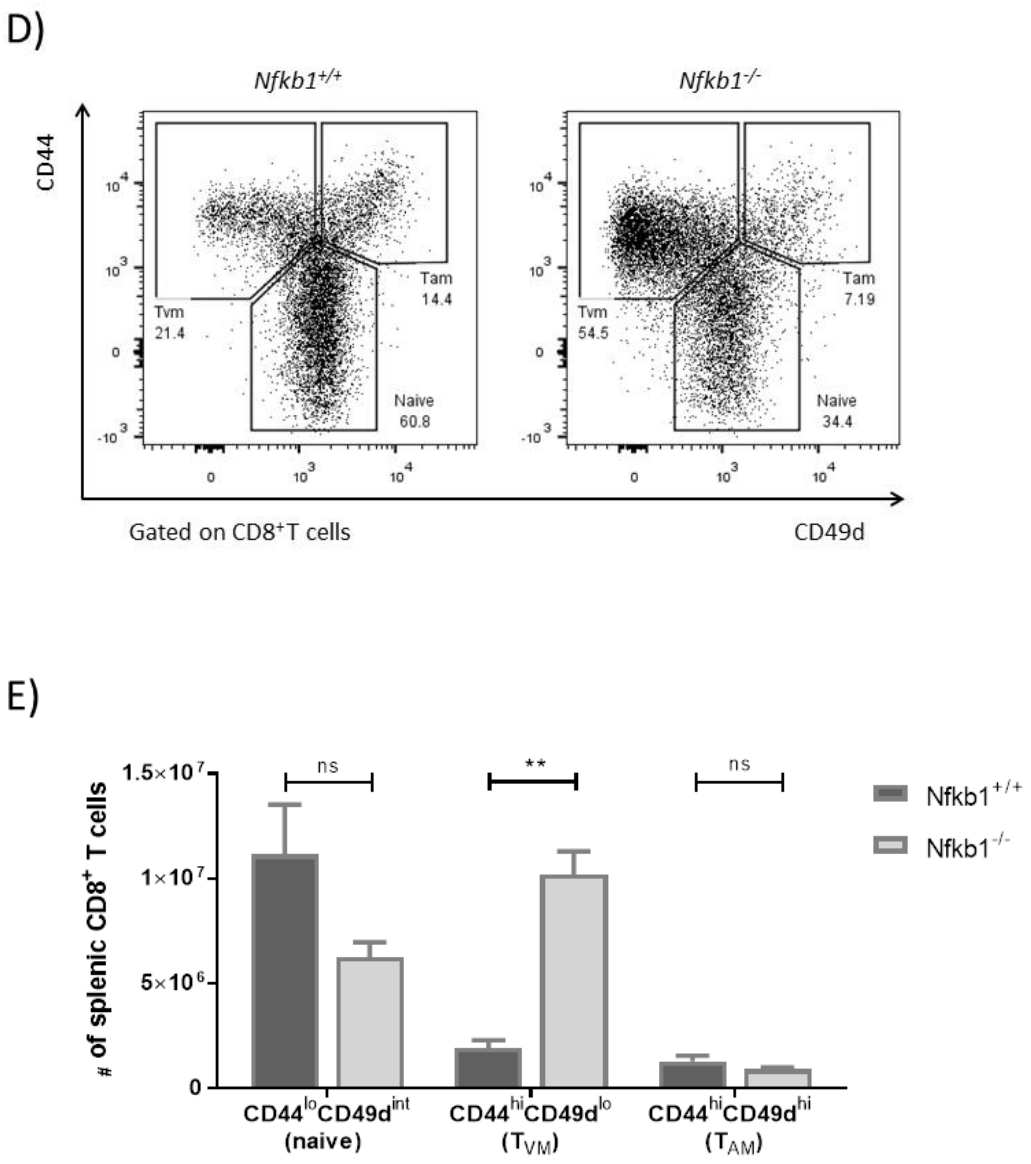


Figure 3.3. *Nfkb1*$^{-/-}$ mice have an expanded T_{VM} cell population compared to age-matched WT control mice. (**A-E**) Flow cytometric analysis of naïve, T_{VM} and T_{AM} $CD8^+$ T cell subsets from spleen, pLN and BM of 6-mo-old *Nfkb1*$^{-/-}$ and *Nfkb1*$^{+/+}$ (WT) control mice (n=5). (**A**) Percentage of T_{VM} cells of $CD8^+$ T cells. (**B**) Percentage of T_{AM} cells of $CD8^+$ T cells. (**C**) Percentage of naïve cells of $CD8^+$ T cells. (**D**) Representative flow cytometry plot of $CD8^+$ T cell subsets in the spleen. (**E**) Total number of $CD8^+$ T cell subsets in the spleen. All graphs presented as the mean +/- SEM, statistical significance was determined using Student's T tests. **, p≤0.01. pLNs=peripheral lymph nodes; BM=bone marrow; naïve=$CD8^+CD44^{lo}CD49d^{int}$ T_{VM}=$CD8^+CD44^{hi}CD49d^{lo}$ Virtual Memory cells; T_{AM}=$CD8^+CD44^{hi}CD49d^{hi}$ conventional antigen-primed memory cells; WT=wild type.

characteristics of being antigen-inexperienced ($CD49d^{lo}$). However, two distinct subsets of antigen-inexperienced MP $CD8^+$ T cells have been described in lymphoreplete mice that both present with a $CD44^{hi}CD49d^{lo}$ cell surface receptor expression signature (White et al, 2017). In addition to T_{VM} cells, 'innate memory' (T_{IM}) $CD8^+$ T cells, which arise during thymocyte development in response to IL-4 produced by $PLZF^+$ T cells (Raberger et al, 2008), emigrate from the thymus and contribute to the peripheral MP $CD8^+$ T cell pool. Expression of the cell surface marker NKG2D, a transmembrane receptor typically expressed by NK cells and particular T cell subsets following activation, has emerged thus far as the best marker to distinguish between these two populations (White et al, 2017). Whilst T_{VM} cells were reported to express NKG2D (White et al, 2016), a property consistent with the capacity of T_{VM} cells to promote antigen-independent by-stander killing, its reputed lack of expression by T_{IM} cells coincides with the inability to kill cells via by-stander mechanisms (Ventre et al, 2012; White et al, 2017). This association between NKG2D expression and T_{VM} cells prompted an examination of NKG2D expression on the antigen-inexperienced MP $CD8^+$ T cell population in *Nfkb1*$^{-/-}$ mice. The findings of this analysis (Fig.3.4A), showing the spleen of 5-month-old *Nfkb1*$^{-/-}$ mice had more than twice the number of $NKG2D^+$ T_{VM} cells when compared to age-matched WT controls established that the expanded MP population in NF-κB1-deficient mice, in part, reflects an expanded T_{VM} cell population. Despite this finding confirming that the loss of NF-κB1 leads to an expanded T_{VM} cell population, this expansion does not fully account for the difference in the sizes of the antigen-inexperienced MP $CD8^+$ T cell population of *Nfkb1*$^{+/+}$ and *Nfkb1*$^{-/-}$ mice. In fact, the greater contribution to the enlarged MP population in *Nfkb1*$^{-/-}$ mice comes from $NKG2D^-$ antigen-inexperienced MP $CD8^+$ T cells (Fig.3.4B), indicating that a lack of NF-κB1 promotes expansion of the total antigen-inexperienced MP $CD8^+$ T cell population. Deciphering whether or not the $NKG2D^-$ antigen-inexperienced MP $CD8^+$ T cell population in NF-κB1-deficient mice exclusively comprises T_{IM} cells, a subset of $NKG2D^-$ T_{VM} cells or a mixture of both populations awaits further advances in the field. Nevertheless, the findings reported here establish that the elevated numbers of MP $CD8^+$ T cells

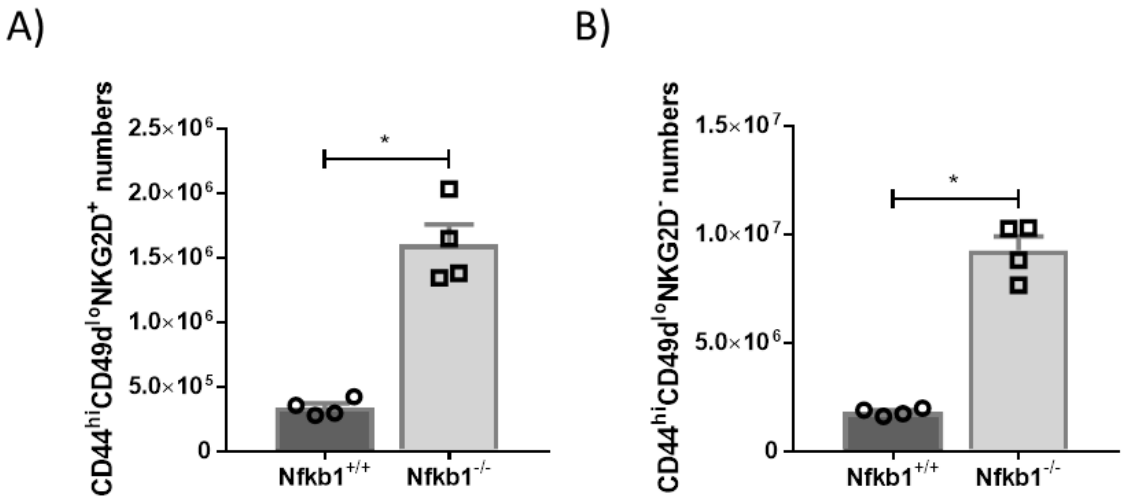


Figure 3.4. *Nfkb1*$^{-/-}$ mice have an increased number of NKG2D^{+} T_{VM} cells and NKG2D^{-} antigen-inexperienced MP CD8^{+} T cells compared to age-matched WT (*Nfkb1*$^{+/+}$) control mice. (**A-B**) Flow cytometric analysis of CD44^{hi}CD49d^{lo}CD8^{+} T cells in the spleen of 5-month-old *Nfkb1*$^{-/-}$ and *Nfkb1*$^{+/+}$ (WT) control mice (n=4). (**A**) Total number of NKG2D^{+} T_{VM} cells in the spleen. (**B**) Total number of NKG2D^{-} MP CD8^{+} T cells in the spleen. All graphs presented as the mean +/- SEM, statistical significance was determined using Student's T tests. *, p≤0.05. T_{VM}=CD8^{+}CD44^{hi}CD49d^{lo} Virtual Memory cells; WT=wild type.

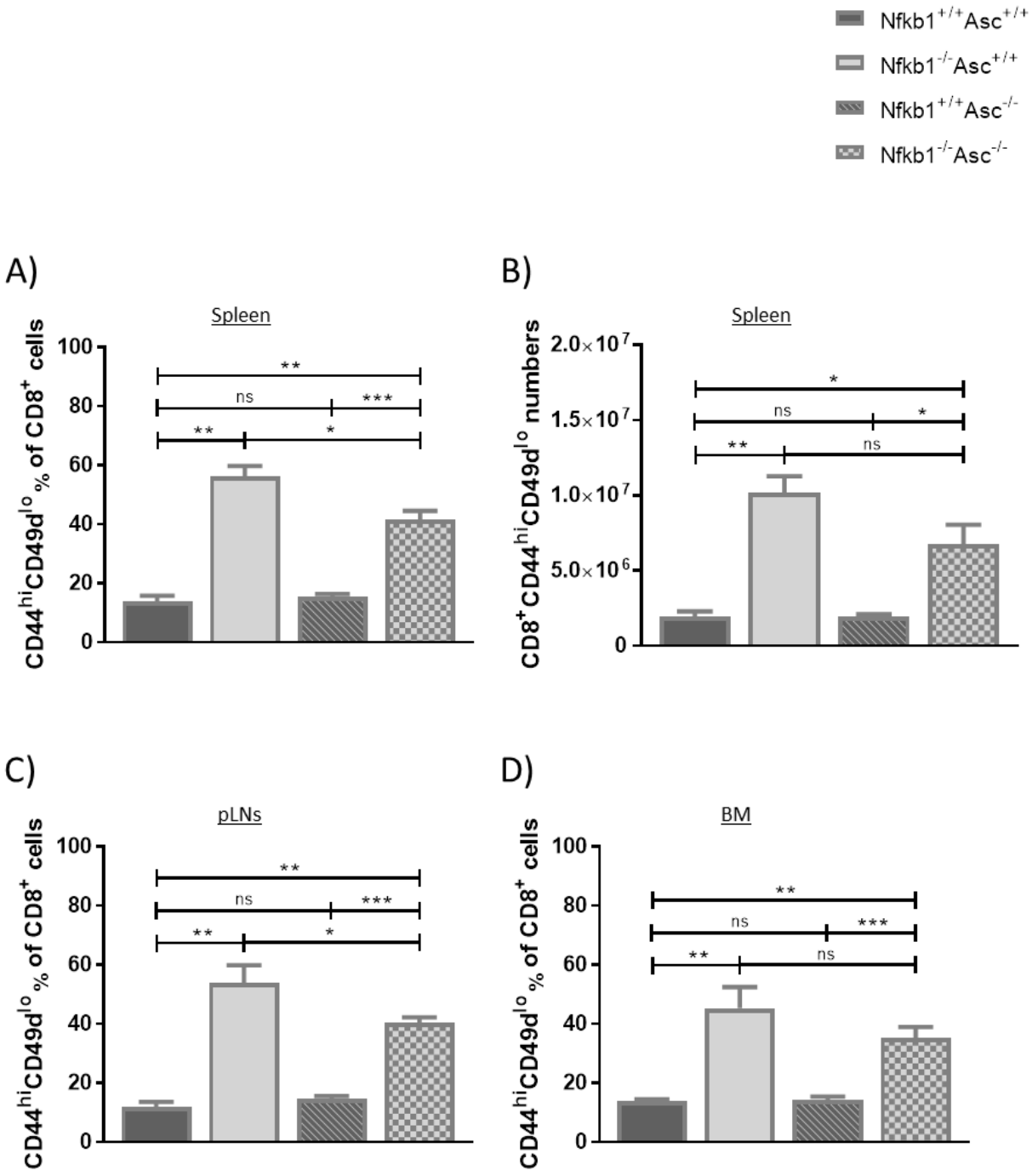
previously reported in *Nfkb1*$^{-/-}$ mice is caused, in part, by an expanded T_{VM} cell population. Interestingly, this analysis also showed that *Nfkb1*$^{+/-}$ mice had a similar sized T_{VM} cell population to that of age-matched WT controls (*Nfkb1*$^{+/+}$ mice) (Supplementary Fig.1), indicating that unlike the certain inflammation associated pathologies documented in *Nfkb1* haplo-insufficient mice, a 50% reduction in NF-κB1 levels does not appear to influence the size of T_{VM} cell population.

3.2.4 A loss of ASC reduces the extent of T_{VM} cell expansion in Nfkb1$^{-/-}$ *mice*

When compared to age-matched WT controls, *Nfkb1*$^{-/-}$ mice aged between 6 and 12-months have been reported to display certain age-related pathologies that appear closely aligned with increased systemic inflammation (Bernal et al, 2014; de Valle et al, 2016; O'Reilly et al, 2018). Following on from previous reports showing that the levels of numerous inflammatory cytokines including TNF, IL-6 and IFN-γ are significantly higher in *Nfkb1*$^{-/-}$ mice (de Valle et al, 2016; O'Reilly et al, 2018), the serum levels of the inflammasome-dependent cytokines IL-1β and IL-18 were also assessed in 5 to 6-month-old *Nfkb1*$^{-/-}$ mice. In addition to confirming that IFN-γ levels, as previously reported by O'Reilly and colleagues (2018), are significantly higher in *Nfkb1*$^{-/-}$ mice, IL-1β and IL-18 levels were also found to be significantly higher in this strain than in age-matched WT control animals (A. Mansell, unpublished results) (Supplementary Fig.2A). Given the emerging evidence that suggests the homeostasis of T_{VM} cells is regulated largely by inflammatory cytokines (Kurzweil et al, 2014; Martinet et al, 2015), coupled with a recent report showing T_{VM} cells express high levels of the genes (*Il18r1* and *Ifngr1*) encoding IL-18R1 and IFNγR1 (White et al., 2016), it was decided to determine if reducing the level of inflammasome generated cytokines in *Nfkb1*$^{-/-}$ mice would impact the size of T_{VM} cell population. As a start to addressing this question, the ASC-dependent production of IL-18 was targeted by introducing a null allele for ASC (apoptosis-associated speck-like protein containing C-terminal caspase recruitment domain [CARD]), onto the *Nfkb1*$^{-/-}$ genetic background. ASC is a subunit of certain inflammasomes, most

notably NALP3, but also NALPX and NALPY, which is essential for the processing of pro-IL-1β and pro-IL-18 into their biologically active forms (Latz et al, 2013). T_{VM} cells appear to express the IL-18R, but not the IL-1R (White et al, 2016), making IL-18 the key inflammasome-induced inflammatory cytokine likely to regulate T_{VM} cell homeostasis. However, it remains a formal possibility that increased levels of IL-1β could indirectly promote T_{VM} cell expansion by establishing an inflammatory cascade that generates other T_{VM} cell responsive cytokines. Notwithstanding this caveat, the focus was on assessing the role ASC-dependent IL-18 production had on T_{VM} homeostasis in *Nfkb1*$^{-/-}$ mice. First, the impact ASC inactivation had on IL-18 production was determined by examining IL-18 serum levels using ELISA. Both *Nfkb1*$^{+/+}$*Asc*$^{-/-}$ and *Nfkb1*$^{-/-}$*Asc*$^{-/-}$ mice had similar IL18 serum levels that were significantly lower than those of WT controls (*Nfkb1*$^{+/+}$*Asc*$^{+/+}$) and much lower than the IL-18 levels detected in *Nfkb1*$^{-/-}$*Asc*$^{+/+}$ mice (A. Mansell, unpublished results) (Supplementary Fig.2B). Although reduced, as expected, IL-18 levels in mice on an *Asc*$^{-/-}$ background were still detectable due to IL-18 production by ASC-independent inflammasomes.

At 6-months of age, T_{VM} cell populations were examined in WT (*Nfkb1*$^{+/+}$*Asc*$^{+/+}$), *Nfkb1*$^{-/-}$*Asc*$^{+/+}$, *Nfkb1*$^{+/+}$*Asc*$^{-/-}$ and *Nfkb1*$^{-/-}$*Asc*$^{-/-}$ mice using flow cytometry (Fig.3.5A-D). WT and *Nfkb1*$^{+/+}$*Asc*$^{-/-}$ mice had T_{VM} cell populations of comparable sizes in all of the lymphoid organs tested, indicating that an absence of ASC does not impair T_{VM} generation per see. Although *Nfkb1*$^{-/-}$*Asc*$^{-/-}$ mice did display an expanded population of T_{VM} cells in the spleen (Fig.3.5A&B), pLNs (Fig.3.5C) and BM (Fig.3.5D) when compared to age-matched WT controls, the proportion of T_{VM} cells was significantly reduced in both the spleen and pLNs when compared to *Nfkb1*$^{-/-}$*Asc*$^{+/+}$ mice. While the inability of ASC inactivation to reduce T_{VM} cell levels to those seen in WT mice is consistent with ASC-independent cytokines contributing to the expansion of T_{VM} cells in *Nfkb1*$^{-/-}$ mice, our data clearly establishes that ASC-dependent inflammation does contribute to the expansion of the T_{VM} cell population in mice that lack NF-κB1.


Nfkb1+/+Asc+/+
Nfkb1-/-Asc+/+
Nfkb1+/+Asc-/-
Nfkb1-/-Asc-/-
A)
Spleen
CD44hiCD49dlo % of CD8+ cells
100
80
60
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B)
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CD8+CD44hiCD49dlo numbers
2.0×10^7
1.5×10^7
1.0×10^7
5.0×10^6
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ns
C)
pLNs
CD44hiCD49dlo % of CD8+ cells
100
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D)
BM
CD44hiCD49dlo % of CD8+ cells
100
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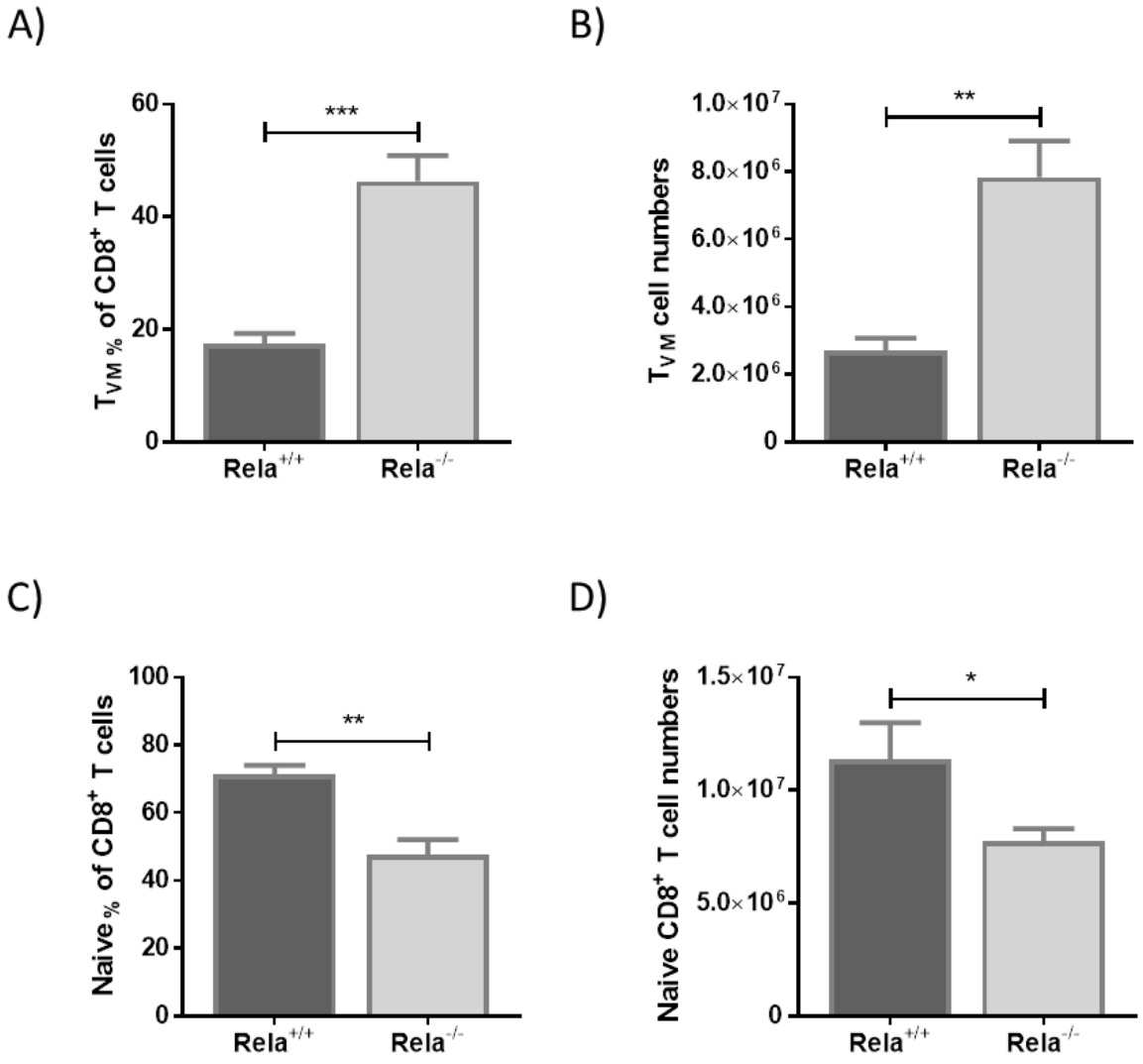
Figure 3.5. Loss of ASC reduces the severity of T_{VM} cell expansion observed in *Nfkb1*$^{-/-}$ mice. (**A-D**) Flow cytometric analysis of T_{VM} ($CD44^{hi}CD49d^{lo}$) $CD8^+$ T cells from spleen, pLN and BM of *Nfkb1*$^{-/-}$*Asc*$^{+/+}$, *Nfkb1*$^{+/+}$*Asc*$^{-/-}$, *Nfkb1*$^{-/-}$*Asc*$^{-/-}$ and *Nfkb1*$^{+/+}$*Asc*$^{+/+}$ (WT) control mice (n=5-7). (**A**) Percentage of T_{VM} cells of $CD8^+$ T cells from spleen. (**B**) Total number of T_{VM} cells in spleen. (**C**) Percentage of T_{VM} cells of $CD8^+$ T cells from pooled pLNs. (**D**) Percentage of T_{VM} cells of $CD8^+$ T cells from BM. All graphs presented as the mean +/- SEM, statistical significance was determined using unpaired Mann-Whitney T tests. *, p≤0.05; **, p≤0.01; ***, p≤ 0.001. pLNs=peripheral lymph nodes; BM=bone marrow; T_{VM}=$CD8^+CD44^{hi}CD49d^{lo}$ Virtual Memory cells; WT=wild type.

3.2.5 Rela$^{-/-}$ HSC chimeric mice fail to maintain a normal sized T_{VM} cell population

While pan-inhibition of the canonical NF-κB pathway has revealed that canonical NF-κB signaling is important in the biology of MP CD8^{+} T cells (Hettmann et al, 2003; Schmidt-Supprian et al 2004), it remains unclear what role each NF-κB subunit plays and the relative importance of each NF-κB family member in different types of MP CD8^{+} T cells. Given the finding that RelA-containing NF-κB heterodimers are selectively expressed in the nucleus of T_{VM} cells, this raised the likelihood that a loss of RelA activity, in part, accounts for the marked reduction in the MP CD8^{+} T cell population of mIκB-α and *Ikkβ* $^{-/-}$ T cell transgenic mice. However, the study of RelA in hematopoiesis comes with technical challenges, given embryonic mice lacking RelA die 'in utero' at approximately E14.5 due to TNF-dependent hepatocyte apoptosis (Beg et al, 1995). In order to overcome this developmental impasse to studying the role of RelA in T_{VM} cells, hematopoietic stem cell (HSC) chimeric mice were generated using E13 to E13.5 *Rela*$^{-/-}$ foetal liver-derived HSCs. Importantly, at this age the viability of *Rela*$^{-/-}$ foetal liver-derived HSC is normal (Doi et al, 1997). Specifically, *Rela*$^{+/-}$ mice on the C57BL/6 background were time-mated, with the resulting pregnant females humanely euthanized when the embryos were aged between E13 and E13.5. Following the PCR genotyping of individual embryos to identify those homozygous for the WT allele (*Rela*$^{+/+}$) or RelA null (*Rela*$^{-/-}$) allele, single cell suspensions of hemopoietic cells from *Rela*$^{+/+}$ and *Rela*$^{-/-}$ foetal livers that contains HSCs were transplanted into lethally irradiated (11Gy) C57BL/6 CD45 congenic recipient mice. This dose of radiation results in the efficient death of mature hematopoietic cells and progenitors, including HSCs in recipient mice, with the donor *Rela*$^{+/+}$ or *Rela*$^{-/-}$ HSCs serving to re-establish the hematopoietic system of the host (Doi et al, 1997). Antibodies that specifically recognize allelic variants of CD45 (CD45.1 and CD45.2), a cell surface marker expressed on all leukocytes, were used to distinguish donor foetal liver-derived cells (CD45.2) from any surviving residual host cells (CD45.1) in the HSC chimeras (Duran-Struuck and Dysko, 2009).

Initially, *Rela*$^{+/+}$ and *Rela*$^{-/-}$ HSC chimeric mice were examined at 6 months post-engraftment, with naïve and T_{VM} cell populations in the spleen, pLNs and BM analyzed by flow cytometry (Fig.3.6A-D). This analysis showed that the number and frequency of particular CD8$^+$ splenic T cell subsets was altered in the *Rela*$^{-/-}$ HSC chimeras. Like mice deficient in NF-κB1, irradiated mice receiving *Rela*$^{-/-}$ HSCs exhibited an expanded T_{VM} cell population when compared to mice receiving *Rela*$^{+/+}$ HSCs (Fig.3.6A&B). In mice receiving *Rela*$^{-/-}$ HSCs, both the proportion (Fig.3.6A) and number (Fig.3.6B) of T_{VM} cells within the splenic CD8$^+$ T cell population was increased. This expansion of T_{VM} cells within mice receiving *Rela*$^{-/-}$ HSCs occurred at the expense of the naïve CD8$^+$ T cell compartment, with *Rela*$^{-/-}$ HSC chimeric mice exhibiting a significant drop in both the proportion (Fig.3.6C) and number (Fig.3.6D) of naïve CD8$^+$ splenic T cells. A similar increase in the proportion of T_{VM} cells within the pLNs (Fig.3.6E) and the BM (Fig.3.6F) was also observed in *Rela*$^{-/-}$ HSC chimeras. These results were unexpected given the working hypothesis that the loss of RelA activity would account, in part, for the reduction in the MP CD8$^+$ T cell population of mice subjected to pan-NF-κB inhibition within the T cell compartment. However, further analysis of the T_{VM} cell population in the *Rela*$^{+/+}$ and *Rela*$^{-/-}$ chimeras using CD45.1 and CD45.2 specific antibodies to distinguish residual host cells (CD45.1$^+$) from HSC donor-derived cells (CD45.2$^+$) showed that the expanded T_{VM} cell population in the *Rela*$^{-/-}$ chimeras was almost entirely comprised of residual host-derived cells (Fig.3.7A-B). This contrasted with the T_{VM} population in the *Rela*$^{+/+}$ HSC chimeras, where a much larger proportion of the T_{VM} cell population was derived from donor HSCs. Similar findings were made for the T_{VM} population in the pLNs and the BM of *Rela*$^{+/+}$ and *Rela*$^{-/-}$ chimeric mice (Fig.3.7B). This outcome is in contrast to the naïve CD8$^+$ T cell populations in the lymphoid organs of both *Rela*$^{+/+}$ and *Rela*$^{-/-}$ HSC chimeras, which are derived almost entirely from donor HSCs (Fig.3.7C). MP T cells are known to be more radio-resistant than naïve T cells (Grayson et al, 2002), so the significant T_{VM} cell populations in the chimeras that are host-derived (CD45.1$^+$) can be accounted for by the T_{VM} cells

(Gated on $CD8^+$ T cells in the spleen)



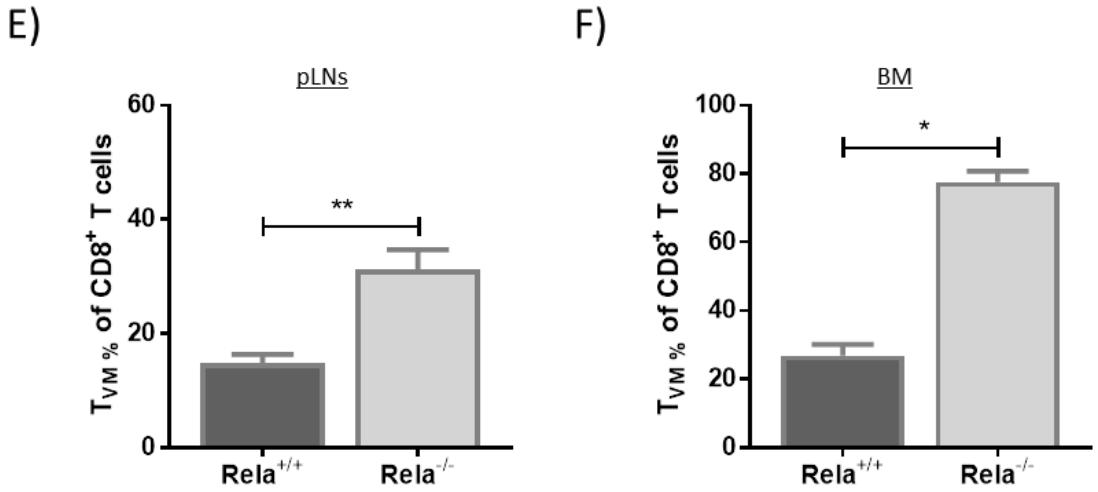
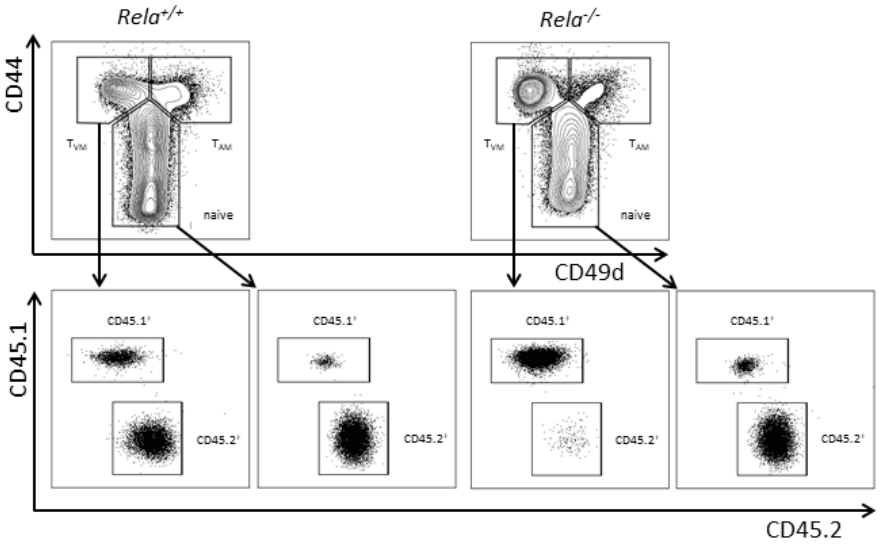


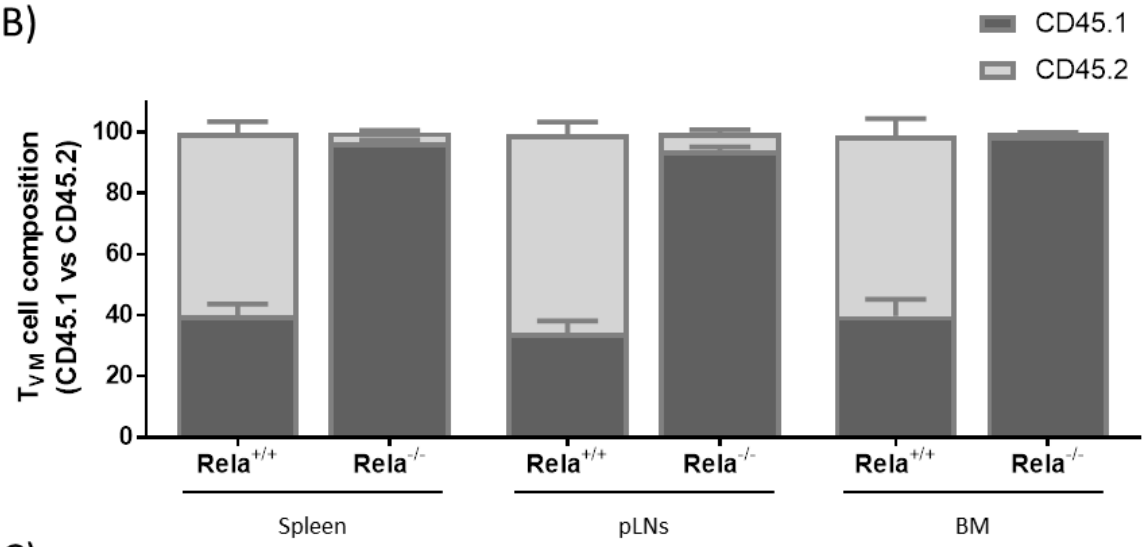
Figure 3.6. *Rela*$^{-/-}$ HSC chimera mice have an expanded T_{VM} cell population. (**A-F**) Flow cytometric analysis of $CD8^+$ T cell subsets from spleen, pLN and BM of *Rela*$^{+/+}$ and *Rela*$^{-/-}$ HSC chimera mice 6-moths post-transplant (n=5-8). (**A**) Percentage of T_{VM} cells of $CD8^+$ T cells in spleen. (**B**) Total number of T_{VM} cells in spleen. (**C**) Percentage of naïve cells of $CD8^+$ T cells in spleen. (**D**) Total number of naïve $CD8^+$ T cells in spleen. (**E**) Percentage of T_{VM} cells of $CD8^+$ T cells in pooled pLNs. (**F**) Percentage of T_{VM} cells of $CD8^+$ T cells in BM. All graphs presented as the mean +/- SEM, statistical significance was determined using Student's T test. *, p≤0.05; **, p≤0.01; ***, p≤ 0.001. pLNs=peripheral lymph nodes; BM=bone marrow; T_{VM}=$CD8^+CD44^{hi}CD49d^{lo}$ Virtual Memory cells.

Figure 3.7. *Rela*$^{-/-}$ HSCs have an intrinsic defect in the generation of T_{VM} cells (**A-C**) Flow cytometric analysis of CD8^{+} T cell subsets from spleen, pLN and BM of *Rela*$^{+/+}$ and *Rela*$^{-/-}$ HSC chimera mice 6-moths post-transplant (n=5-8). (**A**) Representative plot of getting strategy to determine residual (CD45.1) and donor HSC-derived (CD45.2) CD8^{+} T cell proportions. (**B**) T_{VM} cell composition (CD45.1 vs CD45.2) in spleen, pooled pLNs and BM. (**C**) Naïve CD8^{+} T cell composition (CD45.1 vs CD45.2) in spleen, pooled pLNs and BM. pLNs=peripheral lymph nodes; BM=bone marrow; T_{VM}=CD8^{+}CD44^{hi}CD49d^{lo} Virtual Memory cells.

A)


Rela+/+
Rela-/-
CD44
TVM
TAM
naive
CD49d
CD45.1
CD45.2
CD45.1+
CD45.2+

B)


CD45.1
CD45.2
TVM cell composition (CD45.1 vs CD45.2)
100
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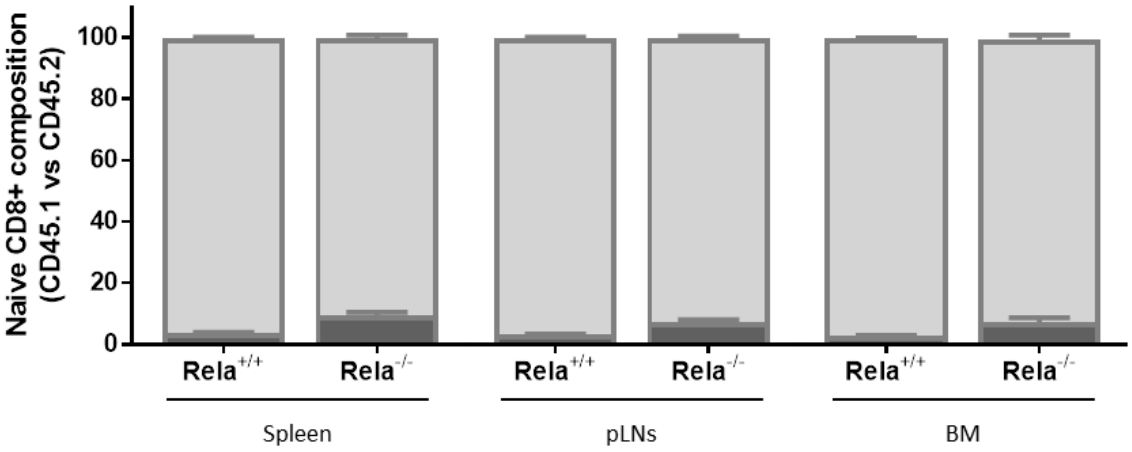
Rela+/+
Rela-/-
Spleen
pLNs
BM

C)


Naive CD8+ composition (CD45.1 vs CD45.2)
100
80
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20
0
Rela+/+
Rela-/-
Spleen
pLNs
BM

that survive radio-ablation undergoing homeostatic expansion in response to the niche created by the death of the remaining host T cells. However, with virtually all naïve $CD8^+$ T cells in *Rela*$^{-/-}$ HSC chimeras derived from donor HSCs (Fig.3.7C), but an almost complete absence of *Rela*$^{-/-}$ donor HSC-derived T_{VM} cells (Fig.3.7B), it appeared that the loss of RelA leads to the impaired generation and/or survival of the T_{VM} cell population, a scenario that would provide residual host-derived *Rela*$^{+/+}$ T_{VM} cells with a competitive advantage.

To better understand how the absence of RelA might influence the dynamics of the T_{VM} cell population in *Rela*$^{-/-}$ HSC chimeras, mice were analyzed at earlier (3 month) and later (11 month) times post-HSC engraftment (Fig.3.8A-B). At 3-months post-HSC engraftment, the number of *Rela*$^{-/-}$ $CD45.2^+$ T_{VM} cell was considerably less than that of the *Rela*$^{+/+}$ T_{VM} counterparts (Fig.3.8A). Between 3 and 6-months post-engraftment, the sizes of both the donor *Rela*$^{+/+}$ and *Rela*$^{-/-}$ T_{VM} populations decreased and continued to do so out to 11-months after HSC engraftment. Unlike the continued presence of donor derived T_{VM} cells in mice receiving *Rela*$^{+/+}$ HSC 11 months after engraftment, in the *Rela*$^{-/-}$ HSC chimeras there was an almost complete absence of donor HSC-derived T_{VM} cells. Given donor HSC-derived *Rela*$^{+/+}$ and *Rela*$^{-/-}$ T_{VM}s appear to decrease at similar rates between 3 and 11 months post-engraftment (Fig.3.8A), the almost complete absence of $CD45.2^+$ *Rela*$^{-/-}$ T_{VM} cells in chimeras at the 11 month time point is likely due, in part, to the *Rela*$^{-/-}$ T_{VM} population already being significantly smaller at the earliest (3 month) post-engraftment time point examined. There are several potential explanations for a smaller T_{VM} cell population in the *Rela*$^{-/-}$ HSC chimeras at the 3 months post-engraftment time point. The most likely reason is that the efficiency with which *Rela*$^{-/-}$ naïve $CD8^+$ precursors become T_{VM} cells is reduced.

3.2.6 Rela$^{-/-}$ T_{VM} cells in HSC chimeras exhibit reduced expression of IL-2Rβ (CD122)

In response to lymphopenia, naïve $CD8^+$ T cells can undergo homeostatic proliferation and differentiate into T_{VM} cells (Kieper and Jameson, 1999; Cho et al, 2000; Goldrath et al, 2000; Haluszczak et al, 2009).

Based on the findings in the *Rela*$^{-/-}$ HSC chimeric mouse model, the generation of T_{VM} cells in a lymphopenic environment did occur, albeit with an apparently reduced efficiency in the absence of RelA. Certainly, the substantial number of donor HSC-derived naïve CD8$^+$ T cells in *Rela*$^{-/-}$ HSC chimeric mice (Fig.3.6D&3.7C) rules out a lack of T_{VM} precursor cells as the reason for fewer *Rela*$^{-/-}$ T_{VM}s. However, a progressive decline in CD45.2$^+$ T_{VM} cell numbers in *Rela*$^{-/-}$ HSC chimeras that is accompanied by an ongoing expansion of residual WT (CD45.1$^+$) T_{VM} cells (Fig.3.8), a phenomenon not seen in the *Rela*$^{+/+}$ HSC chimeras despite the *Rela*$^{+/+}$ CD45.2$^+$ T_{VM} cell population also dropping over time, does suggest *Rela*$^{-/-}$ T_{VM} cells are competitively disadvantaged in a lymphopenic environment containing radio-resistant wild-type CD45.1$^+$ T_{VM} cells. Therefore, RelA could also be contributing to the maintenance of T_{VM} cells. Obvious mechanisms that could account for the reduced competitiveness of *Rela*$^{-/-}$ T_{VM} cells are impaired T_{VM} survival and/or homeostatic proliferation. Much like antigen-induced memory CD8$^+$ (T_{AM}) cells, the maintenance of the peripheral T_{VM} cell population is highly dependent on IL-15 (Judge et al, 2002; Kennedy et al, 2002; Rubinstein et al, 2008; Sosinowski et al, 2013; White et al, 2016). The signals provided by IL-15 are transmitted through the IL-15R, which consists of the IL-15R α-chain (CD215), the common β-chain (CD122) and the common γ-chain (γc; CD132); the latter two of which are shared with the IL-2 receptor (Surh and Sprent, 2008; Castro et al, 2011). As a first pass at understanding why the RelA-deficient T_{VM} cells in *Rela*$^{-/-}$ HSC chimeric mice appear to be outcompeted by the surviving radio-resistant wild-type host T_{VM} cells, the expression of CD122 on CD8$^+$ T cell subsets in the spleen of *Rela*$^{+/+}$ and *Rela*$^{-/-}$ HSC chimeras was examined by flow cytometry. Analysis of CD122 expression on T_{VM} cells in *Rela*$^{+/+}$ or *Rela*$^{-/-}$ chimeric mice 3-months post-engraftment (Fig.3.9A-B) showed as expected that the residual host (CD45.1$^+$) T_{VM} cells in both groups of chimeric mice maintain an equivalent pattern of CD122 expression. By contrast, T_{VM} cells in mice receiving *Rela*$^{-/-}$ HSCs have a sub-population of donor HSC-derived (CD45.2$^+$) T_{VM} cells that express reduced levels of CD122 (Fig.3.9B), whereas donor *Rela*$^{+/+}$ HSC-derived T_{VM} cells display a CD122 expression pattern comparable with that of the residual host

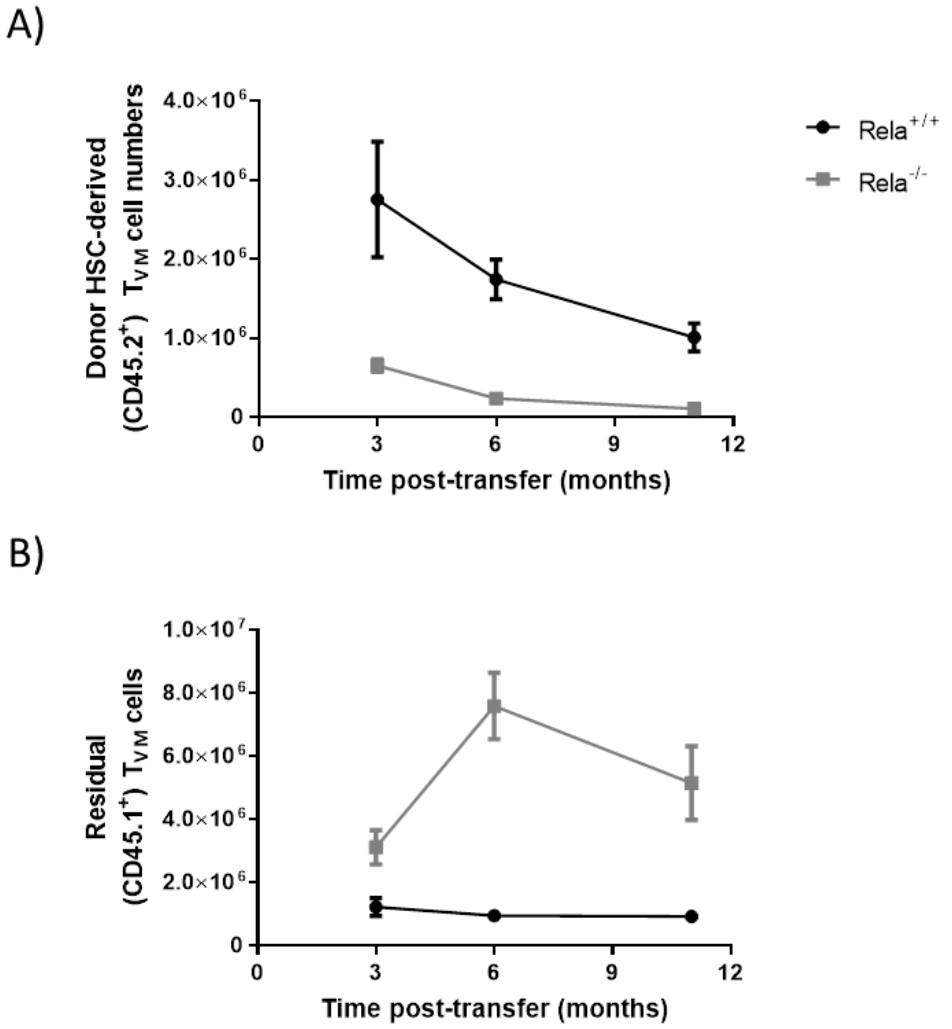


Figure 3.8. *Rela*$^{-/-}$ HSC chimera mice fail to maintain a population of donor HSC-derived T_{VM} cells. (**A-B**) Timecourse analysis of donor HSC-derived (CD45.2) and residual (CD45.1) T_{VM} cell populations in the spleen of *Rela*$^{+/+}$ and *Rela*$^{-/-}$ HSC chimera mice at 3, 6 and 11-moths post-transplant (n=3-8). (**A**) Timecourse analysis of donor HSC-derived (CD45.2^{+}) T_{VM} cell numbers. (**B**) Timecourse analysis of residual (CD45.1^{+}) T_{VM} cell numbers. pLNs=peripheral lymph nodes; BM=bone marrow; T_{VM}=CD8^{+}CD44^{hi}CD49d^{lo} Virtual Memory cells.

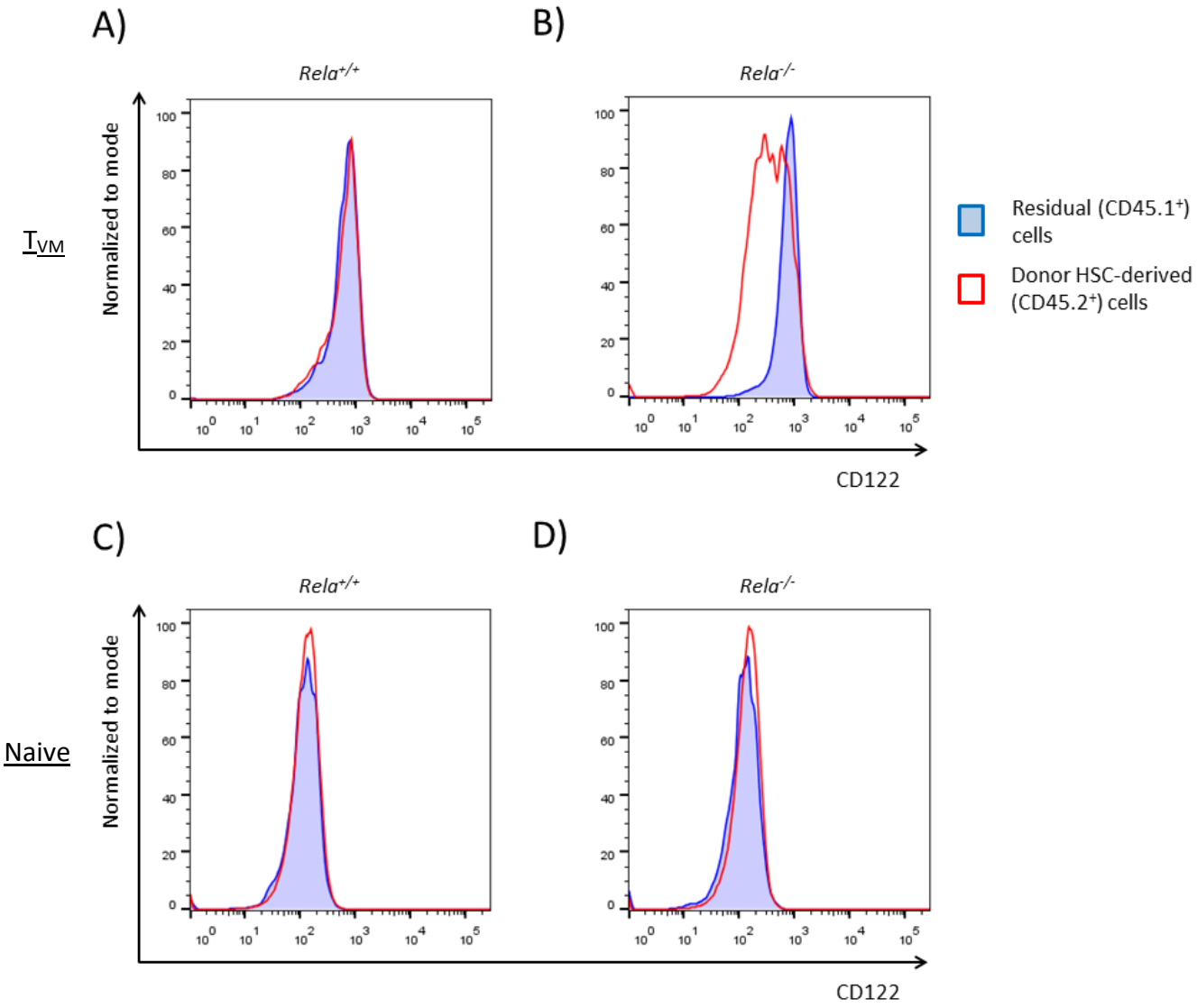


Figure 3.9. *Rela*$^{-/-}$ HSC-derived T_{VM} cells express reduced levels of CD122 (**A-D**) Flow cytometric analysis of residual (CD45.1^{+}) and donor HSC-derived (CD45.2^{+}) T_{VM} and naïve CD8^{+} T cells from the spleen of *Rela*$^{+/+}$ and *Rela*$^{-/-}$ HSC chimera mice 3-months post-transplant (n=3). (**A**) Expression levels of IL-2RB (CD122) on residual (CD45.1) and donor-derived (CD45.2) T_{VM} cells in *Rela*$^{+/+}$ HSC chimeras. (**B**) Expression levels of IL-2RB (CD122) on residual (CD45.1) and donor-derived (CD45.2) T_{VM} cells in *Rela*$^{-/-}$ HSC chimeras.). (**C**) Expression levels of IL-2RB (CD122) on residual (CD45.1) and donor-derived (CD45.2) naïve CD8^{+} T cells in *Rela*$^{+/+}$ HSC chimeras. (**B**) Expression levels of IL-2RB (CD122) on residual (CD45.1) and donor-derived (CD45.2) naïve CD8^{+} T cells in *Rela*$^{-/-}$ HSC chimeras. T_{VM}=CD8^{+}CD44^{hi}CD49d^{lo} Virtual Memory cells.

CD45.1$^+$ T_{VM} cells (Fig.3.9A). The finding that a significant proportion of *Rela*$^{-/-}$ HSC-derived T_{VM} cells exhibit a reduction in CD122 expression may indicate that the loss of *Rela*$^{-/-}$ T_{VM} cells in these mice is, in part, due to an inability of these cells to effectively compete with residual (*Rela*$^{+/+}$) T_{VM} cells for those cytokines that utilize CD122 to maintain T_{VM} cell homeostasis.

3.3 Discussion

3.3.1 NF-κB1 regulates the homeostasis of T_{VM} cells, in part, via mechanisms that serve to suppress inflammation

The role that NF-κB transcription factors play in the development of MP CD8$^+$ T cells has been investigated in a number of studies utilizing various mutant mouse models that employ a 'loss of function' approach. Both mIκB-α and *Ikkβ*$^{-/-}$ mice, exhibit a marked reduction of MP CD8$^+$ T cells in the spleen and pLNs, as well as a failure of naïve CD8$^+$ T cells to expand when adoptively transferred into *Rag1*$^{-/-}$ recipients (Hettmann et al, 2003; Schmidt-Supprian et al, 2004). This is in direct contrast to a more recent study utilizing mice deficient for the NF-κB family member NF-κB1, which exhibit a marked expansion of a MP CD8$^+$ T cell population in the thymus and peripheral immune organs (Gugasyan et al, 2012). In addition to the previous characterization of these cells as a population that express high levels of CD122 on the surface and upregulate the memory T cell-associated transcription factor Eomes (Gugasyan et al, 2012), preliminary data obtained from this research project indicates that a portion of the expanded MP CD8$^+$ T cell population in *Nfkb1*$^{-/-}$ mice phenotypically resemble T_{VM} cells (Fig.3.3). Consistently low expression of CD49d on the majority of the MP cell population in *Nfkb1*$^{-/-}$ mice as well as a marked increase in the number of NKG2D$^+$ antigen-inexperienced MP CD8$^+$ T cells indicates that the loss of NF-κB1 results in a significant expansion of the T_{VM} cell population (Fig.3.4A). However, it would appear that the greatest contribution to the expanded MP CD8$^+$ T cell population in *Nfkb1*$^{-/-}$ mice is by an

increase in the numbers of NKG2D$^-$ antigen-inexperienced MP CD8$^+$ T cells. While the field awaits additional ways of distinguishing T_{VM} and T_{IM} cells, it is difficult to determine, with certainty, whether the loss of NF-κB1 predominantly drives an expansion of the NKG2D$^-$ T_{IM} cell population, or if this expanded MP CD8$^+$ T cells represents a T_{VM} cell population comprised of both NKG2D$^+$ and NKG2D$^-$ subsets. Evidence to support the latter is two-fold. Firstly, the studies outlined above investigating the role of NF-κB1 in T_{VM} cell homeostasis were conducted using mice on a C57BL/6 background, a strain in which T_{IM} cells make up only a minor fraction of the MP CD8$^+$ T cell population (Weinrich et al, 2010). Secondly, the absence of NF-κB1 does not alter the number of IL-4 producing PLZF$^+$ thymocytes, or increase IL-4 output by these cells (Gugasyan et al, 2012), two mechanisms that strongly influence T_{IM} cell development and maintenance (Verykokakis et al, 2010; Weinrich et al, 2010; Lee et al, 2011). Given that the number of NKG2D$^-$ antigen-inexperienced MP CD8$^+$ T cells is increased more than 5-fold in NF-κB1-deficient mice (Fig.3.4B), it therefore seems unlikely that this is just due to an expansion of T_{IM} cells. More likely, NKG2D is a marker upregulated on certain T_{VM} cells in the periphery and the expanded antigen-inexperienced MP CD8$^+$ T cell population in *Nfkb1*$^{-/-}$ mice in the main comprises NKG2D$^+$ and NKG2D$^-$ T_{VM} cells, with potentially some contribution made by NKG2D$^-$ T_{IM} cells.

Recent unpublished findings (S. Gerondakis, personal communication) have shown that *Nfkb1*$^{-/-}$ mice promote the enhanced division of MP CD8$^+$ T cells, irrespective of whether these cells do or do not express NF-κB1. In fact, *Nfkb1*$^{-/-}$ MP CD8$^+$ T cells divide less efficiently than their WT counterparts when adoptively transferred into *Nfkb1*$^{-/-}$ hosts (S. Gerondakis, unpublished results). These collective findings indicate that NF-κB1 may regulate the homeostasis of MP CD8$^+$ T cells via both cell-intrinsic and cell-extrinsic mechanisms. I therefore proposed that the expansion of MP CD8$^+$ T cells in *Nfkb1*$^{-/-}$ mice may be influenced, in part, by T_{VM} cell-extrinsic inflammatory cytokine signals linked to the increased tissue inflammation that has been reported to occur in these mice (Bernal et al, 2014), and supported by our findings showing the inflammasome regulated cytokines IL-1β and IL-18 are also elevated in *Nfkb1*$^{-/-}$

mice (Supplementary Fig.2A). To determine if the T_{VM} cell population is influenced by inflammasome generated cytokines in *Nfkb1*$^{-/-}$ mice, *Nfkb1*$^{-/-}$*Asc*$^{-/-}$ mice were generated. It was found that in the absence of ASC, the increased frequency of T_{VM} cells in *Nfkb1*$^{-/-}$ mice is reduced, but not normalized in both the spleen and pLNs (Fig.3.5A-C). The most plausible explanation for the reduced frequency of T_{VM} cells in *Nfkb1*$^{-/-}$*Asc*$^{-/-}$ mice is that the loss of ASC activity inhibits the T_{VM} cell-extrinsic, ASC-dependent inflammasome production of IL-1β and IL-18, thereby reducing the extent of systemic inflammation that occurs from the loss of NF-κB1. This hypothesis is supported by our finding that both IL-1β and IL-18 levels in the serum of *Nfkb1*$^{-/-}$*Asc*$^{-/-}$ mice are significantly less than those in *Nfkb1*$^{-/-}$*Asc*$^{+/+}$ mice (Supplementary Fig.2B).

However, indirect roles for these ASC-dependent cytokines that promote an inflammatory environment in *Nfkb1*$^{-/-}$ mice, or inflammasome-independent roles for ASC in immune responses could also account for the reduced T_{VM} cell population in the double knockout mice (Ippagunta et al, 2010). In the case of the latter, mice deficient in ASC (*Asc*$^{-/-}$), but not mice lacking caspase-1, or the NLR family member NLRP3 (*Casp1*$^{-/-}$ and *Nlrp3*$^{-/-}$ mice respectively) show impaired granuloma formation and immunity to chronic *Mycobacterium tuberculosis* infections, as well as altered disease progression in experimental models of rheumatoid arthritis and experimental autoimmune encephalomyelitis (EAE) (McElvania Tekippe et al, 2010; Ippagunta et al, 2010; Shaw et al, 2010). *Asc*$^{-/-}$ mice also exhibit impaired antigen presentation by dendritic cells (DCs) and T cell-intrinsic defects such as impaired lymphocyte migration (Ippagunta et al, 2010). Furthermore, interpreting the precise mechanisms responsible for the phenotypes see in our study is further complicated by ASC also being used by the NLRC4 inflammasome to process pro-IL-1β and pro-IL-18 (Guo et al, 2015). Nevertheless, a recent study that suggests T_{VM} cells will be responsive to IL-18 based on the higher expression of *Il18r1* (IL-18R1) and *Il18rap* (IL-18R accessory protein) in T_{VM} cells when compared to naïve $CD8^+$ T cells (White et al, 2016), leads us to favor reduced IL-18 production in in *Nfkb1*$^{-/-}$*Asc*$^{-/-}$ mice as the basis for the reduced frequency of T_{VM} cells in

this strain when compared with *Nfkb1*$^{-/-}$ mice. However, the failure of ASC inactivation to return the T_{VM} cell numbers in *Nfkb1*$^{-/-}$ mice to levels seen in wild-type animals, highlights the likely importance heightened levels of ASC-independent inflammatory cytokines such as TNF, IL-6 and IFN-γ, play in the expansion of T_{VM} cells in *Nfkb1*$^{-/-}$ mice. In conclusion, our findings, in conjunction with unpublished communications from collaborators and published data support our hypothesis that NF-κB1 regulates the homeostasis of T_{VM} cells, in part, via mechanisms that act to suppress ASC-dependent inflammation.

Further work is required to determine the entire gamut of inflammatory signals that promote T_{VM} cell expansion in *Nfkb1*$^{-/-}$ mice, the types of cells that produce these signals, and how $CD8^+$ T cell-intrinsic NF-κB1 expression controls T_{VM} cell generation and maintenance. As many of these questions are already being addressed by our collaborators via means of adoptive transfer studies using donor and recipient mice that have different combinations of wild-type and knockout *Nfkb1* and *Asc* alleles, the remaining studies outlined in this thesis will address the roles of the canonical NF-κB family member RelA in the development and function of T_{VM} cells.

3.3.2 RelA activity is required for the generation and/or homeostatic maintenance of T_{VM} cells in a chronic lymphopenic environment

Contrasting findings that emerge from previous studies investigating the role of the NF-κB pathway in the generation of MP $CD8^+$ T cell using *Nfkb1*$^{-/-}$ mice, or mIκB-α and *Ikkβ*$^{-/-}$ mouse models, in the main appear to reflect the extent to which canonical NF-κB signaling is inhibited (Hettmann et al, 2003; Schmidt-Supprian et al 2004; Gugasyan et al, 2012). Unlike *Nfkb1*$^{-/-}$ mice, mIκB-α and *Ikkβ*$^{-/-}$ mice are unable to activate multiple NF-κB family members, namely the canonical family members NF-κB1, RelA and cRel. Taken together, these studies provide strong evidence for the non-redundant involvement of multiple NF-κB family members in the generation of MP $CD8^+$ T cells. Considering that antigen-inexperienced T_{VM} cells comprise a large proportion of the MP $CD8^+$ T cell population in adult mice

(Halusczak et al, 2009), the reduced number of the MP $CD8^+$ T cells in mIκB-α and *Ikkβ*$^{-/-}$ mice is likely to be attributed, in part, to a reduction in the T_{VM} cell population. Using *Rela*$^{-/-}$ foetal liver-derived HSC chimeric mice, the initial investigation of the influence the canonical NF-κB family member RelA has on the size of the T_{VM} cell population found that RelA makes a significant contribution to the generation of the T_{VM} cell population. Although *Rela*$^{-/-}$ HSC chimeras exhibit a marked expansion of T_{VM} cells when compared to *Rela*$^{+/+}$ HSC chimeras at 6-months post-engraftment, it was found that the resultant T_{VM} cell population in *Rela*$^{-/-}$ HSC chimeras was almost exclusively comprised of endogenous radiation-resistant T_{VM} cells that underwent re-expansion in the lymphopenic environment created by radio-ablation of the hematopoietic system (Fig.3.7B). The donor HSC-derived *Rela*$^{-/-}$ T_{VM} cells that do develop in *Rela*$^{-/-}$ HSC chimeras were severely diminished in number when compared to the equivalent donor HSC-derived *Rela*$^{+/+}$ T_{VM} cell population that developed in *Rela*$^{+/+}$ HSC chimeras. Whilst this finding shows that RelA plays an important role in T_{VM} cell biology, it was unclear if RelA only serves a developmental function, or is also important in the post-developmental homeostasis of T_{VM} cells. One possible explanation for the RelA-dependent T_{VM} cell phenotype is that this canonical transcription factor assists in the induction of a transcriptional program that promotes naïve $CD8^+$ T cell precursors to differentiate into T_{VM} cells. Clearly, the reduced number of *Rela*$^{-/-}$ T_{VM} cells was not caused by a lack of precursor cells (Fig.3.6D & 3.7C). Moreover, the presence of a minor population of *Rela*$^{-/-}$ T_{VM} cells in *Rela*$^{-/-}$ HSC chimeras at earlier times following post-HSC reconstitution analysis showed that RelA is not absolutely essential for T_{VM} cell differentiation. Rather, if RelA is involved in T_{VM} cell differentiation, the evidence suggested that RelA contributes to the efficiency with which naïve $CD8^+$ T cells differentiate into a T_{VM} cell.

Alternatively, but not mutually exclusive of a potential contribution to the initial generation of T_{VM} cells, is the possibility that RelA promotes the post-developmental homeostatic expansion and maintenance of the T_{VM} cell population by controlling survival and/or proliferative responses. In support of a potential role in T_{VM} survival, RelA has been shown to regulate the survival of activated $CD8^+$ T cells both *in vitro*

and *in vivo* (Mondor et al, 2005). $CD8^+$ T cells that over-express a truncated form of RelA (p65TAD) that lacks the two C-terminal transactivation domains (TADs), but retains the Rel homology domain (RHD) were shown to undergo cell death immediately following antigen-induced activation (Mondor et al, 2005). Consistent with previous reports detailing the induction of NF-κB-regulated gene transcription following TCR signalling (Zheng et al, 2003), the impaired up-regulation of the pro-survival factor Bcl_{xL} was found to contribute to the diminished survival of $CD8^+$ T cells transduced with p65TAD (Mondor et al, 2005). Whilst reinforcing the well characterized role of RelA in cell survival (Hsu et al, 1995; Gerondakis et al, 1999; Gerondakis et al, 2006), the available evidence indicated that it would be unlikely that the same mechanism would account for any defect observed in the homeostatic expansion of *Rela*$^{-/-}$ T_{VM} cells. Whilst RelA-containing NF-κB heterodimers were shown to be constitutively expressed in the nucleus of T_{VM} cells (Fig.3.1B), it is unlikely these complexes were induced by signals transduced via the TCR. Evidence for this conclusion is based, in part, on the characteristically low expression of the alpha subunit of the α4β1 integrin (CD49d) on T_{VM} cells, a surface marker upregulated on T cells following antigen-induced T cell activation and sustained TCR signalling (Haluszczak et al, 2009). Nevertheless, it remains a formal possibility that tonic TCR signals received by T_{VM}s in the periphery through contact with pMHCI complexes could be of sufficient strength to promote the activation and nuclear translocation of RelA-containing heterodimers without up-regulating CD49d expression. Stronger tonic TCR signalling in T_{VM} cells is certainly plausible given the T_{VM} cell population has been shown to express a repertoire of TCRs with a greater affinity for pMHCI complexes than their naïve counterparts (Quinn et al 2016; White et al 2016). This issue was addressed by comparing Nur77 expression in immediate *ex-vivo* T_{VM} and naïve $CD8^+$ T cells. The gene encoding Nur77 (*Nr4a1*) is upregulated in developing and mature T cells following TCR stimulation to levels directly reflective of TCR signalling strength (Moran et al, 2011). Despite RelA being selectively expressed in the nucleus of T_{VM} cells, comparable levels of Nur77 in T_{VM} and naïve $CD8^+$ T cells based on the use of the $Nur77^{GFP}$

reporter mouse strain showed that the level of tonic signalling received by these two populations in the periphery were equivalent (Fig.3.2B). This indicated that the select nuclear expression of RelA heterodimers in T_{VM} cells is not linked to these cells receiving stronger tonic TCR signals. Therefore, it would appear that the constitutive expression of RelA heterodimers in the nucleus of T_{VM} cells was induced by a TCR-independent mechanism.

Whilst being unable to assign specific contributions of RelA to each of the distinct processes of T_{VM} cell differentiation, homeostatic proliferation and survival based on the analysis completed thus far, this model of *Rela* gene-inactivation did identify a possible explanation for the reduced number of *Rela*$^{-/-}$ T_{VM} cells in *Rela*$^{-/-}$ HSC chimera mice. When compared to the equivalent donor HSC-derived T_{VM} cell population in *Rela*$^{+/+}$ HSC chimeras, a significant proportion of the *Rela*$^{-/-}$ T_{VM} cell population in *Rela*$^{-/-}$ HSC chimera mice exhibited a significant reduction in the cell surface levels of CD122, an important component of the receptors that transmits survival and proliferative signals provided by IL-2 and IL-15 (Fig.3.9). Importantly, this reduced expression of CD122 on *Rela*$^{-/-}$ T_{VM} cells was not observed on *Rela*$^{+/+}$ donor HSC-derived T_{VM} cells, or on the residual *Rela*$^{+/+}$ T_{VM} cell population that survived in *Rela*$^{-/-}$ HSC chimeras. T_{VM} cells, much like conventional memory $CD8^+$ T cells, are highly reliant on cytokines, in particular IL-15, for survival and homeostatic maintenance (Schlune et al, 2000; Surh and Sprent, 2008). Survival and proliferative responses induced by IL-15 are mediated via two mechanisms. The first involves soluble IL-15 binding to the high affinity IL-15 receptor (IL-15R), a receptor complex comprising the IL-15Rα-chain (CD215), CD122 and CD132 (common-γ-chain; γc). The second and more common mode of IL-15 signalling employs the trans-presentation of IL-15 by CD215 expressed on monocytes and DCs to a low affinity IL-15R consisting of CD122 and CD132 (Colpitts et al, 2012). These cytokine-receptor binding interactions lead to the recruitment of the receptor-associated tyrosine kinases JAK1 and JAK3 to the intracellular domains of CD122 and CD132. The subsequent recruitment, phosphorylation and activation of STAT-5 transcription factors promotes memory $CD8^+$ T cell survival by

inducing expression of the gene encoding the pro-survival factor Bcl-2 (Nelson et al, 1998; Waldman et al, 2006). The importance of this signalling pathway in the generation and homeostatic maintenance of MP $CD8^+$ T cell is exemplified by studies showing that these cells are significantly reduced in both IL-15-deficient mice and mice with T cells lacking CD122 (Kennedy et al, 2000). Therefore, the selective loss of *Rela*$^{-/-}$ T_{VM} cells in *Rela*$^{-/-}$ HSC chimeras that is accompanied by a considerable expansion of the residual *Rela*$^{+/+}$ T_{VM} cell population, that does not occur to the same extent in *Rela*$^{+/+}$ HSC chimeras, may be contributed to, in part, by the reduced expression of CD122, which would impair the capacity of *Rela*$^{-/-}$ T_{VM} cells to compete with the residual *Rela*$^{+/+}$ T_{VM} cells for IL-15. Whilst naïve $CD8^+$ T cells are less dependent on IL-15 for their survival and homeostatic maintenance, relying instead on the availability of IL-7 and contact with self-pMHCI complexes to maintain their numbers (Surh and Sprent, 2008), naïve $CD8^+$ T cells expressing slightly higher levels of CD122 have been shown to preferentially acquire a T_{VM} cell phenotype in a lymphoreplete environment (White et al, 2016). However, the data showing that T_{VM} cells, but not naïve $CD8^+$ T cells express RelA heterodimers in the nucleus (Fig.3.1), when combined with equivalent expression of CD122 on *Rela*$^{+/+}$ and *Rela*$^{-/-}$ naïve $CD8^+$ T cells (Fig.3.9D), reinforces the idea that the loss of RelA specifically impacts the maintenance rather than generation of T_{VM} cells. Given these findings, I propose that RelA is activated in newly generated T_{VM} cells via TCR-independent mechanisms that serve to up-regulate and/or maintain the expression of CD122 to enhance IL-15 responsiveness. Whilst it is presently unclear if RelA performs this function directly or indirectly, one plausible explanation is that RelA promotes CD122 expression by assisting in the induction of Eomes. IL-15-mediated stimulation through CD122 induces Eomes expression in $CD8^+$ T cells, which in a perpetual regulatory feedback loop, acts to enhance responsiveness to IL-15 by further increasing the expression of CD122 (Boyman et al, 2007). In support of this hypothesis, Eomes-deficient memory $CD8^+$ T cells, like *Rela*$^{-/-}$ T_{VM} cells, express reduced levels of CD122 (Banerjee et al, 2010). Further analysis will need to determine whether or not RelA-deficient T_{VM} cells also exhibit a reduction in the expression of Eomes.

Alternatively, the reduced expression of CD122 on *Rela*$^{-/-}$ T_{VM} cells is a result of a T_{VM} cell-extrinsic influence. The loss of RelA from the entire hematopoietic compartment in the chimeric mice is likely to compromise the proper development or function of various types of immune cells. How the T cell-extrinsic loss of RelA might negatively influence the *Rela*$^{-/-}$ T_{VM} cell population in *Rela*$^{-/-}$ HSC chimeras is currently difficult to explain in the context of T_{VM} cell generation, homeostatic expansion and maintenance. However, precedents do exist for how a loss of RelA in other immune cells impacts CD8^{+} T cell homeostasis. CD4 Foxp3^{+} regulatory T cells (Tregs) have recently been shown to require constitutive activation of RelA to maintain Treg stability and to control Treg effector functions, the later including the capacity to suppress the expansion of conventional T cells in *Rag1*$^{-/-}$ mice (Messina et al, 2016). Although the impaired functional capacity of *Rela*$^{-/-}$ Tregs may provide an explanation for the excessive proliferation of residual *Rela*$^{+/+}$ T_{VM} cells in *Rela*$^{-/-}$ HSC chimeras, such a model would still require a T_{VM} cell-intrinsic contribution by RelA to account for the contrasting *Rela*$^{+/+}$ and *Rela*$^{-/-}$ T_{VM} phenotypes seen in these chimeric mice. These findings in the HSC chimera model also highlight the caveats associated with using *Rela*$^{-/-}$ HSC chimera mice as a model of *Rela* gene-inactivation. In addition to the potential impact of other *Rela*$^{-/-}$ hematopoietic cells, the heightened levels of various inflammatory cytokines generated by radiation may differentially impact the RelA-deficient and RelA-sufficient T_{VM} cell populations. For example, the well characterised role RelA plays in counteracting apoptotic signals mediated by TNF (Hsu et al, 1995), a cytokine whole levels are elevated following radio-ablation of the hematopoietic system, could create a survival disadvantage for *Rela*$^{-/-}$ T_{VM} cells. These caveats highlight the various limitations of using *Rela*$^{-/-}$ HSC chimera mice as a model for investigating the roles played by RelA in a specific type of immune cell, reinforcing the need to substantiate the finding on the roles of RelA in T_{VM} cell generation and maintenance obtained from chimera models using alternative approaches (*Chapter 4*).

3.4 Conclusion

In summary, data presented here and in previous studies provide compelling evidence to support the hypothesis that the canonical NF-κB family members NF-κB1 and RelA play opposing regulatory roles in T_{VM} cell generation and homeostatic maintenance. NF-κB1 has been found to regulate the homeostasis of T_{VM} cells, in part, via T cell-extrinsic mechanisms that act to suppress inflammation (S. Gerondakis, unpublished results). In contrast, RelA appears to contribute to the generation and/or homeostatic maintenance of the T_{VM} cell population, in part, by promoting CD122 expression and enhancing the responsiveness of T_{VM} cells to survival and proliferative signals provided by IL-15. Further work is required to determine whether the reduced expression of CD122 on RelA-deficient T_{VM} cells results in decreased responsiveness to IL-15 signals or whether RelA contributes to the generation and/or maintenance of T_{VM} cell via other mechanisms.

Chapter 4:

The selective inactivation of RelA in T cells alters the homeostatic maintenance of T_{VM} cells

4.1 Introduction

The memory-phenotype (MP) $CD8^+$ T cell population in mice is comprised of conventional antigen-primed (T_{AM}) and antigen-inexperienced (T_{VM}) memory T cell subsets. Whereas T_{AM} cells are generated following the expansion and contraction of effector $CD8^+$ T cell responses, T_{VM} cells arise from naïve $CD8^+$ precursors via a process of cytokine-driven homeostatic proliferation. Despite any prior contact with their cognate antigen failing to promote cellular activation, as is typically seen in an immune response to an infectious agent, T_{VM} cells share many characteristics with T_{AM} cells, including expression of the transcription factor Eomes and high cell surface expression of CD122, an essential component of the receptors for the common γ-chain cytokines IL-2 and IL-15 (Haluszczak et al, 2009; Lee et al, 2011; Akue et al, 2012). T_{VM} cells also rapidly proliferate in response to antigen stimulation and are capable of controlling certain infections, such as *Listeria monocytogenes,* albeit not as effectively as T_{AM} cells (Lee et al, 2013). In mice, T_{VM}s can be phenotypically distinguished from their antigen-experienced counterparts by the level of CD49d expression, which is upregulated on T_{AM} cells in response to sustained TCR signaling, but expressed at low levels on antigen-inexperienced T_{VM} cells (Haluszczak et al, 2009). However, unlike other $CD49d^{lo}$ $CD8^+$ MP T cell populations, T_{VM} cells are capable of mediating antigen-independent cytotoxic immunity in response to cytokine stimulation, endowing these T cells with both innate and adaptive immune cell properties (White et al, 2016). The ability of T_{VM} cells to mediate rapid, antigen non-specific protective immunity is thought to provide protection to the host during times of lymphopenia, such as that which occurs naturally in neonates, or develops in old age. An initial study investigating the ontology of T_{VM} cells found that these antigen-inexperienced MP $CD8^+$ T cells first arise in the periphery of neonatal mice as a result of newly generated naïve $CD8^+$ T cells undergoing lymphopenia-induced homeostatic proliferation (Akue et al, 2012). These T_{VM} cells accumulated over a period of 2-3 weeks, peaking at around ~30% of the peripheral $CD8^+$ T cell compartment, after which the population stabilized at between 10-20% of the total $CD8^+$ T cell population by 6-weeks of age (Akue et

al, 2012). Although remaining fairly constant in number throughout most of adult life, the T_{VM} cell population begins to increase in old age to levels reportedly approaching >40% of the $CD8^+$ T cell population in 20-month-old mice (Chiu et al, 2013).

More recently, a study using time-stamping methods that involve the inducible expression of a fluorescent protein (Red Fluorescent Protein, RFP) to identify $CD8^+$ T cells that develop at specific times after birth has challenged the importance lymphopenia-induced proliferation plays in the generation of T_{VM} cells (Smith et al, 2018). Instead, they proposed that naïve $CD8^+$ T cells are generated in distinct waves that have different cell-intrinsic propensities to become T_{VM} cells. At birth, T cells develop from foetal HSCs, whereas after the neonatal period, T cells are derived from adult HSCs (Wang et al, 2016). The first wave of naïve $CD8^+$ T cells that develop at birth were found to acquire a T_{VM} cell phenotype far more readily than those T cells developing post-neonatally (Smith et al, 2018). When time-stamped thymi from newborn mice that produce RFP^+ T cells were transplanted into time-stamped adult mice that generate YFP (Yellow Fluorescent Protein) expressing $CD8^+$ T cells, $CD8^+$ T cells from newborn mice underwent more proliferation and preferentially developed into T_{VM} cells. This indicated that $CD8^+$ T cells of earlier developmental origin had a greater propensity to become T_{VM} cells, a fate that was determined independently of the peripheral environment.

Although T_{VM} cell development occurs independently of activation by cognate antigen, naïve $CD8^+$ T cells expressing TCRs with a greater affinity for self-peptide in complex with MHC class I glycoproteins (pMHC-I) have been shown to preferentially acquire a T_{VM} cell phenotype in the periphery (Quinn et al, 2016; White et al, 2016). CD5, a cell surface protein that serves to negatively regulate TCR signaling, is expressed on T cells at levels that directly correlate with the affinity a TCR has for self pMHC-I, thereby making it a useful surrogate indicator of TCR affinity for self-antigens (Azzam et al, 1998). A recent study from the Kedl laboratory showed that naïve $CD8^+$ T cells with higher affinity for self-antigens ($CD5^{hi}$),

which also display an increased responsiveness to IL-15 as indicated by increased cell surface expression of CD122, more readily acquired a T_{VM} cell phenotype when adoptively transferred into lympho-replete congenic hosts (White et al, 2016). This finding demonstrated that homeostatic proliferation and the differentiation of $CD5^{hi}$ naïve $CD8^+$ T cell into T_{VM} cells can still occur within a lympho-replete environment. Based on these findings, White and colleagues proposed that those $CD8^+$ T cells selected in the thymus bearing TCRs with higher affinity for self-pMHC-I ligands acquired a cellular program that preferentially primed them for T_{VM} cell differentiation.

The enhanced responsiveness of these differentiated T_{VM} cells to various inflammatory cues and their inherent capacity to mediate both cytotoxic and innate-like effector functions in the absence of antigen receptor signaling appears to be driven predominantly by IL-15 (Sosinowski et al, 2013; White et al, 2016). Indeed, the indispensable role of IL-15 in T_{VM} cell development and maintenance is well established, with T_{VM} cell numbers severely reduced in both IL-15-deficient mice and mice with T cells lacking CD122 (Kennedy et al, 2000). IL-15 signaling in naïve $CD8^+$ T cells occurs via two modes. One involves signaling through the high affinity IL-15 receptor (IL-15R), a receptor complex comprising the IL-15Rα-chain (CD215), CD122 and CD132 (common-γ-chain; γc). Alternatively, signaling can occur via the trans-presentation of IL-15 by CD215 expressed on monocytes and DCs to a low affinity IL-15R consisting of CD122 and CD132 expressed on CD8 T cells (Colpitts et al, 2012). IL-15 signaling in naïve $CD8^+$ T cells leads to the induction of the transcription factor Eomes, which in addition to controlling the expression of numerous genes associated with effector CD8 T cell differentiation, promotes the increased cell surface expression of CD122 and enhanced responsiveness to IL-15 (Boyman et al, 2007). Trans-presentation of IL-15 by lymphoid tissue-resident $CD8\alpha^+$ DCs has been identified as the main mechanism driving T_{VM} development from naïve $CD8^+$ T cells in the periphery (Sosinowski et al, 2013). This process of IL-15 trans-presentation by DCs and inflammatory monocytes is highly dependent on type I interferon (IFN) signals (Mattei et al, 2001). A more direct role for type I IFN signaling in T_{VM} cell development has

also been described in which activation of the ISG3 transcriptional complex consisting of STAT1, STAT2 and IFN regulatory factor 9 (IRF9), regulates Eomes expression in CD8 T cells, in turn leading to the induction of CD122 and increased responsiveness to IL-15 (Martinet et al, 2015). In addition to IL-15 and type I IFNs, other cytokines have either been shown to play a role, or are implicated in T_{VM} cell generation and maintenance. IL-7, which is crucial for maintaining naïve T cells numbers under normal non-lymphopenic conditions, becomes elevated following T cell depletion and drives the proliferation and differentiation of naïve $CD8^+$ T cells into cells resembling T_{VM} cells (Schluns et al, 2000). Furthermore, transgenic over-expression of IL-7 in mice, in addition to massively increasing T cell numbers, results in a dominance of the $CD8^+$ T cell compartment by cells with a similar phenotype to T_{VM} cells (Kieper et al, 2002). Crossing this transgenic strain onto an IL-15-deficient background showed that this expanded MP cell population could be generated and maintained in the absence of IL-15, reinforcing the notion that multiple cytokines can control T_{VM} cell development and homeostasis. Another cytokine thought to be involved in T_{VM} cell generation and maintenance is IL-4, a conclusion based on the findings that the frequency of MP $CD8^+$ T cells is reduced in mice lacking either IL-4 or the IL-4R, but conversely is increased in mice overexpressing IL-4 (Akue et al, 2012; Kurzweil et al, 2014). However, there is uncertainty in the field about the contribution IL-4 makes to T_{VM} cell homeostasis, given the existence of another population of antigen-inexperienced MP $CD8^+$ T cells in mice, termed 'innate memory' (T_{IM}) $CD8^+$ T cells, that have been shown to be highly dependent on IL-4 (Weinreich et al, 2010; Gordon et al, 2011; Lee et al, 2011). Despite the striking resemblance to T_{VM} cells, T_{IM} cells are a distinct subset of antigen-inexperienced MP cell that arise in lympho-replete mice. To date, perhaps the differences that best distinguish T_{VM} and T_{IM} cells is their distinct sites of development and their dependency on different common γ-chain cytokines (White et al, 2017). Whereas T_{VM}s develop in the periphery from naïve $CD8^+$ T cells in response to IL-15, T_{IM} cells are generated in the thymus from CD8 SP thymocytes in response to IL-4 produced by $PLZF^+$ NKT cells (Raberger et al, 2008; Weinreich et al, 2010).

The dependency of T_{IM} cell development on IL-4 produced by $PLZF^+$ NKT cells is exemplified by findings that these cells are absent in the thymi of mice deficient for IL-4, PLZF or NKT cells (Verykokakis et al, 2010; Weinrich et al, 2010). In fact, numerous studies have shown that the frequency of T_{IM} cells in the thymus of various strains of transgenic and non-transgenic mice is directly dependent on the availability of IL-4 produced by $PLZF^+$ NKT cells. For example, higher numbers of T_{IM} cells in BALB/C compared with C57BL/6 mice coincide with three to five times more IL-4-producing $PLZF^+$ cells being present in the thymus of a BALB/C mouse (Weinrich et al, 2010). Moreover, the frequency of T_{IM} cells was found to be increased in C57BL/6 mice deficient for Kruppel-like factor 2 (KLF2), CREB binding protein (CBP), Inhibitor of DNA binding 3 (Id3), or the Tec family of non-receptor tyrosine kinases Itk and Rlk (Atherly et al, 2006; Broussard et al, 2006; Berg et al, 2007; Lee et al, 2011). All of these genetic changes coincide with a relative increase in the number of $PLZF^+$ cells residing in the thymus, which in turn drives the expansion of T_{IM} cells through increased IL-4 production (Raberger et al, 2008; Lee et al, 2011). IL-4 was found to promote a memory phenotype in developing CD8 SP thymocytes via a 'by-stander effect' which induced Eomes expression through the Akt and STAT-6 signaling pathways (Carty et al, 2014). Despite its crucial role in promoting T_{IM} cell development, the influence of IL-4 on the generation, expansion and homeostatic maintenance of T_{VM} cells in the periphery is less clear. The reduced number of peripheral MP $CD8^+$ T cells in IL-4-deficient mice suggests that IL-4 may play some role in regulating the T_{VM} cell population (Akue et al, 201), although the absence of a peripheral MP $CD8^+$ T cell population in IL-15-deficient mice questions the capacity of IL-4 alone to drive T_{VM} cell development (Kennedy et al, 2000; White et al, 2017). Instead, exposure to IL-4 in the thymus may enact a cellular program in developing CD8 T cells that enhances sensitivity to IL-15 (Carty et al, 2014; White et al, 2017). Much like the mechanism by which type I IFN signaling promotes T_{VM} cell development, exposure to IL-4 may increase CD122 expression via the induction of Eomes, making newly generated naïve $CD8^+$ T cell more responsive to IL-15 stimulation in the periphery (Carty et al, 2014; Martinet et al, 2015). In addition, but

not mutually exclusive of a role in priming T_{VM} cell development, there is evidence to suggest that IL-4 can promote the expansion of existing T_{VM} cells. Transcription of the gene encoding the IL-4 Rα-chain (CD124) is significantly increased in differentiated T_{VM} cells compared to naïve $CD8^+$ T cell precursors, indicating a likelihood of greater sensitivity to signals provided by IL-4 (White et al, 2016). Furthermore, the peripheral MP $CD8^+$ T cell population is expanded in mice that either overexpress IL-4, or are administered exogenous IL-4 complexes (Kurzweil et al, 2014). However, given the high degree of similarity in the cell surface receptor expression signature between T_{VM} and T_{IM} cells ($CD44^{hi}CD122^{hi}CD49d^{lo}$), the rate at which T_{IM} cells emigrate from the thymus and contribute to the peripheral antigen-inexperienced MP $CD8^+$ T cell pool remains to be determined. Consequently, it is difficult to conclude whether changes to the peripheral MP $CD8^+$ T cell populations of mice in studies investigating the influence of IL-4 are due to alterations in the homeostasis of T_{VM} cells, T_{IM} cells, or both. Currently, the clearest means of distinguishing between T_{VM} and T_{IM} cells is the cell surface marker NKG2D, which has only recently been reported to be expressed on T_{VM}s, but not T_{IM} cells (White et al, 2017). Using this criterion in future studies to identify T_{VM} and T_{IM} cells can hopefully clarify the distinct roles IL-4 serves in regulating these two antigen-inexperienced MP $CD8^+$ T cell populations, as well as determining with greater accuracy the various factors that influence the development and maintenance of T_{VM} cells. Albeit, the practicality of using NKG2D to distinguish T_{VM} and T_{IM} cells is yet to be determined. Using next-generation transcriptome sequencing (RNAseq), White and colleagues (2016) showed that T_{VM} ($CD8^+CD44^{hi}CD49d^{lo}$) cells within the spleens of C57BL/6 WT mice expressed a significantly higher number of transcripts for the gene encoding NKG2D when compared to naïve ($CD44^{lo}$) $CD8^+$ T cells. However, neither this group, nor any other research group has published data showing NKG2D expression on a per cell basis within the $CD8^+CD44^{hi}CD49d^{lo}$ T cell population to validate the proposal that NKG2D distinguishes all T_{VM} cells from T_{IM} cells. Given that T_{IM} cells have been shown to represent only a minor fraction of the MP $CD8^+$ T cell population in C57BL/6 mice (Weinrich et al,

2010), further use of the T_{VM} cell terminology, unless specified, will refer to the total peripheral $CD8^{+}CD44^{hi}CD49d^{lo}$ T cell population.

As detailed above, numerous cytokines have either been shown to definitely play a role, or have been implicated in T_{VM} cell generation and maintenance. In addition to the catalogue of cytokines already shown to regulate T_{VM} cell homeostasis, it is clear that there are many more yet to be investigated in detail. For example, high cell surface expression of the receptors for IL-18 and TNF on T_{VM} cells suggests a strong influence of these inflammatory cytokines on the T_{VM} cell population (White et al, 2016). This finding fits well with the recent characterization of T_{VM} cells as a population of early immune effectors that possess a heightened capacity to sense and promote inflammation (White et al, 2017). However, whereas the importance of different cytokines in T_{VM} cells homeostasis is well understood, the transcription factors that promote the development and maintenance of the T_{VM} cell population remain less well defined. Notwithstanding the well characterized role of Eomes, which acts to increase the transcription and cell surface expression of CD122 to enhance IL-15 responsiveness (Intlekofer et al, 2005; Banerjee et al, 2010), not much is known about other transcription factors that regulate T_{VM} cell generation and maintenance. Given the clear involvement of type I IFNs and yc cytokines in the homeostasis of T_{VM} cells (Surh and Sprent, 2008; Martinet et al, 2015), it is likely that the JAK-STAT signaling networks activated downstream of the receptors for these cytokines play an important role. The initial findings of this study have now identified RelA, a member of the NF-κB family of transcription factors, as playing a crucial role in the generation and/or maintenance of T_{VM} cells. Processes and functions regulated by the NF-κB family member RelA have been extensively described in numerous types of immune cells, but not T_{VM}s. For example, RelA has been shown to protect macrophages, B cells and T cells from TNF-induced toxicity by regulating the transcription of genes encoding anti-apoptotic proteins that include c-FLIP, cIAP1, cIAP2 and A20 (Gerondakis et al, 2006). In addition, CD4 $Foxp3^{+}$ regulatory T cells (Tregs) have recently been shown to require constitutive RelA activity to maintain Treg

stability and to control Treg effector functions (Messina et al, 2016). Herein, I propose a novel role for RelA in regulating the homeostatic maintenance of the T_{VM} cell population based on data initially obtained from the investigation of RelA-deficient HSC chimeric mice. In this model of *Rela* gene-inactivation, reconstituting the immune system of recipient mice with *Rela*$^{-/-}$ HSCs following a lethal dose of irradiation results in RelA being absent in the entire hematopoietic cell compartment. Although these *Rela*$^{-/-}$ HSCs are able to initially generate a population of RelA-deficient T_{VM} cells, the lack of RelA appears to impair the capacity of these T_{VM} cells to be maintained within these chimeric mice (*Chapter 3.2.5*). An investigation found that a proportion of *Rela*$^{-/-}$ donor HSC-derived T_{VM} cells exhibit significantly reduced expression of CD122 (*Chapter 3.2.6*), which I propose leads to these cells being progressively lost as a result of being outcompeted by residual radio-resistant *Rela*$^{+/+}$ T_{VM} cells. Whilst this experimental model overcomes the issue of embryonic lethality resulting from systemic *Rela* gene-inactivation, thereby allowing the study of RelA function in T cells, the loss of RelA in other immune cells may indirectly influence any T cell-intrinsic role RelA plays in controlling homeostasis of the T_{VM} cell population. In fact, there are several issues associated with the use of HSC chimeric mice that could complicate the interpretation of the findings on RelA-deficient T_{VM} cells. These include the lymphopenic environment and heightened inflammatory cytokine levels created in irradiated recipient mice that in turn are likely to impact the T_{VM} cell population and confound the results of the study. Furthermore, a lethal dose of irradiation does not completely ablate the entire hematopoietic compartment of the recipient mice; in the case of T_{VM} cells this is demonstrated by a gradual resurgence over time of endogenous *Rela*$^{+/+}$ T_{VM} cells in *Rela*$^{-/-}$ HSC chimeras (*Chapter 3.2.5*).

To better understand the cell-intrinsic role of RelA in T_{VM} cells, an alternative method of gene-inactivation was employed. Using Cre-loxP dependent gene targeting, the lethality of RelA-deficient embryos can be overcome through the conditional inactivation of RelA in a cell-specific manner. In the *Rela*^{flox} strain (Algul et al, 2007), loxP sites consisting of 34-bp non-palindromic sequences have been

inserted with the *Rela* gene, flanking exons 7 and 10 respectively. When mice expressing the Cre recombinase, in this case expressed under control of the T cell-specific *Lck* proximal promoter, are inter-crossed with $Rela^{flox}$ mice, it promotes efficient recombination between the loxP sites, thereby excising the loxP-flanked region of *Rela* in a T cell lineage restricted manner. In addition to preventing embryonic lethality associated with the systemic loss of RelA, the inactivation of *Rela* following the onset of *Lck* expression during thymic T cell development overcomes the aforementioned limitations associated with the use of $Rela^{-/-}$ HSC chimeras, thereby allowing an investigation into the T cell-intrinsic role(s) of RelA in T_{VM} cell development and maintenance. In this chapter, I describe the initial investigation of our alternative model of *Rela* gene-inactivation. Data obtained from this investigation, presented in the findings below, provide further evidence that the canonical NF-κB family member RelA plays a crucial, T cell-intrinsic role in the homeostatic maintenance of T_{VM} cells.

4.2 Results

4.2.1 The T_{VM} cell population is reduced throughout the adult life of $Lck^{cre}Rela^{fl/fl}$ mice

To date, my initial findings have revealed that RelA-containing NF-κB heterodimers (p50/p65) are present in the nucleus of peripheral $CD8^+$ T_{VM} cells and that an analysis of $Rela^{-/-}$ foetal liver HSC chimeric mice has identified a crucial, non-redundant role for RelA in the generation and/or maintenance of T_{VM} cells. However, results obtained using $Rela^{-/-}$ HSC chimeras do not rule out the possible impact an absence of RelA in other types of immune cells might have on the T_{VM} phenotype seen in these mice. To eliminate any involvement other non-T lineage immune cells play in the reduced size of the $Rela^{-/-}$ T_{VM} cell population of HSC chimeras, T cell restricted conditional gene targeting of *Rela* was employed. Specifically, mice heterozygous for a floxed allele of *Rela* ($Rela^{fl/wt}$) were intercrossed with the Lck^{cre} deleter strain that expresses a Cre recombinase transgene under the transcriptional control of the T cell

specific *Lck* proximal promoter (Algul et al, 2007). *Lck*^{cre} is expressed early in thymocyte development, thereby ensuring the efficient targeting of genes in all conventional T-lineage cells (Bolen et al, 1991). In all experiments conducted using *Lck*^{cre} targeting of floxed RelA, *Lck*^{cre}*Rela*$^{wt/fl}$ mice were mated to generate litter-matched mice homozygous for the wild-type (*Lck*^{cre}*Rela*$^{wt/wt}$) or floxed (*Lck*^{cre}*Rela*$^{fl/fl}$) *Rela* gene. To ensure that RelA was efficiently inactivated in the T_{VM} cells of *Lck*^{cre}*Rela*$^{fl/fl}$ mice, sorted samples of T_{VM} cells ($CD8^{+}CD44^{hi}Cd49d^{lo}$) were isolated from 12-week-old *Lck*^{cre}*Rela*$^{wt/wt}$ and *Lck*^{cre}*Rela*$^{fl/fl}$ mice, as described previously (*Chapter 2.3.4*), and nuclear extracts then subjected to EMSA analysis (*Chapter 2.5.3 & 3.2.1*). The results from this experiment confirm that RelA expression is undetectable in *Lck*^{cre}*Rela*$^{fl/fl}$ T_{VM} cells (Supplementary Fig.3).

A comparison of *Lck*^{cre}*Rela*$^{wt/wt}$ and *Lck*^{cre}*Rela*$^{fl/fl}$ mice was then undertaken using flow cytometry to determine what effect the T cell-specific loss of RelA had on the size of the T_{VM} cell population within the spleen, pLNs and BM. An initial investigation of 12-week-old *Lck*^{cre}*Rela*$^{fl/fl}$ mice revealed a significant reduction in the T_{VM} cell population when compared to age-matched littermate controls (*Lck*^{cre}*Rela*$^{wt/wt}$ mice). The spleen of 12-week-old *Lck*^{cre}*Rela*$^{fl/fl}$ mice displayed a significant reduction in both the total number of T_{VM} cells (Fig.4.1C) and the proportion of T_{VM} cells within the total $CD8^{+}$ T cell population (Fig.4.1B). This decrease in the proportion of T_{VM} cells was also observed within pLNs (Fig.4.1D) and the BM (Fig.4.1E) of 12-week-old *Lck*^{cre}*Rela*$^{fl/fl}$ mice. Coupled with the results obtained from the *Rela*$^{-/-}$ HSC chimeras, these data confirm that RelA serves a T cell-intrinsic role in the generation and/or maintenance of T_{VM} cells.

A recent study by White et al (2016) revealed a unique signature of chemokine receptor expression on T_{VM} cells was that was suggestive of preferential trafficking to the liver. Given IL-15, to which T_{VM} cells are particularly responsive is highly expressed in the liver (Golden-Mason et al, 2004), the liver may therefore act as a site where T_{VM}s preferentially reside to mediate antigen-specific and bystander

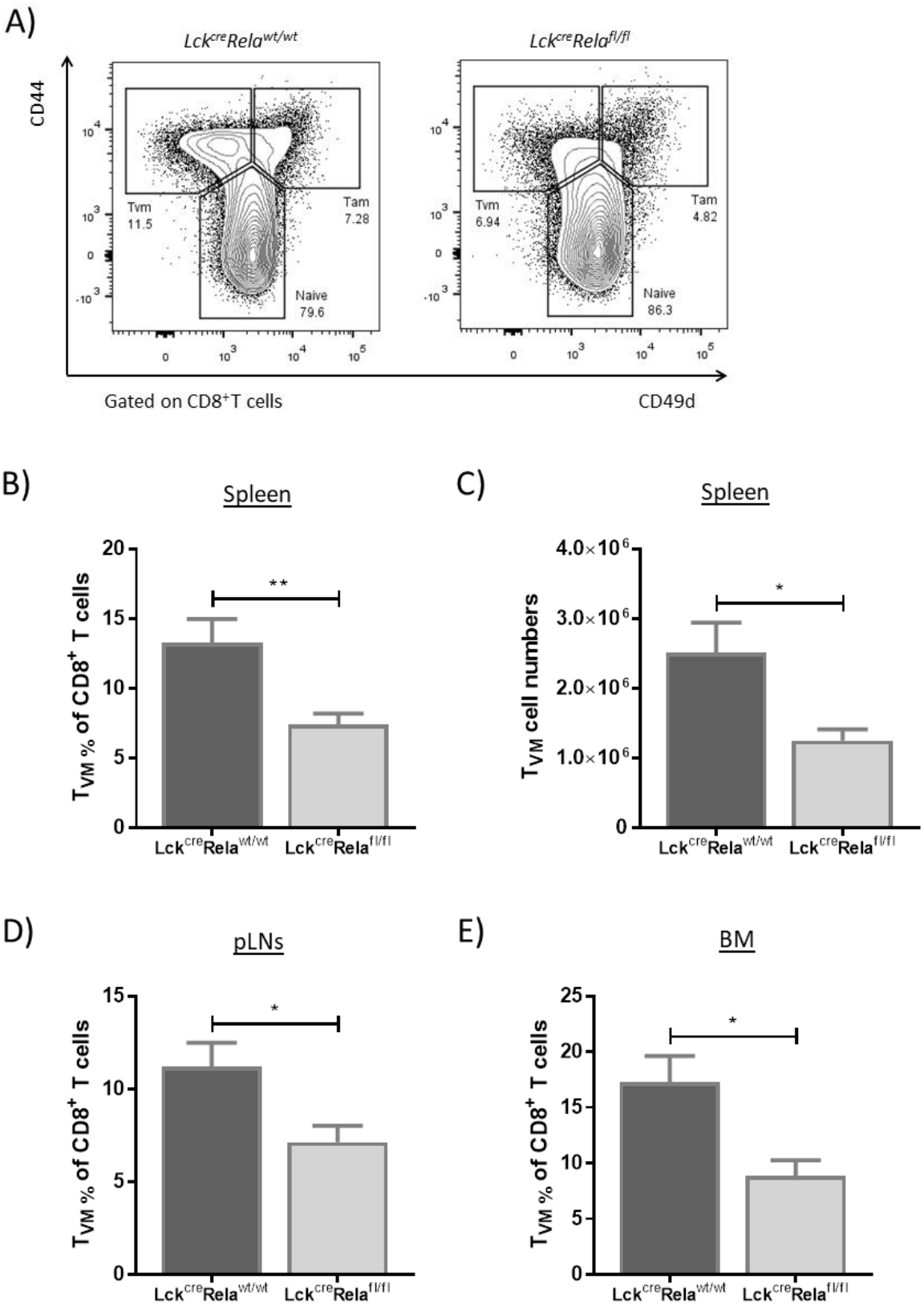

A)
Lck^cre Rela^wt/wt
Lck^cre Rela^fl/fl
CD44
Tvm 11.5
Tam 7.28
Naive 79.6
Tvm 6.94
Tam 4.82
Naive 86.3
Gated on CD8+T cells
CD49d
B)
Spleen
T_VM % of CD8+ T cells
**
C)
Spleen
T_VM cell numbers
*
D)
pLNs
T_VM % of CD8+ T cells
*
E)
BM
T_VM % of CD8+ T cells
*
Lck^cre Rela^wt/wt
Lck^cre Rela^fl/fl

Figure 4.1. *Lck*^{cre}*Rela*$^{fl/fl}$ mice have a reduced T_{VM} cell population compared to *Lck*^{cre}*Rela*$^{wt/wt}$ mice. (**A-F**) Flow cytometric analysis of T_{VM} cells from spleen, pLN and BM of 12-week-old *Lck*^{cre}*Rela*$^{wt/wt}$ and *Lck*^{cre}*Rela*$^{fl/fl}$ mice (n=6). (**A**) Representative plot of VM gating strategy on $CD8^+$ gated cells from spleen sample. (**B**) Percentage of T_{VM} cells of $CD8^+$ T cells in spleen. (**C**) Total number of T_{VM} cells in spleen. (**D**) Percentage of T_{VM} cells of $CD8^+$ T cells in pooled pLNs. (**E**) Percentage of T_{VM} cells of $CD8^+$ T cells in BM. All graphs presented as the mean +/- SEM, statistical significance was determined using Student's T test. *, p≤0.05; **, p≤0.01; ***, p≤ 0.001. pLNs=peripheral lymph nodes; BM=bone marrow; T_{VM}=$CD8^+CD44^{hi}CD49d^{lo}$ Virtual Memory cells.

immunity. Accordingly, the impact a T cell-specific loss of RelA had on the liver-resident T_{VM} cell population was investigated. Livers of 24-week-old *Lck*^{cre}*Rela*$^{wt/wt}$ and *Lck*^{cre}*Rela*$^{fl/fl}$ mice were perfused 'in situ' and processed tissue subjected to Percoll gradient enrichment to separate leukocytes from hepatocytes. Flow cytometry was then used to determine the relative sizes of the T_{VM} cell populations within the liver of these mice. Similar to results obtained from the analysis of T_{VM}s in various lymphoid organs, *Lck*^{cre}*Rela*$^{fl/fl}$ mice also show a reduction in the proportion of T_{VM} cells within liver tissue relative to WT littermate controls (*Lck*^{cre}*Rela*$^{wt/wt}$) (Fig.4.2). This finding demonstrates that the T cell-specific loss of RelA affects the T_{VM} cell population within both lymphoid and non-lymphoid tissues.

To better understand how RelA regulates the T_{VM} cell population and potentially other peripheral $CD8^+$ T cells post-natally and subsequently thereafter throughout adult life, an analysis was undertaken using *Lck*^{cre}*Rela*$^{wt/wt}$ and *Lck*^{cre}*Rela*$^{fl/fl}$ mice of various ages, where the proportion and number of total $CD8^+$ T cells, naïve $CD8^+$ T cells, T_{AM} cells and T_{VM} cells was quantified at 3, 6, 12, 24, 48 and 72-weeks of age (Fig.4.3). This analysis showed that up to 6-weeks of age, the sizes of all the splenic $CD8^+$ T cell populations were equivalent in *Lck*^{cre}*Rela*$^{wt/wt}$ and *Lck*^{cre}*Rela*$^{fl/fl}$ mice. However, between 6 and 12-weeks of age, *Lck*^{cre}*Rela*$^{fl/fl}$ mice exhibit a significant drop (proportion and total number) in the T_{VM} cell population when compared to age-matched WT littermate controls (*Lck*^{cre}*Rela*$^{wt/wt}$) (Fig.4.3E-F). Moreover, this reduction in the T_{VM} cell population of *Lck*^{cre}*Rela*$^{fl/fl}$ mice is maintained thereafter throughout adult life into old age (72-weeks of age). In contrast, the proportion and total number of naïve and T_{AM} $CD8^+$ splenic T cells is largely comparable between *Lck*^{cre}*Rela*$^{wt/wt}$ and *Lck*^{cre}*Rela*$^{fl/fl}$ mice at all of the ages that were examined (Fig.4.3C-D & G-H, respectively).

Levels of NF-κB activity manipulated through altering gene copy number have been shown to impact the numerous NF-κB regulated functions (Grigoriadis et al, 2011; O'Reilly et al, 2018). Given the significant reduction in the size of the T_{VM} cell population in the lymphoid organs and liver of *Lck*^{cre}*Rela*$^{fl/fl}$ mice, it

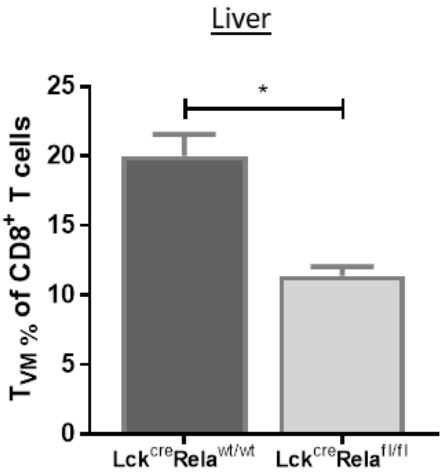
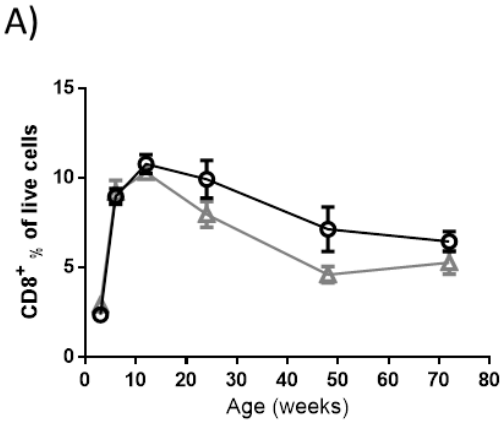
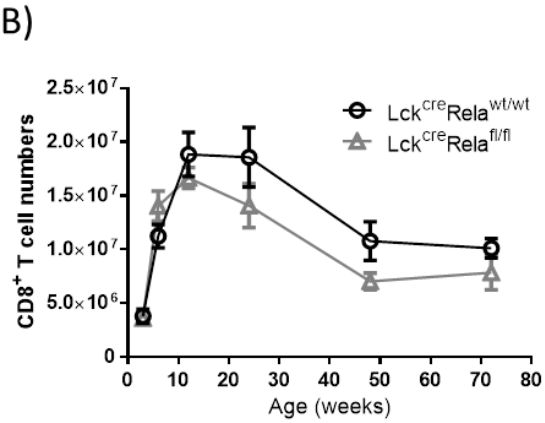


Figure 4.2. The proportion of T_{VM} cells is reduced in the liver of *$Lck^{cre}Rela^{fl/fl}$* compared to *$Lck^{cre}Rela^{wt/wt}$* mice. Flow cytometric analysis of T_{VM} cells within the liver of 12-week-old *$Lck^{cre}Rela^{wt/wt}$* and *$Lck^{cre}Rela^{fl/fl}$* mice (n=5). Percentage of T_{VM} cells within the $CD8^+$ T cell compartment of the liver. Graph presented as the mean +/- SEM, statistical significance was determined using Student's T test. *, p≤0.05. T_{VM}=$CD8^+CD44^{hi}CD49d^{lo}$ Virtual Memory cells.

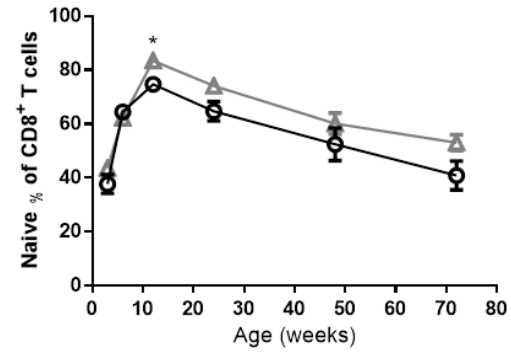
Figure 4.3. *Lck*^{cre}*Rela*$^{fl/fl}$ mice develop a reduction in T_{VM} cell numbers between 6-12-weeks of age that is sustained throughout adult life. (**A-H**) Timecourse analysis (6, 12, 24, 48 and 72-weeks-of-age) of proportion and total number of splenic CD8^{+} T cells (**A-B**) and subsets: Naïve, CD44^{lo}CD49d^{int} (**C-D**); $T_{VM,}$ CD44^{hi}CD49d^{lo} (**E-F**); T_{AM}, CD44^{hi}CD49d^{hi} (**G-H**) within spleens of *Lck*^{cre}*Rela*$^{wt/wt}$ and *Lck*^{cre}*Rela*$^{fl/fl}$ mice (n=6). All graphs presented as the mean +/- SEM. *, p≤0.05, **, p≤0.01. pLNs=peripheral lymph nodes; BM=bone marrow; naïve=CD8^{+}CD44^{lo}CD49d^{int} T_{VM}=CD8^{+}CD44^{hi}CD49d^{lo} Virtual Memory cells; T_{AM}=CD8^{+}CD44^{hi}CD49d^{hi} conventional antigen-primed memory cells; WT=wild type.

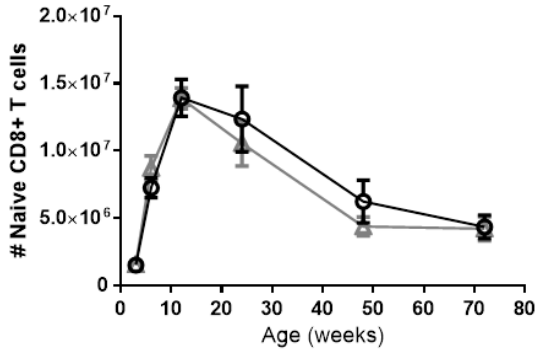
All $CD8^+$ T cells



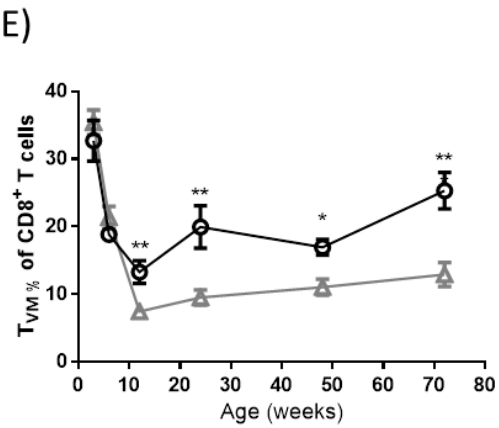


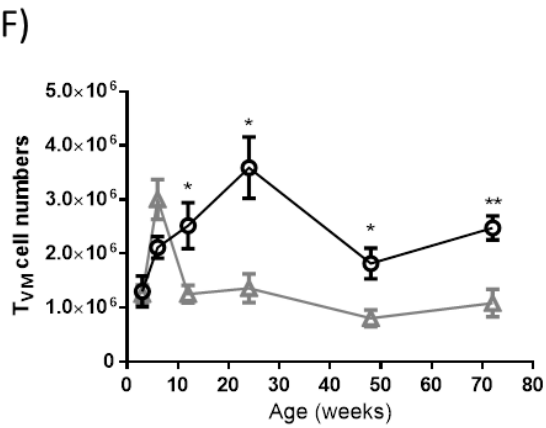
Naïve $CD8^+$ T cells



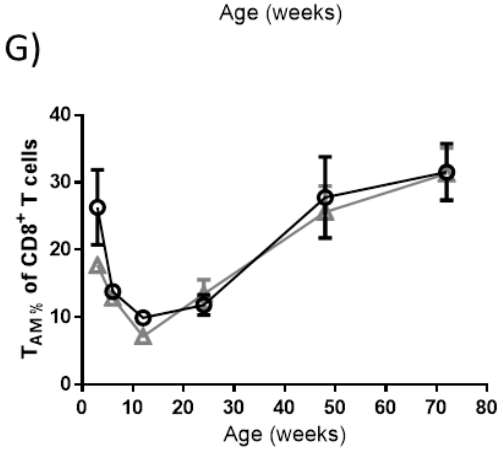


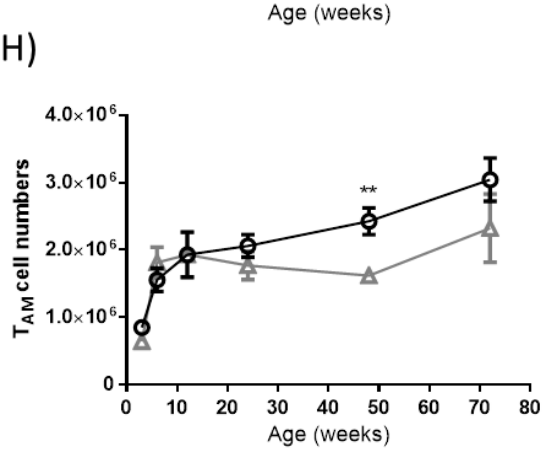
T_{VM} cells





T_{AM} cells





was of interest to investigate what effect, if any, gene dosage had on the T_{VM} cell population. An analysis of the proportion and number of total $CD8^+$ T cells, naïve $CD8^+$ T cells, T_{AM} cells and T_{VM} cells in 12-week-old *Lck*^{cre}*Rela*$^{wt/fl}$ mice found that the sizes (total number and proportion) of all these splenic $CD8^+$ T cell subsets was comparable with that of age-matched WT littermate controls (*Lck*^{cre}*Rela*$^{wt/wt}$) (data not shown). This establishes that the resulting defect in the T_{VM} cell population following the loss of RelA is not subject to haplo-insufficiency.

4.2.2 The NKG2D$^+$ T_{VM} cell population is reduced in the spleen of Lck^{cre}Rela$^{fl/fl}$ mice

An examination *of Lck*^{Cre}*Rela*$^{fl/fl}$ mice found that the T cell-specific loss of RelA causes a significant reduction in the peripheral $CD8^+CD122^{hi}CD44^{hi}CD49d^{lo}$ T cell population between 6 and 12 weeks of age and remains at ~50% of normal levels thereafter throughout adult life. This reduction in *Rela*$^{-/-}$ antigen-inexperienced MP $CD8^+$ T cells occurs in both lymphoid and non-lymphoid tissues that were examined, which includes the spleen, pLNs, BM and liver. This indicates that RelA plays a non-redundant role in the generation and/or maintenance of this MP population. In addition to T_{VM} cells, there is another subset of antigen-inexperienced MP $CD8^+$ T cells in lympho-replete mice that also presents as a $CD8^+CD122^{hi}CD44^{hi}CD49d^{lo}$ population. These other antigen-inexperienced 'innate memory' (T_{IM}) $CD8^+$ T cells which develop in the thymus in response to IL-4 produced by $PLZF^+$ cells (Raberger et al, 2008; Lee et al, 2011), emigrate from the thymus and contribute to the peripheral T cell pool. This common $CD8^+CD122^{hi}CD44^{hi}CD49d^{lo}$ cell surface phenotype shared by T_{VM} and T_{IM} $CD8^+$ T cells highlights the need to determine whether the T cell-specific loss of RelA impacts either T_{VM}s, T_{IM}s, or both populations. To date, the clearest distinction between T_{VM} and T_{IM} cells is the cell surface marker NKG2D, which is expressed on T_{VM}s, but not T_{IM} cells (White et al, 2017). NKG2D is a type II membrane receptor present on all NK cells, as well as being expressed on γδ T cells and certain $CD8^+$ T cell subsets following activation (Wensveen et al, 2018; Perez et al, 2019). Ligation of NKG2D by a variety of stress ligands,

including members of the RAE-1 (α-ε) and H60 (a, b and c) protein families in mice, induces PI3K and Vav-SOS signaling through recruitment of DAP10 to enhance $CD8^+$ T cell effector functions and promote memory cell formation (Bauer et al, 1999; Wensveen et al, 2013; Prajapati et al, 2018). The link between the select expression of NKG2D and T_{VM}s prompted an examination of NKG2D expression on the antigen-inexperienced MP ($CD8^+CD122^{hi}CD44^{hi}CD49d^{lo}$) population in *Lck*^{cre}*Rela*$^{wt/wt}$ and *Lck*^{cre}*Rela*$^{fl/fl}$ mice at 6, 12 and 24-weeks of age using flow cytometry. Analysis of NKG2D expression on antigen-inexperienced MP $CD8^+$ T cells in the spleen of *Lck*^{cre}*Rela*$^{wt/wt}$ and *Lck*^{cre}*Rela*$^{fl/fl}$ mice revealed that the T cell-specific loss of RelA indeed impacts the T_{VM} subset (Fig.4.4). Whereas *Lck*^{cre}*Rela*$^{wt/wt}$ mice exhibit a steady increase in the frequency of $NKG2D^+$ antigen-inexperienced MP (T_{VM}) cells between 6 and 24-weeks of age (3, 6 and 14% $NKG2D^+$ cells respectively at 6, 12 and 24-weeks of age), in *Lck*^{cre}*Rela*$^{fl/fl}$ mice there is a delay in the rate at which the $NKG2D^+$ MP $CD8^+$ T cell population develops during this time frame. At 6 and 12-weeks of age, the frequency of $NKG2D^+$ cells in *Lck*^{cre}*Rela*$^{fl/fl}$ mice is ~1 and ~3% respectively. Despite substantial variability in the frequency of $NKG2D^+$ cells observed in individual *Lck*^{cre}*Rela*$^{fl/fl}$ mice at 24-weeks of age, overall the frequency of $NKG2D^+$ cells in *Lck*^{cre}*Rela*$^{wt/wt}$ and *Lck*^{cre}*Rela*$^{fl/fl}$ mice is now similar (Fig.4.4A). By contrast, a comparison of splenic $NKG2D^+$ MP $CD8^+$ T cell numbers in these two genotypes reveals that there are significantly fewer $NKG2D^+$ T_{VM} cells at all ages in *Lck*^{cre}*Rela*$^{fl/fl}$ mice (Fig.4.4B). While a reduction in the $NKG2D^+$ population that coincides with the decrease in the size of the T_{VM} cell population of *Lck*^{cre}*Rela*$^{fl/fl}$ mice reinforces the notion that RelA is important in T_{VM} cell generation and/or maintenance, this drop in the $NKG2D^+$ MP $CD8^+$ T cell population alone does not account for the overall difference in the sizes of the $CD8^+CD122^{hi}CD44^{hi}CD49d^{lo}$ populations of *Lck*^{cre}*Rela*$^{wt/wt}$ and *Lck*^{cre}*Rela*$^{fl/fl}$ mice at equivalent ages. While this finding cannot eliminate the possibility that the loss of RelA also impacts the T_{IM} population, a more likely explanation is that the T cell-specific loss of RelA reduces both the NKG2D positive and negative T_{VM} cells.

4.2.3 Reduced expression of γc cytokine receptor subunits on RelA-deficient T_{VM} cells

Given a similar phenotype was observed for T_{VM} cells in both the *Rela*$^{-/-}$ HSC chimera and *Lck*^{cre}*Rela*$^{fl/fl}$ mouse models, the finding that the donor HSC-derived T_{VM} cells in the spleen of *Rela*$^{-/-}$ HSC chimeric mice expressed a reduced level of CD122, prompted a comparison of CD122 expression on CD8^{+} T cells in *Lck*^{cre}*Rela*$^{wt/wt}$ and *Lck*^{cre}*Rela*$^{fl/fl}$ mice. This analysis was initially performed on 6 and 12-week-old mice, an age range during which the T_{VM} cell population decreases in *Lck*^{cre}*Rela*$^{fl/fl}$ mice. Flow cytometric analysis of CD122 expression on naïve CD8^{+} T cells, T_{AM} cells and T_{VM} cells from the spleens of *Lck*^{cre}*Rela*$^{wt/wt}$ and *Lck*^{cre}*Rela*$^{fl/fl}$ mice showed that at both 6 and 12-weeks of age, a proportion of the T_{AM} and T_{VM} cell populations in *Lck*^{cre}*Rela*$^{fl/fl}$ mice exhibit significantly reduced levels of CD122 expression when compared to their counterparts in WT littermate controls (*Lck*^{cre}*Rela*$^{wt/wt}$) (Fig.4.5). In contrast, the relative expression of CD122 on naïve CD8^{+} T cells from *Lck*^{cre}*Rela*$^{fl/fl}$ mice at both 6 and 12-weeks of age was significantly higher on these cells than in control mice. CD122, together with CD132 and CD215 form the IL-15R (Castro et al, 2011; Colpitts et al, 2012). The observed changes in CD122 expression on naïve, T_{AM} and T_{VM} *Rela*$^{-/-}$ CD8 T cells prompted an examination of the other IL-15R subunits on *Rela*$^{-/-}$ CD8 T cells taken from 6 and 12-week-old *Lck*^{cre}*Rela*$^{wt/wt}$ and *Lck*^{cre}*Rela*$^{fl/fl}$ mice. Compared with age-matched WT controls, CD132 expression was reduced on T_{VM} cells from 6-week-old *Lck*^{cre}*Rela*$^{fl/fl}$ mice, but unchanged on T_{VM} cells from 12-week-old mice (Fig.4.6). CD132 expression was also consistently lower on naïve *Rela*$^{-/-}$ CD8 T cells taken from 6 and 12-week-old mice. By contrast and somewhat unexpectedly, despite the expression of CD132 on T_{AM} cells from 6-week-old *Lck*^{cre}*Rela*$^{wt/wt}$ and *Lck*^{cre}*Rela*$^{fl/fl}$ mice being comparable, CD132 expression was significantly increased on *Rela*$^{-/-}$ T_{AM} cells at 12-weeks of age. T_{AM} cells from 12-week-old *Lck*^{cre}*Rela*$^{fl/fl}$ mice also showed a significantly higher expression of CD215 when compared to T_{AM} cells taken from age-matched WT controls (Fig.4.7). Neither T_{VM} nor naïve CD8 T cells from 6 and 12-week-old *Lck*^{cre}*Rela*$^{fl/fl}$ mice displayed changes in CD215 when compared to WT littermate controls. Based on the evidence presented here, the defect in the T_{VM} cell population of *Lck*^{cre}*Rela*$^{fl/fl}$

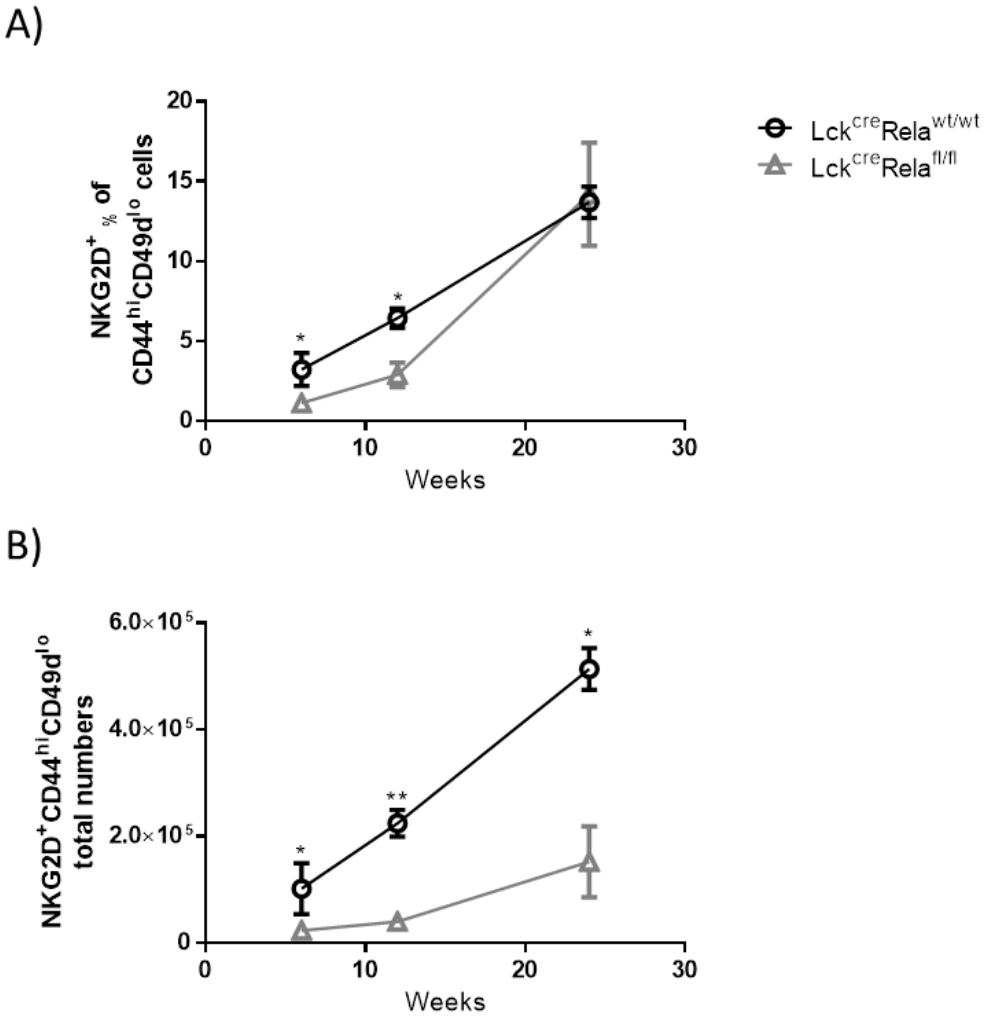


Figure 4.4. *Lck^{cre}Rela$^{fl/fl}$* mice have a reduction in the NKG2D$^+$ antigen-inexperienced MP CD8$^+$ T cell population. (**A-B**) Timecourse analysis (6, 12 and 24-weeks-of-age) of proportion and total number of antigen-inexperienced MP CD8$^+$ T cells (CD44^{hi}CD49d^{lo}) expressing NKG2D in the spleen of *Lck^{cre}Rela$^{wt/wt}$* and *Lck^{cre}Rela$^{fl/fl}$* mice (n=6). (**A**) Percentage of NKG2D$^+$ cells of antigen-inexperienced MP CD8$^+$ T cells within the spleen. (**C**) Total number NKG2D$^+$ antigen-inexperienced MP CD8$^+$ T cells within the spleen. All graphs presented as the mean +/- SEM. *, p≤0.05; **, p≤0.01.

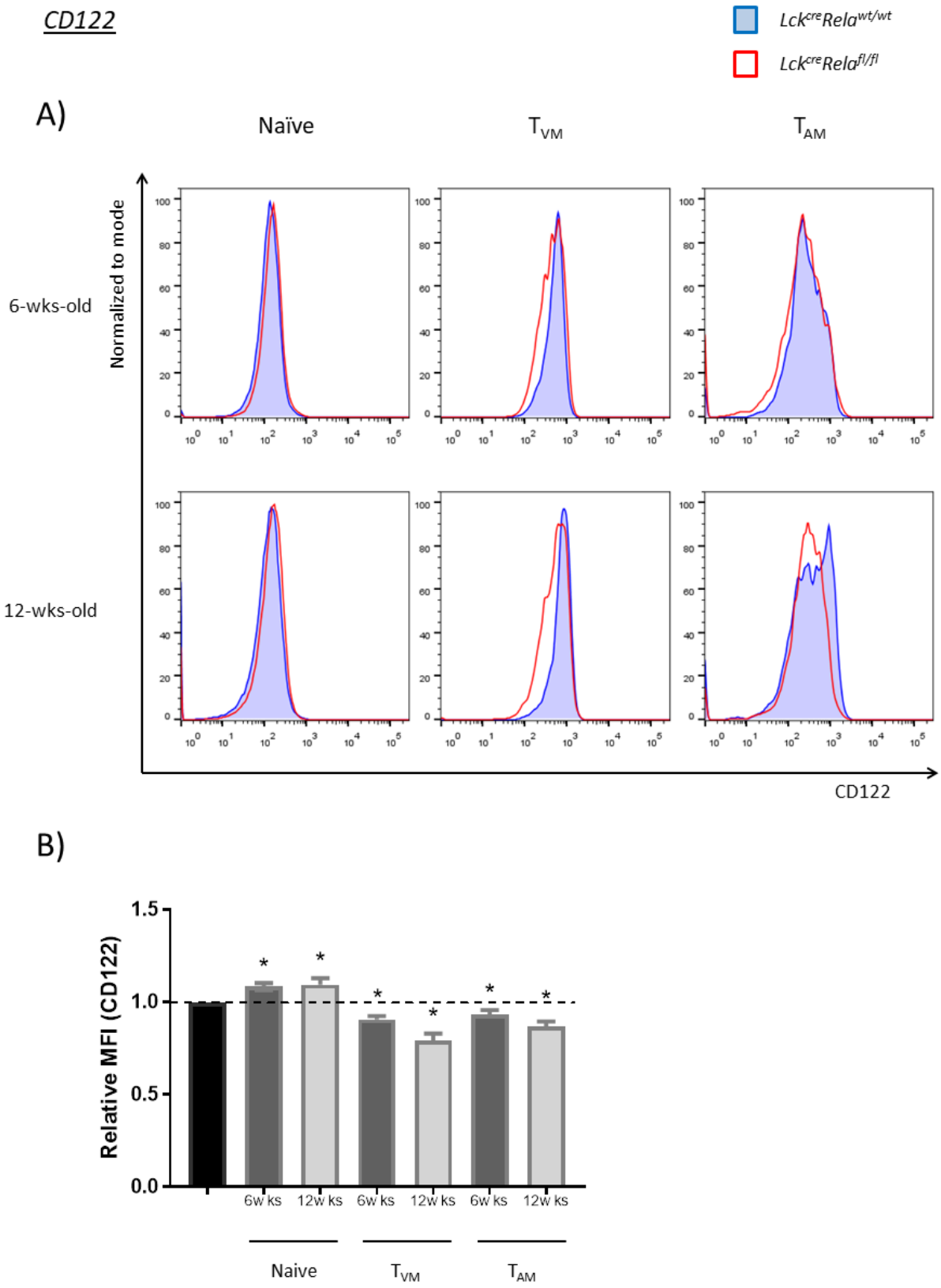

CD122
Lck^cre Rela^wt/wt
Lck^cre Rela^fl/fl
A)
Naïve
T_VM
T_AM
6-wks-old
12-wks-old
Normalized to mode
CD122
B)
Relative MFI (CD122)
1.5
1.0
0.5
0.0
6w ks
12w ks
6w ks
12w ks
6w ks
12w ks
Naive
T_VM
T_AM

Figure 4.5. CD122 expression levels are altered on CD8$^+$ T cell subsets in *Lck^{cre}Rela$^{fl/fl}$* mice. (**A-B**) Flow cytometric analysis of naïve, T_{VM} and T_{AM} CD8$^+$ T cell subsets from spleens of 6 and 12-week-old *Lck^{cre}Rela$^{wt/wt}$* and *Lck^{cre}Rela$^{fl/fl}$* mice (n=6-8) measuring CD122 surface expression levels. (**A**) Representative histogram of CD122 expression on naïve, T_{VM} and T_{AM} CD8$^+$ T cells. (**B**) MFI of CD122 on splenic CD8$^+$ subsets in *Lck^{cre}Rela$^{fl/fl}$* to relative to MFI of CD122 on equivalent CD8$^+$ T cell subsets in age-matched WT control (*Lck^{cre}Rela$^{wt/wt}$*) mice. Relative MFI graph presented as the mean +/- SEM. *, p≤0.05. naïve=CD8$^+$CD44^{lo}CD49d^{int}; T_{VM}=CD8$^+$CD44^{hi}CD49d^{lo} Virtual Memory cells; T_{AM}=CD8$^+$CD44^{hi}CD49d^{hi} conventional antigen-primed memory cells; MFI=median fluorescent intensity; WT=wild type.

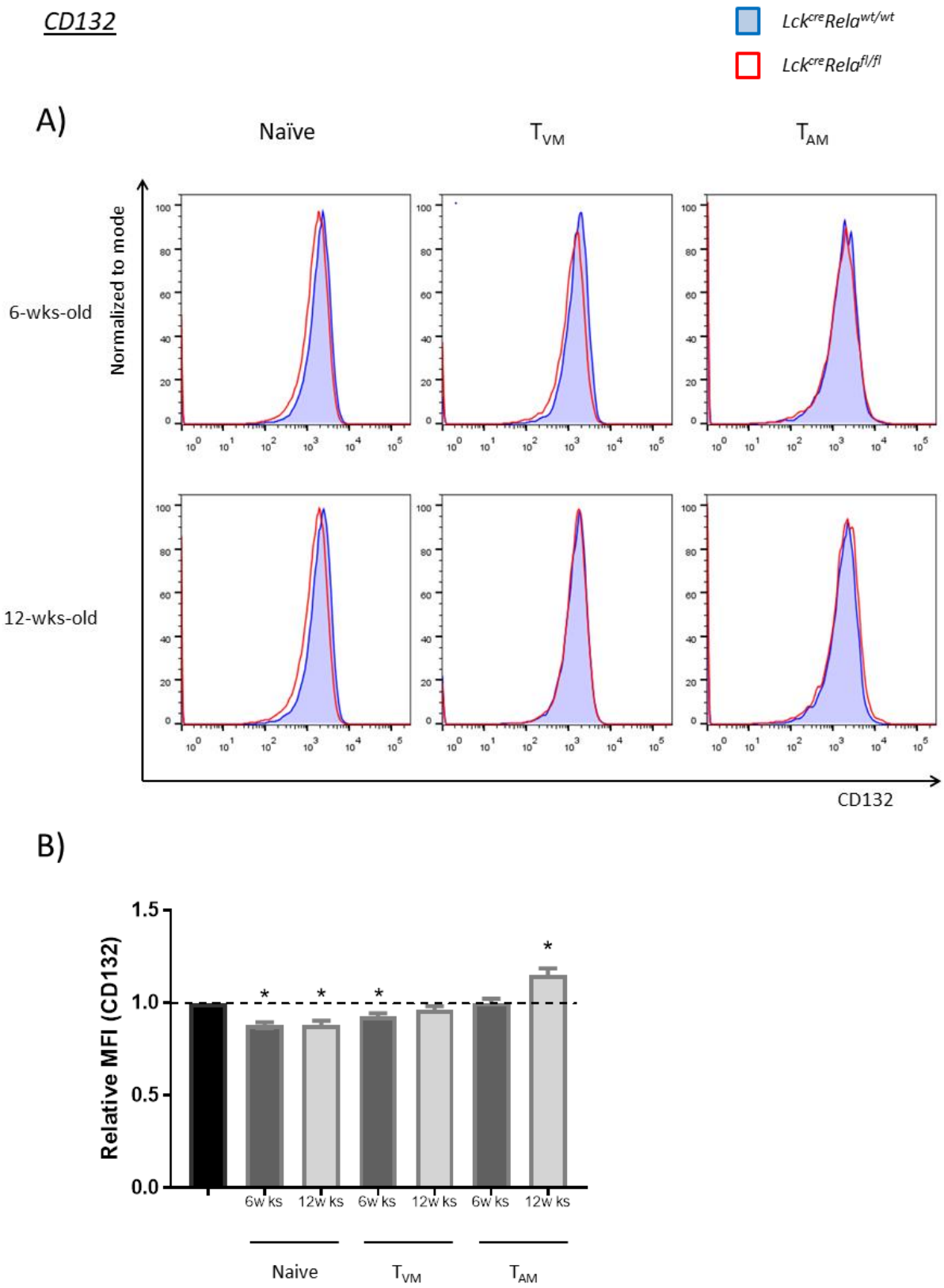
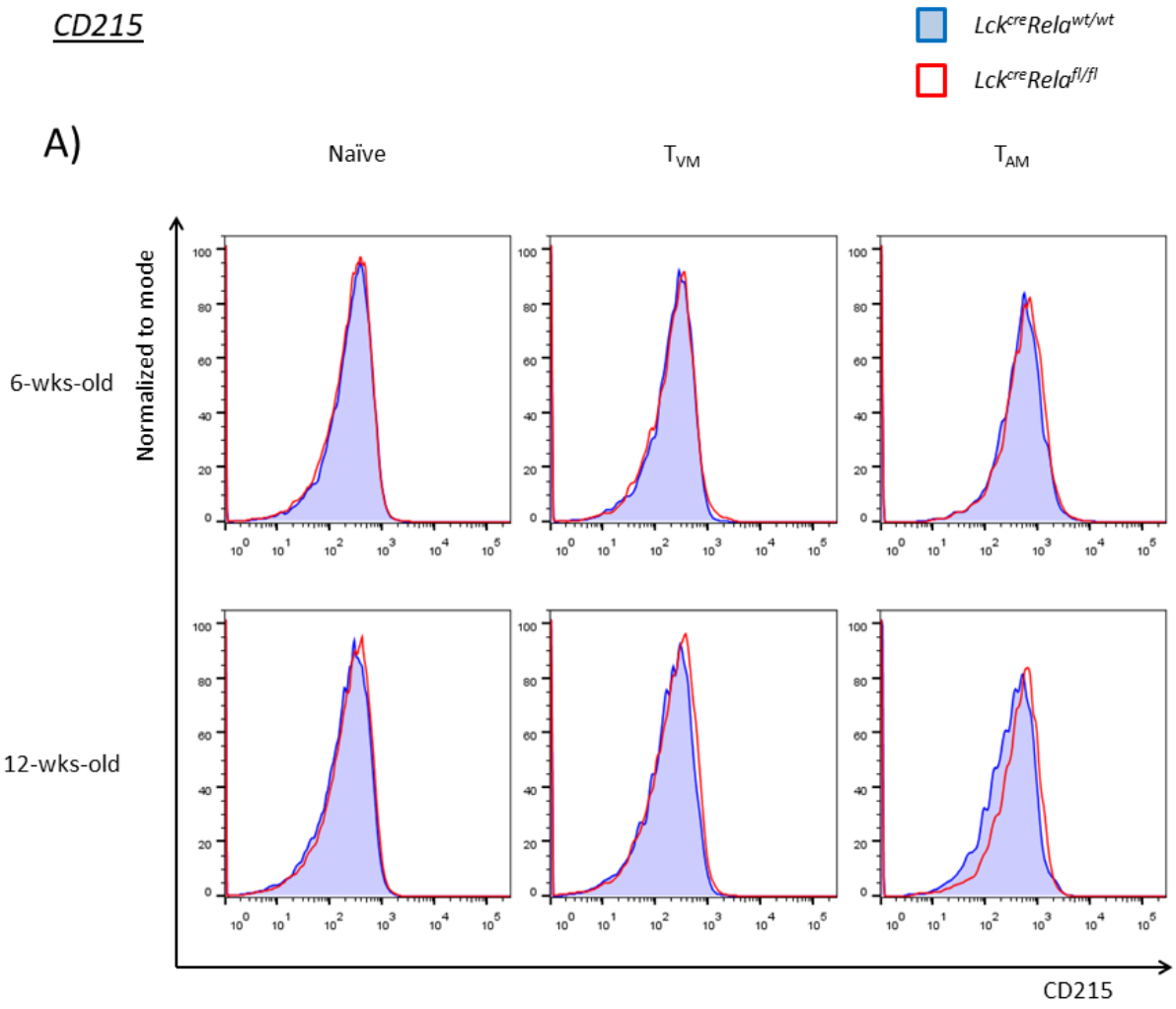

CD132
Lck^cre Rela^wt/wt
Lck^cre Rela^fl/fl
A)
Naïve
T_VM
T_AM
6-wks-old
12-wks-old
Normalized to mode
CD132
B)
Relative MFI (CD132)
1.5
1.0
0.5
0.0
6w ks
12w ks
6w ks
12w ks
6w ks
12w ks
Naive
T_VM
T_AM

Figure 4.6. CD132 expression levels are altered on CD8$^+$ T cell subsets in *Lck^{cre}Rela$^{fl/fl}$* mice. (**A-B**) Flow cytometric analysis of naïve, T_{VM} and T_{AM} CD8$^+$ T cell subsets from spleens of 6 and 12-week-old *Lck^{cre}Rela$^{wt/wt}$* and *Lck^{cre}Rela$^{fl/fl}$* mice (n=6-8) measuring CD132 surface expression levels. (**A**) Representative histogram of CD132 expression on naïve, T_{VM} and T_{AM} CD8$^+$ T cells. (**B**) MFI of CD132 on splenic CD8$^+$ subsets in *Lck^{cre}Rela$^{fl/fl}$* to relative to MFI of CD132 on equivalent CD8$^+$ T cell subsets in age-matched WT control (*Lck^{cre}Rela$^{wt/wt}$*) mice. Relative MFI graph presented as the mean +/- SEM. *, p≤0.05. naïve=CD8$^+$CD44^{lo}CD49d^{int}; T_{VM}=CD8$^+$CD44^{hi}CD49d^{lo} Virtual Memory cells; T_{AM}=CD8$^+$CD44^{hi}CD49d^{hi} conventional antigen-primed memory cells; MFI=median fluorescent intensity; WT=wild type.


CD215
Lck^cre Rela^wt/wt
Lck^cre Rela^fl/fl
A)
Naïve
T_VM
T_AM
6-wks-old
12-wks-old
Normalized to mode
CD215

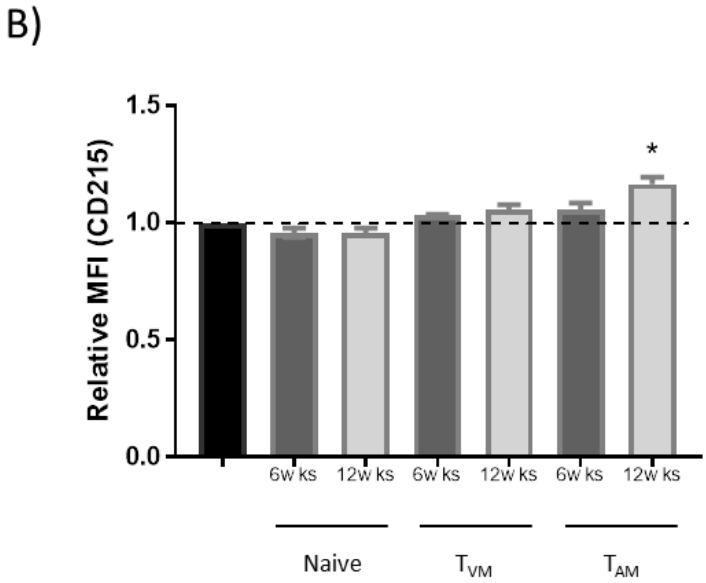

B)
Relative MFI (CD215)
1.5
1.0
0.5
0.0
*
6w ks
12w ks
6w ks
12w ks
6w ks
12w ks
Naive
T_VM
T_AM

Figure 4.7. CD215 expression levels are increased on T_{AM} cells in 12-week-old *Lck^{cre}Rela$^{fl/fl}$* mice. (**A-B**) Flow cytometric analysis of naïve, T_{VM} and T_{AM} $CD8^+$ T cell subsets from spleens of 6 and 12-week-old *Lck^{cre}Rela$^{wt/wt}$* and *Lck^{cre}Rela$^{fl/fl}$* mice (n=6-8) measuring CD215 surface expression levels. (**A**) Representative histogram of CD215 expression on naïve, T_{VM} and T_{AM} $CD8^+$ T cells. (**B**) MFI of CD215 on splenic $CD8^+$ subsets in *Lck^{cre}Rela$^{fl/fl}$* to relative to MFI of CD215 on equivalent $CD8^+$ T cell subsets in age-matched WT control (*Lck^{cre}Rela$^{wt/wt}$*) mice. Relative MFI graph presented as the mean +/- SEM. *, p≤0.05. naïve=$CD8^+CD44^{lo}CD49d^{int}$; T_{VM}=$CD8^+CD44^{hi}CD49d^{lo}$ Virtual Memory cells; T_{AM}=$CD8^+CD44^{hi}CD49d^{hi}$ conventional antigen-primed memory cells; MFI=median fluorescent intensity; WT=wild type.

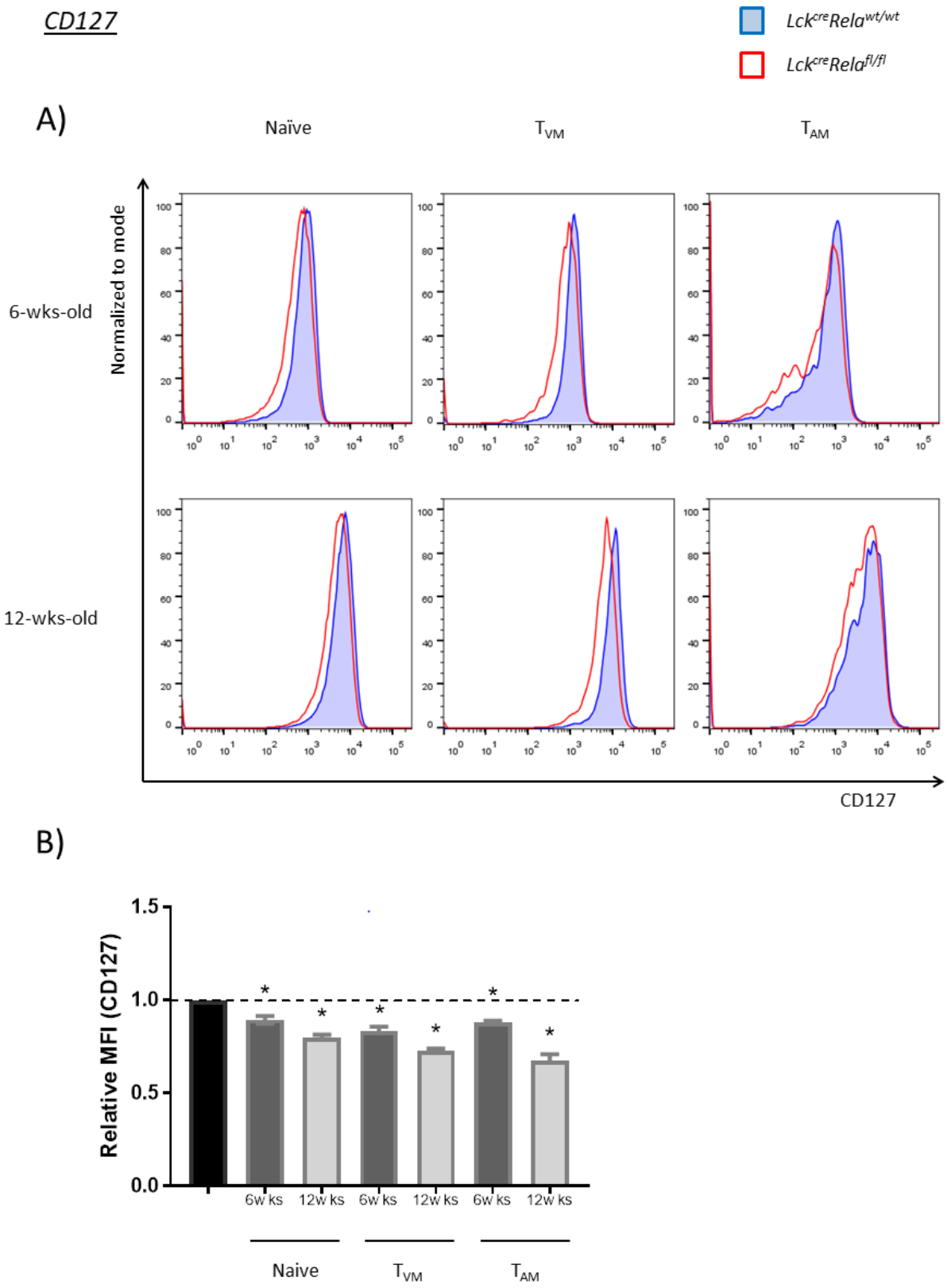

CD127
Lck^cre Rela^wt/wt
Lck^cre Rela^fl/fl
A)
Naïve
T_VM
T_AM
6-wks-old
12-wks-old
Normalized to mode
CD127
B)
Relative MFI (CD127)
1.5
1.0
0.5
0.0
6w ks
12w ks
6w ks
12w ks
6w ks
12w ks
Naive
T_VM
T_AM

Figure 4.8. CD127 expression levels are reduced on $CD8^+$ T cell subsets in *$Lck^{cre}Rela^{fl/fl}$* mice. (**A-B**) Flow cytometric analysis of naïve, T_{VM} and T_{AM} $CD8^+$ T cell subsets from spleens of 6 and 12-week-old *$Lck^{cre}Rela^{wt/wt}$* and *$Lck^{cre}Rela^{fl/fl}$* mice (n=6-8) measuring CD127 surface expression levels. (**A**) Representative histogram of CD127 expression on naïve, T_{VM} and T_{AM} $CD8^+$ T cells. (**B**) MFI of CD127 on splenic $CD8^+$ subsets in *$Lck^{cre}Rela^{fl/fl}$* to relative to MFI of CD127 on equivalent $CD8^+$ T cell subsets in age-matched WT control (*$Lck^{cre}Rela^{wt/wt}$*) mice. Relative MFI graph presented as the mean +/- SEM. *, p≤0.05. naïve=$CD8^+CD44^{lo}CD49d^{int}$; T_{VM}=$CD8^+CD44^{hi}CD49d^{lo}$ Virtual Memory cells; T_{AM}=$CD8^+CD44^{hi}CD49d^{hi}$ conventional antigen-primed memory cells; MFI=median fluorescent intensity; WT=wild type.

mice may be explained, in part, by a reduced capacity of RelA-deficient T_{VM} cells to respond to homeostatic maintenance signals provided by IL-15. These data also suggest a compensatory mechanism exists for RelA-deficient T_{AM} cells responding to signals provided by IL-15, whereby changes in the expression of other IL-15R subunits help maintain these cells in *Lck*^{cre}*Rela*$^{fl/fl}$ mice, despite CD122 being expressed at reduced levels.

Notwithstanding the likely impact altered IL-15 receptor subunit expression has on peripheral *Rela*$^{-/-}$ naïve and MP CD8^{+} T cell populations, the established involvement of IL-7 signaling via CD127 (IL-7Rα-chain) and CD132 heterodimer receptors in promoting the homeostatic maintenance of CD8 T cells (Schluns et al, 2000; Carrio et al, 2007; Boyman et al, 2009), also led to an examination of CD127 expression on these *Rela*$^{-/-}$ CD8^{+} T cell populations. In the spleen of 6-week-old *Lck*^{cre}*Rela*$^{fl/fl}$ mice, it was found that the expression of CD127 was decreased on the entire T_{VM} cell population when compared to WT littermate controls (*Lck*^{cre}*Rela*$^{wt/wt}$) (Fig.4.8). A similar decrease in CD127 expression on these cells was also observed in the spleen of 12-week-old *Lck*^{cre}*Rela*$^{fl/fl}$ mice. In contrast to the increased expression of CD132 and CD215 seen on *Rela*$^{-/-}$ T_{AM} cells in 12-week-old mice, CD127 expression was reduced on these cells at both 6 and 12-weeks of age. Moreover, like the *Rela*$^{-/-}$ T_{VM} and T_{AM} cells, the naïve CD8 T cell population in *Lck*^{cre}*Rela*$^{fl/fl}$ mice also expressed reduced levels of CD127 at both 6 and 12-weeks of age.

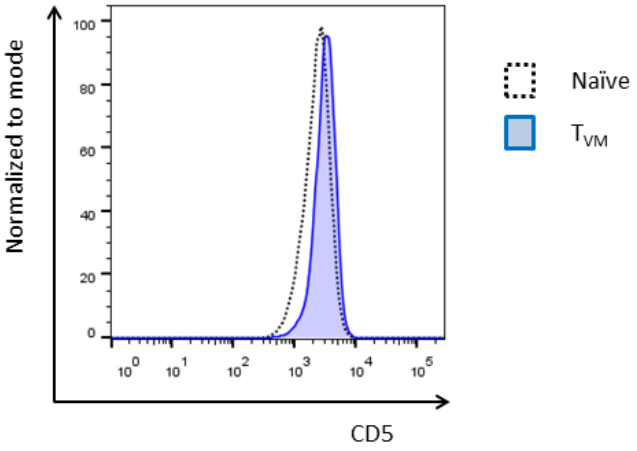
In summary, the conditional deletion of RelA in T cells resulted in a sub-population of T_{VM}s expressing reduced levels of CD122. Furthermore, the T_{VM} cell population in *Lck*^{cre}*Rela*$^{fl/fl}$ mice also presents with an overall reduction in the expression of CD127. Given the importance IL-15 and to a lesser extent IL-7 play in MP CD8^{+} T cell homeostasis (Schluns et al, 2000; Judge et al, 2002; Carrio et al, 2007; Boyman et al, 2009), the differential changes in the patterns of specific cytokine receptor subunit expression seen on *Rela*$^{-/-}$ T_{VM} versus T_{AM} cells not only suggests that impaired cytokine signaling contributes to the defect in

T_{VM} cell homeostasis observed in *Lck^{cre}Rela$^{fl/fl}$* mice, but also offers a potential explanation for why the $T_{VM,}$ but not the T_{AM} population is reduced in these mice.

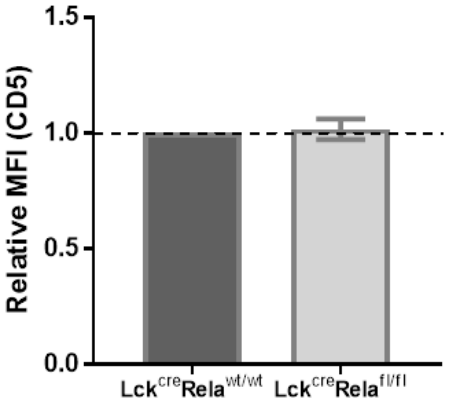
4.2.4 Equivalent CD5 expression on T_{VM} cells in Lck^{cre}Rela$^{wt/wt}$ and Lck^{cre}Rela$^{fl/fl}$ mice

Naïve CD8 T cells expressing TCRs with a greater affinity for self-peptide bound to MHC class I glycoproteins (pMHC-I) preferentially acquire a T_{VM} cell phenotype in the periphery (Quinn et al, 2016; White et al, 2016). This disposition to become a T_{VM} cell is proposed to occur within the thymus where developing $CD8^+$ thymocytes expressing a TCR with higher affinity for self-pMHC-I acquire a cellular program that primes naïve $CD8^+$ T cells to undergo T_{VM} cell differentiation (White et al, 2017). This program promotes the differentiation of newly generated naïve CD8 T cell into a T_{VM} phenotype by increasing the cell surface expression levels of CD122, endowing these cells with a greater affinity for self-pMHC-I and the capacity to respond more effectively to IL-15 in the periphery. Whilst the signal(s) that enact this cellular program are yet to be fully described, one possible model invokes the level of signaling received through the TCR of developing $CD8^+$ thymocytes as dictating the capacity of newly generated naïve CD8 T cells to differentiate into a T_{VM} phenotype (White et al, 2016). Given that NF-κB promotes the survival of developing $CD8^+$ thymocytes during MHC-I-dependent positive selection via the induction of the gene encoding Bcl-2 (Jimi et al, 2008), the reduced surface expression levels of CD122 on T_{VM} cells in *Lck^{cre}Rela$^{fl/fl}$* mice may potentially be attributable to a developmental issue in which the conditional deletion of RelA in T-lineage cells results in the death of $CD8^+$ thymocytes expressing TCRs with a higher affinity for self-pMHC-I complexes. This prompted an analysis of the overall affinity of the TCR repertoire of T_{VM} cell populations taken from the spleen of *Lck^{cre}Rela$^{wt/wt}$* and *Lck^{cre}Rela$^{fl/fl}$* mice. CD5, a protein marker on the surface of T cells that serves to negatively regulate TCR signaling, is expressed at levels that directly correlate with the affinity a TCR has for pMHC-I complexes, thereby making it a useful indicator of TCR affinity for self-antigens (Azzam et al, 1998). Accordingly, the mean affinity of the TCR

A)


Normalized to mode
100
80
60
40
20
0
10^0 10^1 10^2 10^3 10^4 10^5
CD5
Naïve
T_{VM}

B)


6-weeks-old
Relative MFI (CD5)
1.5
1.0
0.5
0.0
Lck^{cre}Rela$^{wt/wt}$
Lck^{cre}Rela$^{fl/fl}$

C)

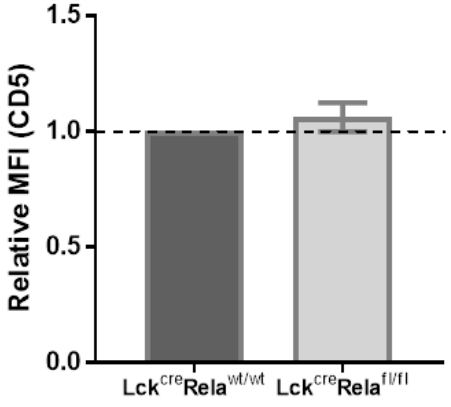

52-weeks-old
Relative MFI (CD5)
1.5
1.0
0.5
0.0
Lck^{cre}Rela$^{wt/wt}$
Lck^{cre}Rela$^{fl/fl}$

Figure 4.9. T_{VM} cells in $Lck^{cre}Rela^{wt/wt}$ and $Lck^{cre}Rela^{fl/fl}$ mice express equivalent levels of CD5. (**A-C**) Flow cytometric analysis of naïve and T_{VM} $CD8^+$ T cell subsets from spleens of 6 and 52-week-old *$Lck^{cre}Rela^{wt/wt}$* and *$Lck^{cre}Rela^{fl/fl}$* mice (n=5) measuring CD5 surface expression levels. (**A**) Expression levels of CD5 on naïve and T_{VM} $CD8^+$ T cell subsets in the spleen of 6-week-old *$Lck^{cre}Rela^{wt/wt}$* mice showing T_{VM} cells express higher levels of CD5 compared to naïve $CD8^+$ T cells. (**B**) MFI of CD5 on T_{VM} cells in the spleen of 6-week-old *$Lck^{cre}Rela^{fl/fl}$* mice relative to MFI of CD5 on T_{VM} cells in the spleen of age-matched WT control (*$Lck^{cre}Rela^{wt/wt}$*) mice. (**C**) MFI of CD5 on T_{VM} cells in the spleen of 52-week-old *$Lck^{cre}Rela^{fl/fl}$* mice relative to MFI of CD5 on T_{VM} cells in the spleen of age-matched WT control (*$Lck^{cre}Rela^{wt/wt}$*) mice. Relative MFI graph presented as the mean +/- SEM. naïve=$CD8^+CD44^{lo}CD49d^{int}$; T_{VM}=$CD8^+CD44^{hi}CD49d^{lo}$ Virtual Memory cells; MFI=median fluorescent intensity; WT=wild type.

repertoire of splenic T_{VM} cells in 6 and 52-week old *Lck*^{cre}*Rela*$^{wt/wt}$ and *Lck*^{cre}*Rela*$^{fl/fl}$ mice was determined by measuring CD5 expression levels by flow cytometry. In addition to confirming that T_{VM} cells in C57BL/6 mice express a repertoire of TCRs with greater affinity for pMHC-I than the naïve CD8 T cell population (Fig.4.9A), the median fluorescence intensity (MFI) of CD5 was found to be comparable on the splenic T_{VM} cell populations taken from *Lck*^{cre}*Rela*$^{wt/wt}$ and *Lck*^{cre}*Rela*$^{fl/fl}$ mice at both ages (Fig.4.9B-C). This finding indicates that the reduced surface expression levels of CD122 on RelA-deficient T_{VM} cells is not caused by a loss of $CD8^+$ thymocytes during development that express TCRs with a higher affinity for self-pMHC-I complexes.

4.2.5 The expression of Eomes is comparable between T_{VM} cells in Lck^{cre}*Rela*$^{wt/wt}$ and *Lck*^{cre}*Rela*$^{fl/fl}$ *mice*

The responsiveness of $CD8^+$ T cells to IL-15 is highly dependent on the transcription factor Eomes (Pearce et al, 2003; Intlekofer et al, 2005; Boyman et al, 2007; Banerjee et al, 2010). This process appears to be regulated by a perpetual regulatory feedback loop, whereby IL-15-mediated stimulation through CD122 promotes the induction of Eomes, which in turn enhances the expression of CD122 (Boyman et al, 2007). This model is supported by the finding that Eomes-deficient memory $CD8^+$ T cells show a marked reduction in CD122 expression (Banerjee et al, 2010). Given that in both of the *Rela* gene-inactivation models exploited in this study, T_{VM} cells exhibit a similar reduction in CD122 expression, one potential explanation for these findings is that RelA promotes T_{VM} cell homeostasis by assisting in the induction of *Eomes* transcription. Accordingly, the level of Eomes expression in the splenic T_{VM} cell populations of 12-week-old *Lck*^{cre}*Rela*$^{wt/wt}$ and *Lck*^{cre}*Rela*$^{fl/fl}$ mice was measured by intracellular anti-Eomes staining. The mean fluorescence intensity (MFI) of Eomes was found to be comparable in T_{VM} cells isolated from *Lck*^{cre}*Rela*$^{wt/wt}$ and *Lck*^{cre}*Rela*$^{fl/fl}$ mice (Fig.4.10), indicating that RelA does not control functions in T_{VM} cell by regulating the transcription of *Eomes*.

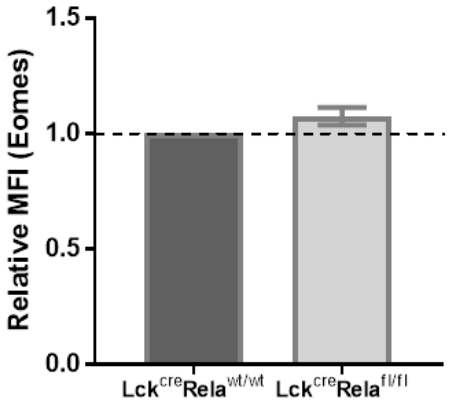


Figure 4.10. T_{VM} cells in *Lck^{cre}Rela$^{wt/wt}$* and *Lck^{cre}Rela$^{fl/fl}$* mice express equivalent levels of Eomes. Flow cytometric analysis of T_{VM} cells within spleens of 12-week-old *Lck^{cre}Rela$^{wt/wt}$* and *Lck^{cre}Rela$^{fl/fl}$* mice (n=5), measuring intracellular Eomes expression levels. MFI of Eomes in T_{VM} cells of 12-week-old *Lck^{cre}Rela$^{fl/fl}$* mice relative to MFI of Eomes in T_{VM} cells of age-matched WT control (*Lck^{cre}Rela$^{wt/wt}$*) mice. Graph presented as the mean +/- SEM. naïve=CD8^{+}CD44^{lo}CD49d^{int}; T_{VM}=CD8^{+}CD44^{hi}CD49d^{lo} Virtual Memory cells; MFI=median fluorescent intensity; WT=wild type.

4.3 Discussion

4.3.1 Lck^{cre}Rela$^{fl/fl}$ cKO mice develop a T_{VM} cell deficiency

The various limitations associated with the use of *RelA*$^{-/-}$ HSC chimeric mice highlighted the need for an alternative model of *Rela* gene-inactivation to better understand the influence RelA has on T_{VM} cell biology. To more accurately discern the T cell-intrinsic roles controlled by RelA in the generation, homeostatic expansion and maintenance of the T_{VM} cell population, Cre-loxP-dependent gene targeting was employed to achieve the T cell specific inactivation of RelA. While the use of a RelA conditional knockout (cKO) model (*Lck*^{cre}*Rela*$^{fl/fl}$ mice) overcomes some of the drawbacks associated with the use of radiation chimeras, such as eliminating the confounding influence chronic lymphopenia and heightened inflammatory cytokine levels have on T_{VM} cell generation and maintenance, it does not resolve all of the limitations of using *RelA*$^{-/-}$ HSC chimeric mice. For example, use of the *Lck* proximal promoter to drive Cre expression results in the loss of RelA from all T-lineage cells, including CD4^{+}Foxp3^{+} Tregs, which require constitutive activation of RelA to promote Treg effector functions (Messina et al, 2016). Therefore, like the analysis of *RelA*$^{-/-}$ HSC chimeric mice, how the altered properties of *Rela*$^{-/-}$ Tregs or other T cells influences T_{VM} cell biology also needs to be considered when interpreting T_{VM} cell data obtained from the investigation of *Lck*^{cre}*Rela*$^{fl/fl}$ cKO mice. Despite this caveat, the use of a T cell-specific promoter to drive Cre expression that restricts RelA inactivation only to T-lineage cells does limit the extent extrinsic factors associated with the loss of RelA in other types of immune cells has on the T_{VM} cell population. Finally, given the ontogeny of T_{VM} cells and the age-associated changes that occur early in life and into old age (Akue et al, 2012; Lee et al, 2013), the use of a cKO model allows for an investigation of the role played by RelA at both these time-points, an aim that is very difficult to achieve using HSC radiation chimeras.

Initial experiments utilizing T cell-specific *Rela* cKO mice yielded preliminary results not unlike those seen in mIκB-α and *Ikk2*$^{-/-}$ mice (Hettmann et al, 2003; Schmidt-Supprian et al, 2004), where a marked reduction in the number of MP CD8$^+$ T cells was also recorded in both strains. Between 6 and 12-weeks of age, *Lck*^{cre}*Rela*$^{fl/fl}$ mice exhibit an approximate 50% reduction in the antigen-inexperienced MP CD8$^+$ T cell population when compared to WT littermate controls. Moreover, this MP CD8$^+$ T cell deficit is sustained throughout adult life and into old age (Fig.4.3). In addition to T_{VM} cells, the antigen-inexperienced MP CD8$^+$ T cell pool in mice also contains a minor population of T_{IM} cells that arise from developing CD8 SP thymocytes in response to IL-4 produced by PLZF$^+$ NKT cells (Raberger et al, 2008; Lee et al, 2011). Although sharing a high degree of similarity in their cell surface receptor expression signature, it has been proposed that T_{IM}s can be distinguished from T_{VM}s by the T_{VM} cell specific expression of the cell surface marker NKG2D; a type II membrane receptor (White et al, 2017). This difference in NKG2D expression is accredited in part to the effect IL-4 has on CD8$^+$ T cells. While essential for the development of T_{IM} cells, exposure of CD8 SP thymocytes to IL-4 in the thymus down regulates NKG2D expression via a STAT-6-dependent mechanism (Ventre et al, 2012). The observation that there is indeed a marked reduction of antigen-inexperienced MP CD8$^+$ T cells expressing NKG2D in *Lck*^{cre}*Rela*$^{fl/fl}$ mice confirms that the T cell-conditional loss of RelA does impact the T_{VM} cell population (Fig.4.4). However, the reduction in NKG2D$^+$ T_{VM} cell numbers does not fully account for the overall difference in the sizes of the antigen-inexperienced MP CD8$^+$ T cell populations of age-matched *Lck*^{cre}*Rela*$^{wt/wt}$ and *Lck*^{cre}*Rela*$^{fl/fl}$ mice. This indicates that the T cell-specific loss of RelA also impacts the NKG2D$^-$ antigen-inexperienced MP CD8$^+$ T cell population. As previously stated (*Chapter 3.3.1*), given the high degree of similarity between T_{VM} and T_{IM} cells, it is currently unclear precisely what contribution each of these subsets make to the overall NKG2D$^-$ antigen-inexperienced MP CD8$^+$ T cell pool, thereby making it difficult to determine whether the T cell-conditional loss of RelA impacts exclusively T_{VM} cells, or both T_{VM} and T_{IM} cells. However, given that T_{IM} cells only make up a minor percentage of the MP CD8$^+$

T cell population of C57BL/6 mice (Weinrich et al, 2010), coupled with the finding that the antigen-inexperienced MP $CD8^+$ T cell population in C57BL/6 mice is largely $NKG2D^-$ (Fig.4.4), on balance, I propose that the T_{VM} cell population in mice comprises both $NKG2D^+$ and $NKG2D^-$ subsets. The well characterized role of the NKG2D receptor in enhancing antigen non-specific cytotoxicity immunity (Lanier et al, 2015) may indicate that NKG2D expression identifies a more mature, or activated subset of T_{VM} cells with enhanced effector capacity. This could explain the lack of NKG2D expression within the antigen-inexperienced MP $CD8^+$ T cell population during early life and the gradual accumulation of $NKG2D^+$ T_{VM} cells with age (Fig.4.4). Alternatively, the T_{VM} cell population is truly heterogeneous, with some cells capable of delivering cytotoxic functions, whereas others perform non-cytotoxic immune effector roles. Thus, while additional means of distinguishing T_{VM} and T_{IM} cells is ultimately required to accurately discern the impact the loss of RelA specifically has on the $NKG2D^-$ T_{VM} and T_{IM} populations, here it is confirmed that the conditional deletion of RelA impairs the generation and/or maintenance of $NKG2D^+$ T_{VM} cells.

Comparable sizes of both the naïve and T_{AM} $CD8^+$ T cell subsets in $Lck^{cre}Rela^{wt/wt}$ and $Lck^{cre}Rela^{fl/fl}$ mice indicate that in the CD8 T cell lineage, RelA is selectively indispensable for T_{VM} cells. In particular, this highlights a disparity between T_{VM}s and their antigen-experienced (T_{AM}) counterparts (Fig.4.3). This finding is consistent with the electrophoretic mobility gel shift analysis (EMSA) of nuclear NF-κB DNA binding activity in immediate 'ex-vivo' naïve, T_{AM} and T_{VM} $CD8^+$ T cells, where only T_{VM} cells were found to express significant nuclear RelA activity (*Chapter 3.2.1*). Taken together, these data not only support the hypothesis that RelA plays a crucial role in the development of T_{VM} cells, but also suggest that the marked reduction of MP $CD8^+$ T cells in mIκB-α and $Ikk2^{-/-}$ can be attributed, in part, to a reduction in the T_{VM} cell population. Furthermore, given naïve $CD8^+$ T cell numbers in $Lck^{cre}Rela^{fl/fl}$ mice are normal, coupled with a drop in the T_{VM} population of these mice only occurring during a narrow window between 6 and 12-weeks of age-when neonatal lymphopenia-induced proliferation is largely complete,

suggests that the differentiation of RelA-deficient T_{VM} cells from naïve cells is not markedly impaired and that RelA instead is primarily required for the homeostatic maintenance of T_{VM} cells.

4.3.2 RelA-deficient T_{VM} cells exhibit altered expression of the receptors for IL-15 and IL-7

The homeostatic maintenance of conventional memory (T_{AM}) $CD8^+$ T cells, unlike their naïve counterparts, is not reliant on signals from self-peptide/MHC complexes (Schluns et al, 2000). Rather, antigen-induced T_{AM} cells rely on cytokines for their survival and homeostatic proliferation, in particular a combination of IL-7 and IL-15 (Boyman et al, 2009). The same appears to be the case for T_{VM} cells, which rely heavily on IL-15 signaling for their generation and homeostatic maintenance (Rubinstein et al, 2008; Sosinowski et al, 2013; White et al, 2016), a conclusion supported by data showing MP $CD8^+$ T cell numbers are significantly reduced in IL-15-deficient mice (Kennedy et al, 2000). IL-15 signaling in MP $CD8^+$ T cells occurs predominantly via the *trans*-presentation of IL-15 bound to CD215 expressed on the surface of neighboring monocytes and DCs to the low affinity IL-15R, a signaling complex comprising a heterodimer of CD122 and CD132. In addition to the *trans*-presentation of IL-15 by monocytes and DCs, MP $CD8^+$ T cells can also respond directly to IL-15 via the high-affinity IL-15R. For this alternate mode of IL-15 signaling, CD215 expressed on the surface of MP $CD8^+$ T cells binds soluble IL-15, which is then presented in *cis* to the adjoining CD122/CD132 signaling complex. Whilst there is evidence to support both these modes of IL-15 signaling playing a role in MP $CD8^+$ T cell homeostasis (Dubois et al, 2002; Sosinowski et al, 2013), a prior study using CD215-deficient mice showed that MP $CD8^+$ T cells have a greater dependency on IL-15 *trans*-presented by monocytes and DCs than on IL-15 *cis*-presented on the cell surface (Berkett et al, 2002). CD215, whilst significantly enhancing the binding affinity of the IL-15R, lacks an intracellular signaling domain and its expression on the surface on MP $CD8^+$ T cells is largely dispensable for their generation and maintenance (Berkett et al, 2002). Conversely, CD215 expression on other hematopoietic cells, most notably monocytes and DCs, is crucial for maintaining MP $CD8^+$ T cell

numbers through the *trans*-presentation of IL-15 (Berkett et al, 2002). These findings not only demonstrate the importance of IL-15 *trans*-presentation in MP $CD8^+$ T cell maintenance, but also offer some insight into the differential dependency of these cells on the surface expression of the individual subunits that collectively comprise the IL-15R. Unlike CD215, the expression of CD122 on the surface of MP $CD8^+$ T cells is absolutely essential for maintaining their numbers as demonstrated by the profound MP $CD8^+$ T cell deficiency in mice with T cells lacking CD122, a phenotype akin to that of IL-15-deficient mice (Kennedy et al, 2000). Although possessing a lower binding affinity for IL-15 than CD215, CD122 contains an intracellular signaling domain that transduces signals following both the *cis* and *trans*-presentation of IL-15 (Nelson et al, 1998; Waldmann, 2006; Castro et al, 2011). In response to IL-15, dimerization of CD122 with CD132 activates STAT-5 transcription factors through induction of the receptor-associated tyrosine kinases JAK1 and JAK3. Subsequent translocation of phosphorylated STAT-5 into the nucleus results in the induction of numerous target genes, including those that encode the pro-survival factors Bcl-2 and Bcl-xL (*Bcl2* and *Bcl2l1* respectively), as well as a number of genes encoding proteins that promote cell cycle progression and division (*cdk4*, *sipa1l3* and *c-myc*) (Moriggl et al, 1999; Nosaka et al, 1999; Nelson et al, 2004; Rawlings et al, 2004).

The initial investigation into the influence RelA has on the development of MP T_{VM} cells using *Rela*$^{-/-}$ HSC chimeric mice found that reconstitution of the entire hematopoietic system of lethally irradiated C57BL/6 mice with *Rela*$^{-/-}$ foetal liver-derived HSCs, failed to properly generate or maintain a population of RelA-deficient T_{VM} cells (*Chapter 3.2.5*). A plausible explanation for the lack of donor HSC-derived T_{VM} cells in *Rela*$^{-/-}$ HSC chimeras was the altered expression levels of CD122, which were found to be significantly reduced on the *Rela*$^{-/-}$ T_{VM} cell population when compared to the residual, radio-resistant *Rela*$^{+/+}$ T_{VM} cells that re-expand in these mice (*Chapter 3.2.6*). This suggested that the progressive loss of *Rela*$^{-/-}$ T_{VM} cells in *Rela*$^{-/-}$ HSC chimeras may have occurred, in part, because of a reduced capacity of these cells to compete with the residual *Rela*$^{+/+}$ T_{VM} cells for IL-15. Subsequent investigation into the

contribution of RelA in T_{VM} cell development using a cKO model (*Lck*^{cre}*Rela*$^{fl/fl}$ mice), in the main produced results similar to that obtained using *Rela*$^{-/-}$ HSC chimeras. However, unlike the *Rela*$^{-/-}$ HSC chimeric model, where *Rela*$^{-/-}$ T_{VM} cell numbers continue to decrease with time, in *Lck*^{cre}*Rela*$^{fl/fl}$ mice, T_{VM} cell numbers only decrease between 6 and 12-weeks of age and remain stable thereafter throughout adult life (Fig.4.3). Like the *Rela*$^{-/-}$ T_{VM} cells in the HSC chimeras, the T_{VM} cell population that persists in the *Lck*^{cre}*Rela*$^{fl/fl}$ mice after 6 weeks of age also exhibits reduced cell surface expression levels of CD122 (Fig.4.5). Notably, this reduction in CD122 expression was first observed in *Lck*^{cre}*Rela*$^{fl/fl}$ mice at 6-weeks of age, coinciding precisely with the age at which the T_{VM} cell population starts to decline in this strain. Moreover, at 3 weeks of age, when the sizes of the T_{VM} cell populations in *Lck*^{cre}*Rela*$^{wt/wt}$ and *Lck*^{cre}*Rela*$^{fl/fl}$ mice are comparable, cell surface expression levels of CD122 are equivalent (Supplementary Fig.4). MP $CD8^+$ T cells expressing low levels of CD122 respond poorly to IL-15 stimulation both *in vitro* and *in vivo* (Judge et al, 2002), suggesting that the drop in T_{VM} cell numbers observed in *Lck*^{cre}*Rela*$^{fl/fl}$ mice between 6 and 12-weeks of age does occur, in part, due to this change in CD122 surface expression levels, thereby reducing the capacity of these cells to respond to IL-15, a factor essential in maintaining MP T_{VM} cell numbers.

Whilst IL-15 is the primary cytokine involved in T_{VM} cell generation and maintenance (White et al, 2016), IL-7 has also been shown to influence the size of the T_{VM} cell population. Besides the non-redundant role of IL-7 in MP $CD8^+$ T cell survival (Carrio et al, 2007; Boyman et al, 2009), elevated levels of IL-7 following T cell depletion drive the proliferation and differentiation of naïve $CD8^+$ T cells into T_{VM} cells (Schluns et al, 2000). Moreover, overexpression of IL-7 is able to adequately restore the depleted MP $CD8^+$ T cell compartment in IL-15$^{-/-}$ mice, showing that the dependency of these cells on IL-15 can be overcome with a greater availability of IL-7 (Kieper et al, 2002). IL-7 signaling occurs through the IL-7R, a heterodimer complex comprising the IL-7Rα-chain (CD127) and CD132 (Schluns et al, 2000; Carrio et al, 2007; Boyman et al, 2009). Activation of this receptor following IL-7 ligation promotes cell survival via the

induction of *Bcl2* gene transcription (Schluns et al, 2000). Whilst the drop in T_{VM} cell numbers in *Lck*^{cre}*Rela*$^{fl/fl}$ mice after 6-weeks of age is most likely to be attributable to a reduction in the surface expression levels of CD122, it was found that T_{VM} cells taken from this model of *Rela* gene-inactivation also displayed significantly reduced surface expression levels of CD127. Moreover, T_{VM} cells from 6-week old *Lck*^{cre}*Rela*$^{fl/fl}$ mice also exhibited a marked reduction in the surface expression levels of CD132, a receptor subunit important for eliciting signal transduction responses to a number of cytokines, including both IL-15 and IL-7. Given the well characterized roles of IL-15 and IL-7 in regulating MP CD8$^+$ T cell survival and homeostatic proliferation (Judge et al, 2002; Kieper et al, 2002), these collective findings presented in this thesis support the hypothesis that RelA plays a key role in maintaining the T_{VM} cell population by regulating the expression of various receptor subunits that determine the capacity of these cells to respond to multiple homeostatic promoting cytokines. Somewhat unexpected was the finding that T_{AM} cells in *Lck*^{cre}*Rela*$^{fl/fl}$ mice, despite being maintained at ostensibly normal numbers throughout life, also exhibit a marked reduction in the cell surface expression levels of CD122 and CD127 (Fig.4.5 & 4.8). The disparity in the homeostatic maintenance of T_{VM} and T_{AM} cells may be partly attributed to the different signals responsible for generating and maintaining these distinct populations of MP CD8$^+$ T cells. For example, a study comparing the gene transcription profiles of MP CD8$^+$ T cells stimulated with IL-15, or anti-CD3, found that despite a high degree of similarity, there were a significant number of genes differentially expressed in the two populations (Liu et al, 2002). Therefore it is plausible that cognate antigen stimulation of the TCR on T_{AM} cells may induce the transcription of specific genes that act to maintain long-term survival of T_{AM} cells, in turn making them less reliant on survival signals provided by IL-7 and IL-15 when compared to T_{VM} cells. An alternative explanation is that the reduced expression levels of CD122 on the RelA-deficient T_{AM} cell population is counter balanced by a change in the expression levels of other IL-15R subunits, thereby maintaining a high responsiveness to IL-15. Indeed, T_{AM} cells taken from 12-week old *Lck*^{cre}*Rela*$^{fl/fl}$ mice were found to express higher levels of

both CD132 and CD215 when compared to the equivalent cell population in WT littermate controls (Fig.4.6 & 4.7). CD132 also contains an intracellular signaling domain that may potentially serve to compensate for the reduced expression levels of CD122 by amplifying STAT-5 activity following IL-15 stimulation. Moreover, the increased cell surface expression levels of CD215, in addition to increasing the overall binding affinity of the IL-15R and therefore the capacity of T_{AM} cells in *Lck*^{cre}*Rela*$^{fl/fl}$ mice to respond to soluble IL-15, may in turn serve an additional role in maintaining the long-term survival of these cells. CD215 expressed on the surface of MP CD8^{+} T cells is able to form stable complexes with soluble IL-15 due to the high affinity binding interaction of these two molecules. This cytokine-receptor binding interaction induces the internalization and subsequent *trans*-endosomal recycling of these complexes (Dubois et al, 2002). The eventual reappearance of IL-15 bound to CD215 on the cell surface is able to re-stimulate CD122/CD132 signaling that promotes the long-term survival of MP CD8^{+} T cells even in the absence of IL-15 (Dubois et al, 2002; Sato et al, 2007).

Based on the data obtained from a primary analysis of *Lck*^{cre}*Rela*$^{fl/fl}$ mice, it would appear that RelA plays an essential role in the homeostatic maintenance of the T_{VM} cell population by regulating the appropriate expression levels of various γc cytokine receptor subunits important for conveying survival and proliferative responses elicited by IL-15 and IL-7. It remains to be formally shown if a reduction in the cell surface expression levels of CD122, CD127 and CD132 associated with the loss of RelA leads to reduced responsiveness to these homeostatic cytokines. Furthermore, the mechanisms by which RelA functions to up-regulate and/or maintain the expression of these receptor subunits is unclear. However, NF-κB transcription factors have previously been shown to play an essential role in naïve T cell homeostasis by enhancing transcription of the gene encoding CD127 (Miller et al, 2014). Low basal NF-κB activity regulates CD127 expression in quiescent naïve T cells as a result of RelA/NF-κB1 heterodimers binding to an evolutionarily conserved region located 3.6 kb upstream of *Il7r*. These RelA heterodimers enhance *Il7r* transcription by cooperating with a number of other transcription factors, namely Foxo1

and Ets1 (Kerdiles et al, 2009; Ouyang et al, 2009; Miller et al, 2014). This role for RelA-containing NF-κB heterodimers regulating CD127 expression appears to occur during T cell development, given that post-thymic inhibition of the canonical NF-κB signaling pathway in T cells has no effect on the surface expression levels of CD127 (Silva et al, 2014). Taken together, these collective findings may help to explain the defect in CD127 expression levels on RelA-deficient T_{VM} cells. In addition to T_{VM} cells, T_{AM} and naïve $CD8^+$ T cells in *Lck*^{cre}*Rela*$^{fl/fl}$ mice exhibit a reduction in the level of CD127 expression at all the ages examined when compared to their counterparts in WT littermate controls. This consistency between all $CD8^+$ T cell subsets is indicative of a defect arising during T cell development and maintained thereafter. Moreover, the available data for NF-kB activity during thymocyte development (Miller et al, 2014; Silva et al, 2014) suggests that the loss of RelA in the $CD8^+$ T-lineage cells of the cKO model used in this study results in the defective induction of *Il7r* during the double negative (DN) stage of T cell development. Whilst a role for RelA in regulating CD127 expression levels in T cells has been already been reported (Miller et al, 2014), there is no previously described link between NF-κB/RelA activity and CD122 expression. One possible explanation for the reduced CD122 cell surface expression levels on RelA-deficient T_{VM} cells was that RelA assists in the induction of Eomes, a transcription factor intimately associated with the regulation of CD122 expression on MP $CD8^+$ T cells (Pearce et al, 2003; Intlekofer et al, 2005; Boyman et al, 2007; Martinet et al, 2015). However, a direct comparison of Eomes expression levels in T_{VM} cells taken from *Lck*^{cre}*Rela*$^{wt/wt}$ and *Lck*^{cre}*Rela*$^{fl/fl}$ mice found no difference in the levels of this transcription factor (Fig.4.10), indicating that RelA does not appear to control T_{VM} cell functions by regulating Eomes expression. Another plausible model was that RelA inactivation negatively impacts the generation of naïve $CD8^+$ T cells expressing TCRs with higher affinity for self-pMHC-I, cells that have also recently been postulated to express higher levels of CD122 and preferentially acquire a T_{VM} cell phenotype in the periphery (White et al, 2017). Whilst NF-κB promotes the survival of developing $CD8^+$ thymocytes undergoing positive selection (Jimi et al, 2008), the loss of RelA did not appear to impact the

breadth of TCR affinity for self-pMHC-I complexes, which based on CD5 levels, were comparable between *Lck*^{cre}*Rela*$^{fl/fl}$ mice and WT littermate controls (Fig.4.9). Therefore, it remains to be determined how the loss of RelA leads to a reduction in the cell surface expression of CD122 and whether this occurs a result of direct or indirect mechanisms.

4.4 Conclusion

In summary, subsequent investigation of a secondary model of *Rela* gene-inactivation provides additional evidence to support the hypothesis that RelA plays a crucial, non-redundant role in T_{VM} cells. Conditional inactivation of RelA in T cells (*Lck*^{cre}*Rela*$^{fl/fl}$ mice) reveals an issue in T_{VM} cell maintenance with these mice exhibiting a decline in the T_{VM} cell population from 6 weeks of age to ~50% of normal numbers by 12 weeks, after which this reduced level of T_{VM} cells is maintained throughout adult life. In addition to a reduced expression of CD122, IL-7 receptor α-chain (CD127) expression is lower on *Lck*^{cre}*Rela*$^{fl/fl}$ T_{VM} cells when compared to age-matched littermate controls (*Lck*^{cre}*RelA*$^{wt/wt}$ mice), a finding suggestive of an IL-7 survival defect. Whilst a role for RelA in promoting CD127 expression on developing DN thymocytes has previously been described, how the lack of RelA in T_{VM} cells brings about a change in CD122 expression remains unresolved. Albeit, this change in CD122 expression levels does not appear to be caused by a disruption in normal expression levels of Eomes, or a loss of naïve $CD8^+$ T cells expressing TCRs with higher affinity for self-pMHC-I that preferentially differentiate into T_{VM} cells. Whilst it remains to be proven whether the reduced expression of CD122 and CD127 on RelA-deficient T_{VM} cells confers a decreased responsiveness to IL-15 or IL-7 signals, it would appear that RelA plays an essential role in the homeostatic maintenance of the T_{VM} cell population by regulating the appropriate expression levels of γc cytokine receptor subunits important for conveying homeostatic responses elicited by IL-15 and IL-7.

Chapter 5:

T_{VM} cells lacking RelA have impaired IL-7 and IL-15-induced homeostatic responses

5.1 Introduction

T_{VM} cells develop from naïve $CD8^+$ T cell precursors through cytokine-driven homeostatic proliferation (HP), a process primarily understood from studies investigating lymphopenic animal models (Surh and Sprent, 2000). In these experiments, it was shown that naïve $CD8^+$ T cells transferred into a T cell-deficient host proliferated rapidly and acquired features reminiscent of memory cells, including the up-regulation of memory cell surface markers CD44, CD122 and Ly6C, increased expression of the T-box transcription factor Eomes, and the ability to rapidly produce IFN-γ upon activation (Goldrath et al, 2004). However, unlike conventional antigen-primed memory (T_{AM}) $CD8^+$ T cells that exhibit high expression of the lymphocyte homing receptor CD49d, the antigen-independent generation of memory phenotype (MP) $CD8^+$ T cells that arise from naïve $CD8^+$ T cells undergoing HP within lymphopenic hosts maintain low surface expression levels of CD49d (Haluszczak et al, 2009). CD49d is up-regulated on antigen-primed T_{AM} cells following sustained TCR signalling, indicating that the HP of naïve $CD8^+$ T cells in lymphopenic hosts is driven by cytokine stimuli, rather than a dependence on cognate antigen signaling. In fact, the signals that drive HP are akin to those that maintain naïve $CD8^+$ T cells at constant numbers under non-lymphopenic conditions. That being said, low level tonic signaling via the TCR, received through interaction with self-peptide in complex with MHC class I glycoproteins (self-pMHC-I), combined with IL-7 promotes the survival of both naïve and memory $CD8^+$ T cell subsets by up-regulating the expression of the survival-associated transcription factor Kruppel-Like Factor 2 (KLF2) and the pro-survival factors Bcl-2 and Mcl-1 (Kuo et al, 1997; Kieper and Jameson, 1999; Schober et al, 1999; Schluns et al, 2000; Takada and Jameson, 2009). Both chronic lymphopenia, such as that experienced by mice deficient for recombination activating gene-1 (*Rag1*), as well as acute lymphopenia that occurs following irradiation, increases the availability of common γ-chain (γc) cytokines, including IL-2, IL-4, IL-7 and IL-15 (Mombaerts et al, 1992; Tan et al, 2001). Of these cytokines, only IL-7 has been shown to be essential for HP in lymphopenic mice. However, the mechanisms that drive naïve $CD8^+$ T cells to proliferate in

response to increased levels of IL-7 have not been clearly established. One possibility is that the increased availability of IL-7 amplifies TCR recognition of self-pMHC-I complexes, enhancing TCR signaling and triggering naïve $CD8^+$ T cell proliferation (Kieper et al, 2002). In a process known as 'co-receptor tuning', IL-7 signaling increases CD8 co-receptor expression on naïve $CD8^+$ T cells. Up-regulation of CD8 facilitates TCR engagement of self-pMHC-I complexes, allowing naïve $CD8^+$ T cells to receive tonic signals through the TCR to maintain cell survival. TCR-signaling disrupts the IL-7 signaling pathway and down-regulates CD8 co-receptor expression, allowing disengagement of the TCR from self-ligands (Park et al, 2007). IL-7 signaling is restored and the process repeats in a continuous feedback loop that maintains naïve $CD8^+$ T cell numbers under normal lymphoreplete conditions. A similar mechanism may account for the proliferation of naïve $CD8^+$ T cells in lymphopenic hosts, whereby increased availability of IL-7 amplifies TCR signalling to levels sufficient to induce cellular division. However, it has also been shown that naïve $CD8^+$ T cell HP within a procedurally-induced lymphopenic environment could be induced by cytokine signalling alone (Kimura et al, 2013). In response to IL-7, $CD8^+$ T cells down-regulate expression of the IL-7R α-chain (CD127) on the surface to decrease responsiveness to IL-7 in a mechanism proposed to optimize the homeostatic maintenance of the IL-7-dependent immune compartment (Park et al, 2004). $CD8^+$ T cells taken from transgenic mice that are refractory to IL-7R down-regulation were able to proliferate when adoptively transferred into procedurally-induced lymphopenic MHC-I-deficient syngeneic hosts, demonstrating that naïve $CD8^+$ T cell HP can occur independently of TCR signalling and in response to IL-7 signalling alone (Kimura et al, 2013). Binding of IL-7 to the IL-7R, which comprises CD127 and the common gamma chain (CD132) activates STAT-5 transcription factors (Lin et al, 1995; Pallard et al, 1999; Mazzucchelli and Durum, 2007). In addition to promoting the transcription of the pro-survival factors Bcl-2 and Mcl-1, STAT-5 also regulates the proliferation of a number of cell types (Bashman et al, 2008). It is also likely that high levels of IL-7 can drive naïve $CD8^+$ T cells to proliferate through the STAT-5-mediated induction of genes that promote

entry into the cell cycle. However, whilst lymphopenia-induced proliferation may initially rely on IL-7 signalling, the progressive differentiation of naïve cells to a MP shifts the dependency of these dividing cells to other maintenance factors (Surh and Sprent, 2008). Similar to the signals that maintain the steady turn-over of conventional memory cells, cytokine-driven MP $CD8^+$ T cells, including naturally arising T_{VM} cells, require IL-15 for their sustained proliferation (Sandau et al, 2007). IL-15, like IL-7, regulates cell survival and proliferation by signalling through STAT-5 transcription factors. IL-15 signaling in naïve and MP $CD8^+$ T cells occurs either through the binding of soluble IL-15 to the high affinity IL-15 receptor (IL-15R) composed of the IL-15Rα-chain (CD215), CD122 and CD132, or more commonly through the trans-presentation of IL-15 by CD215 expressed on monocytes and DCs to the low affinity IL-15R consisting of CD122 and CD132 (Colpitts et al, 2012). In response to IL-15, tyrosine kinases JAK1 and JAK3, non-covalently associated with the intracellular region of CD122 and CD132 respectively, phosphorylate and activate one another. Binding to the intracellular region of the IL-15R via their SH2 domain, STAT-5 transcription factors are phosphorylated by the activated tyrosine kinases, JAK1/3. Phosphorylated STAT-5 is then able to form either homodimers or heterodimers with other STAT family members before translocating into the nucleus to promote gene–transcription. Genes controlled by STAT-5 transcription factors influence various cellular responses including cell growth and division, plus differentiation and survival, the latter being regulated in the main by the induction of the pro-survival factor Bcl-2 (Nelson et al, 1998; Waldmann et al, 2006). Although yet to be formally shown, given the well-characterized roles of both IL-7 and IL-15 in T_{VM} cell biology, STAT-5 transcription factors are likely to play an important role in T_{VM} cell generation and maintenance.

Since the initial characterization of T_{VM} cells as a subset of MP $CD8^+$ T cells that develop through a process of IL-15-driven HP that is independent of strong antigen signals, a plethora of studies investigating T_{VM} cells and their properties have produced a better understanding of T_{VM} cell generation, maintenance and function (Lee et al, 2013; Akue et al, 2014; Wong et al, 2016; Smith et al, 2018). Having

been shown to arise during the period of neonatal lymphopenia, the generation of T_{VM} cells from newly developed naïve $CD8^+$ T cells undergoing homeostatic proliferation was originally proposed as a mechanism for helping establish the peripheral T cell compartment in early life (Surh and Sprent, 2000). However, more recently a study by Smith and colleagues (2018) has provided a deeper insight into T_{VM} cell development, in which they propose that it is the heterogeneity of early T cell precursors rather than lymphopenia-induced proliferation that dictates $CD8^+$ T cell fate decisions, including T_{VM} cell differentiation. Immediately following birth, naïve $CD8^+$ T cell development in the thymus was found to occur in two distinct waves, the first arising from neonatal HSCs that originate in the foetal liver, and then the second wave emanating from adult HSCs that originate in the bone marrow (BM) (Wong et al, 2016). These two precursor populations of distinct developmental origins are metabolically and epigenetically distinct, and possess different cell-intrinsic properties that influence the differentiation process. Using time-stamping methodology involving the inducible expression of either a red or yellow fluorescent protein (RFP and YFP respectively), it was shown that the initial wave of neonatal HSCs that seed the thymus and develop into $CD8^+$ SP thymocytes immediately following birth, possess a greater proliferative potential (Wong et al, 2016) and preferentially acquire a T_{VM} cell phenotype in the periphery (Smith et al, 2018). Whilst BM HSC-derived naïve $CD8^+$ T cells were also able to acquire a T_{VM} cell phenotype, demonstrating that the T_{VM} cell population is not entirely derived from foetal liver HSCs, a direct comparison of these two progenitor populations that can become T_{VM} cells within lymphoreplete adult mice revealed that adult HSCs undergo T_{VM} cell differentiation at a much lower frequency (Smith et al, 2018). In disagreement with the pre-existing dogma that neonatal lymphopenia is primarily responsible for driving T_{VM} cell development, the propensity of foetal liver HSC-derived naïve $CD8^+$ T cells to differentiate into T_{VM}s occurred independently of the peripheral environment. Instead, the cell-intrinsic properties of these distinct progenitor populations, appears to be the strongest determinant of T_{VM} cell development, as foetal liver HSC-derived naïve $CD8^+$ T cells transferred into adult

hosts maintained a high rate of T_{VM} cell differentiation, despite the apparent lack of lymphopenia (Smith et al, 2018).

Although the number of T_{VM} cells remains relatively stable throughout most of adult life (Lee et al, 2013), data presented here and elsewhere reveal a drastic age-associated increase in the numbers of these cells, with T_{VM} cells accounting for >40% of the $CD8^+$ T cell compartment of 20-month-old mice (Chiu et al, 2013). This discovery marked a significant turning point in the field of T cell biology, facilitating a paradigm shift in our understanding of immune memory and aging. Previously believed to be the result of a lifetime of antigenic stimulation, the accumulation of MP $CD8^+$ T cells throughout adult life that leads to a dominance of $CD44^{hi}$ cells within the $CD8^+$ T cell compartment of aged mice and humans, is now known to be largely attributed to an increase in the number of antigen-inexperienced T_{VM} cells (Chiu et al, 2013). The significance of this discovery is reinforced by the knowledge that decreases in primary $CD8^+$ T cell responses to newly encountered pathogens in old age coincides with T_{VM} cell accumulation (Jiange et al, 2011; Treanor et al, 2012; Briceno et al, 2016; Quinn et al, 2018). Whilst the age-associated decline in $CD8^+$ T cell immunity can be partly attributed to a reduction in naïve $CD8^+$ T cell numbers emanating from thymic involution (Aspinall and Andrew, 2000), $CD8^+$ T cell dysfunction can now also be associated with a loss of proliferative potential in the expanded T_{VM} cell population (Quinn et al, 2018). Whilst T_{VM} cells in young mice are highly proliferative, the T_{VM} cells of older mice are severely impaired in their capacity to undergo rapid cell division following stimulation. This dysfunction of aged T_{VM} cells arises from excessive cell division and the acquisition of a terminal differentiation state known as senescence. T cell senescence occurs in response to DNA damage following repetitive, yet intermittent stimulation over a prolonged period of time. A recent study conducted by Quinn and colleagues (2018) demonstrated that T_{VM} cells in aged mice display signs of senescence, including cell cycle arrest through increased expression of cyclin-dependent kinase inhibitor (Cdkn) p21, failure to accumulate cyclin D1, and dysregulated MAPK signalling. Furthermore, these senescent T_{VM} cells

accumulate in old age by increasing expression of the pro-survival factor Bcl-2 (Campisi and d'Adda di Fagagna, 2007; Quinn et al, 2018). The outcome of this senescent state of differentiation within the T_{VM} cell population of aged mice may serve to counteract the reduction in naïve $CD8^+$ T cell numbers resulting from thymic involution. For example, the increased survival of the T_{VM} cell population may act to maintain homeostasis of the overall T cell compartment by compensating for the reduction in the proliferative capacity of aged T_{VM}s, as well as the decreased thymic output of naïve $CD8^+$ T cells. The homeostatic maintenance of the T cell compartment, like any immune cell population, is regulated by both the rate of survival and the rate of proliferation. A drop in the number of cells within a population, such as that observed within the T_{VM} cell population of the *Rela* gene-inactivation models described in this thesis, are likely to be attributed to perturbations in cell survival and/or proliferation. In addition to cellular senescence, another state of differentiation that can reduce the proliferative potential of T cells is exhaustion. Unlike senescence that arises over a prolonged period following sporadic stimulation, T cell exhaustion typically occurs in response to chronic infections, whereby excessive and continuous stimulation via the TCR leads to a progressive loss of effector and memory function (Akbar et al, 2011). Although there are a number of similarities between senescent and exhausted T cells, clear differences in the transcriptional profiles, as well as phenotypic and functional characteristics identify clear distinctions between these two states of differentiation. T cell exhaustion is characterized by the reduced expression of memory cell surface markers (CD44, CD122, CD127 and CD62L) and a reduced responsiveness to cytokines that maintain MP $CD8^+$ T cell homeostasis, such as IL-7 and IL-15. Exhausted T cells are also identified by increased expression of inhibitory receptors (PD-1, Lag-3, CTLA-4 and CD160), decreased expression of NK cell-specific markers and a loss of effector function (Wherry and Kurachi et al, 2015).

My investigation into the role of RelA in T_{VM} cell development thus far has shown that T_{VM} cells lacking RelA are impaired in their capacity to maintain population homeostasis. In addition to exhibiting a

significant reduction in T_{VM} cell numbers, the remaining T_{VM} cell population also displays some characteristic signs of T cell exhaustion. Most notable of these characteristics in *Lck*^{cre}*Rela*$^{fl/fl}$ mice was a significant reduction in the number of cells expressing the NK cell-specific marker NKG2D, and a marked reduction in the surface expression levels of CD122 and CD127. In this chapter, a reduction in the surface expression levels of CD122 and CD127 on RelA-deficient T_{VM} cells is shown to coincide with a reduced responsiveness to survival signals provided by IL-7 and IL-15, as well as a delayed proliferative response to higher concentrations of IL-15, both features consistent with an exhausted phenotype. Moreover, T cells lacking RelA were found to be impaired in their capacity to produce IFN-γ following bystander activation *in vitro*, while a comparison of the gene expression profiles of WT and RelA-deficient T_{VM} cells taken from 12 week old mice revealed higher transcription of genes encoding the inhibitory receptors CTLA-4 and Lag-3 in T_{VM} cells lacking RelA. Finally, whilst these data are consistent with elements of an exhausted phenotype, RNA sequencing (RNAseq) analysis also demonstrates differences in the transcription profile of WT and RelA-deficient T_{VM} cells that may suggest the defect in the homeostatic maintenance of T_{VM} cell numbers in *Lck*^{cre}*Rela*$^{fl/fl}$ mice also involves a preferential loss of T_{VM} cells that develop from foetal-liver derived HSCs.

5.2 Results

5.2.1 Cultured Rela$^{-/-}$ *T_{VM} cells display a reduced survival and proliferative response to IL-7 and IL-15*

The discovery that T_{VM} cells taken from *Lck*^{cre}*Rela*$^{fl/fl}$ mice exhibit reduced expression of the γc cytokine receptor subunits CD122, CD127 and CD132, prompted an investigation into the capacity of these cells to respond to survival and/or proliferation signals elicited by various γc cytokines. Initially, splenic $CD8^{+}CD44^{hi}CD49d^{lo}$ T_{VM} cells were isolated by FACS from 12-week-old *Lck*^{cre}*Rela*$^{wt/wt}$ and *Lck*^{cre}*Rela*$^{fl/fl}$ mice and incubated for 3 days in the absence or presence of recombinant IL-2 (10ng/mL), IL-7 (2ng/mL)

or IL-15 (50ng/mL). After 1 or 3-days incubation, cells were stained with the viability dye, LiveDead Aqua and analyzed by flow cytometry to determine cell viability. The presence of IL-2 significantly increased the survival of T_{VM} cells after both 1 and 3-days, with comparable survival rates observed for *Rela*$^{+/+}$ and *Rela*$^{-/-}$ T_{VM} cells at both time-points (Fig.5.1B). In the presence of IL-7, *Rela*$^{-/-}$ T_{VM} cells displayed a modest, yet significant reduction in survival after both 1 and 3-days incubation when compared with *Rela*$^{+/+}$ T_{VM} cells (Fig.5.1C). A more dramatic survival outcome was observed using IL-15, where after 1 or 3-days of cell culture (Fig.5.1D), *Rela*$^{-/-}$ T_{VM}s had a significantly decreased rate of survival when compared to *Rela*$^{+/+}$ T_{VM} counterparts.

These preliminary findings on the responsiveness of *Rela*$^{-/-}$ T_{VM} cells to IL-15 prompted a more detailed investigation of the role RelA plays in mediating the IL-15-dependent survival and proliferation of T_{VM}s. In these experiments, splenic $CD8^{+}CD44^{hi}CD49d^{lo}$ T_{VM} cells isolated by FACS from 12-week-old *Lck*^{cre}*Rela*$^{wt/wt}$ and *Lck*^{cre}*Rela*$^{fl/fl}$ mice were pre-stained with the cell division monitoring dye, Cell Trace Violet (CTV), and then incubated with various concentrations (15, 50 or 150ng/mL) of recombinant IL-15. After 1, 3 and 5-days incubation, cells were stained with the viability dye, LiveDead Aqua and then analyzed by flow cytometry to quantify cell survival and proliferation. At the lowest concentration of IL-15 (15ng/mL), only after 3-days incubation was there a noticeable impact on T_{VM} cell survival when compared with cultures lacking cytokine. Although the survival of both *Rela*$^{+/+}$ and *Rela*$^{-/-}$ T_{VM} cells increased significantly in the presence of 15ng/mL IL-15 after 3-days, no difference was observed in the relative survival rates of *Rela*$^{+/+}$ and *Rela*$^{-/-}$ T_{VM}s at this time point (Fig.5.2B). Whilst the survival of both *Rela*$^{+/+}$ and *Rela*$^{-/-}$ T_{VM} cells was further increased in the presence of an intermediate concentration of IL-15 (50ng/mL) throughout the duration of the assay when compared with cultures containing 15ng/mL, after 3 and 5-days incubation, the survival of RelA-deficient T_{VM} cells was significantly reduced when compared with WT control cultures (*Rela*$^{+/+}$ T_{VM}s) (Fig.5.2C). At the highest concentration of IL-15 (150ng/mL), *Rela*$^{+/+}$ T_{VM} cell viability remained high and relatively constant over a 5 day period (Fig.5.2D).

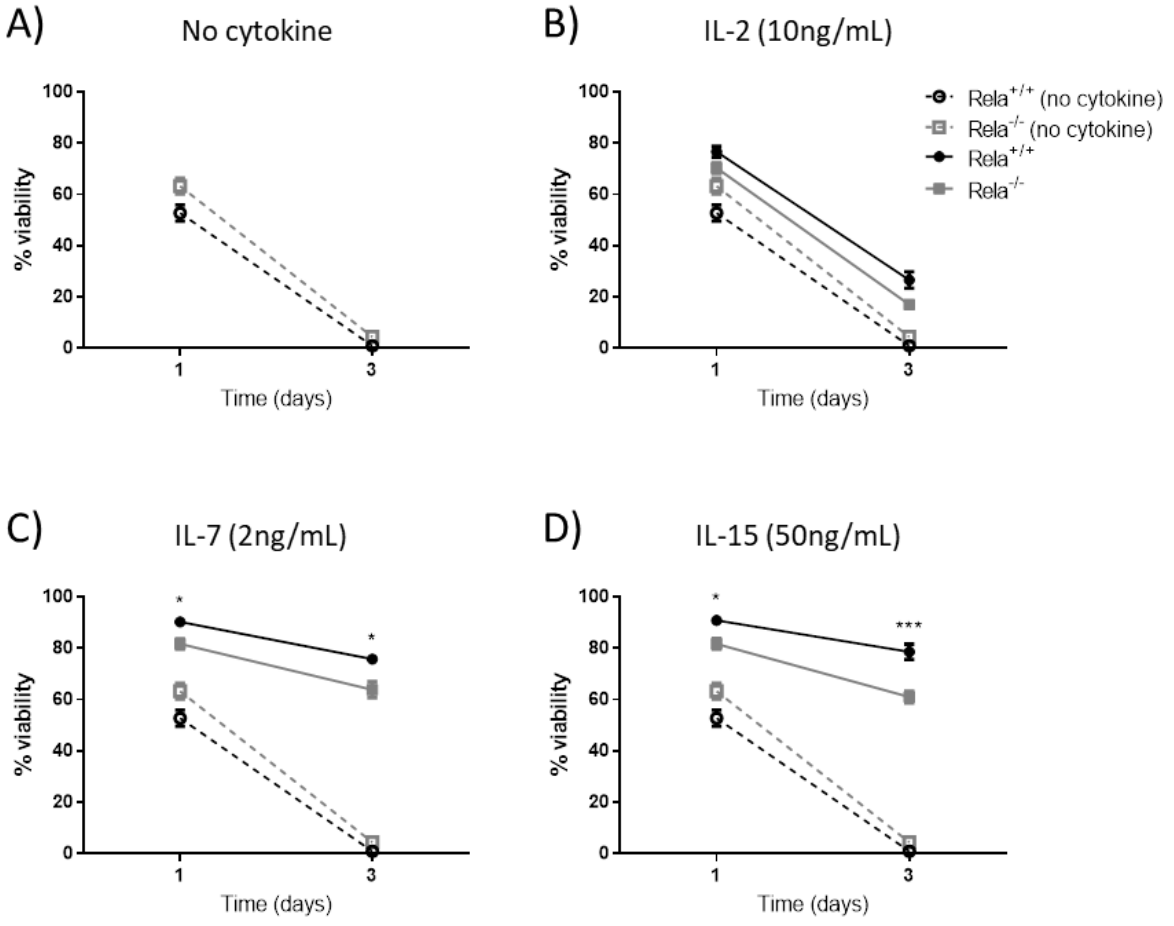


Figure 5.1. *Rela*$^{-/-}$ T_{VM} cells have a reduced viability when cultured in the presence of IL-7 or IL-15. (**A-D**) $CD8^{+}CD44^{hi}CD49d^{lo}$ T_{VM} cells isolated from 12-week-old *Lck*^{cre}*Rela*$^{wt/wt}$ and *Lck*^{cre}*Rela*$^{fl/fl}$ mice were stimulated *in vitro* with cytokine and viability measured after 1 and 3-days by LiveDead aqua staining. (**A**) The frequency of viable *Rela*$^{+/+}$ and *Rela*$^{-/-}$ T_{VM}s in the absence of cytokine (n=4). (**B**) The frequency of viable *Rela*$^{+/+}$ and *Rela*$^{-/-}$ T_{VM}s stimulated with 10ng/mL IL-2 (n=4). (**C**) The frequency of viable *Rela*$^{+/+}$ and *Rela*$^{-/-}$ T_{VM}s stimulated with 2ng/mL IL-7 (n=4). (**D**) The frequency of viable *Rela*$^{+/+}$ and *Rela*$^{-/-}$ T_{VM}s stimulated with 50ng/mL IL-15 (n=8). All graphs presented as the mean +/- SEM, all cytokine-stimulated graphs compared to same no cytokine control assay to demonstrate survival-promoting effect of each cytokine, n numbers represent independent assays taken as average of triplicate repeat wells, statistical significance was determined using Multiple T test. *, p≤0.05; *** p≤0.001. T_{VM}=$CD8^{+}CD44^{hi}CD49d^{lo}$ Virtual Memory cells; CTV=cell trace violet.

By contrast, *Rela*$^{-/-}$ T_{VM}s exhibited a significant reduction in survival following 3 and 5-days of cell culture (Fig.5.2D). Given that higher concentrations of IL-15 can induce T_{VM}s to undergo cellular division (Boyman et al, 2009), it was also important to consider what impact the absence of RelA had on IL-15-induced T_{VM} cell proliferation. The cell tracking dye CTV was used to determine the number of divisions *Rela*$^{+/+}$ and *Rela*$^{-/-}$ T_{VM} cells underwent in response to different concentrations of IL-15. At the lowest concentration of IL-15 (15ng/mL), no proliferation was observed throughout the duration of the assay (data not shown). At the intermediate concentration of IL-15 (50ng/mL), a modest, but comparable level of proliferation was observed for *Rela*$^{+/+}$ and *Rela*$^{-/-}$ T_{VM}s over the initial 3 days of cell culture (Fig.5.3A). However, after 5-days incubation in the presence of the intermediate concentration of IL-15, *Rela*$^{-/-}$ T_{VM} cell division was comparatively slower. At the highest levels of IL-15 (150ng/mL), an impaired *Rela*$^{-/-}$ T_{VM} cell proliferative response was evident at an earlier juncture (day 3) and sustained at day 5 (Fig.5.3B). In summary, these data indicate that at higher concentrations of IL-15, RelA-deficient T_{VM}s exhibit reduced survival and an impaired proliferative capacity.

5.2.2 STAT-5 phosphorylation following IL-15 stimulation is equivalent in WT and Rela$^{-/-}$ *T_{VM} cells*

The survival and homeostatic proliferation of MP CD8$^+$ T cells is heavily reliant on IL-15 (Jameson et al, 2002; Surh and Sprent, 2005). The findings of this study thus far have shown that the cell surface expression levels of CD122 is reduced on T_{VM} cells lacking RelA, a defect found to coincide with the reduced capacity of cultured *Rela*$^{-/-}$ T_{VM} cells to respond to IL-15 survival and proliferative cues. IL-15 promotes MP CD8$^+$ T cell survival by up-regulating the pro-survival factor Bcl-2 via the JAK1,3/STAT-5 signaling pathway (Nelson et al, 1998; Waldmann et al, 2006). Given the reduced expression of CD122 on the surface of T_{VM} cells in *Lck*^{cre}*Rela*$^{fl/fl}$ mice, it was important to determine if the impaired IL-15-mediated survival of cultured RelA-deficient T_{VM} cells coincided with a reduction in STAT-5 phosphorylation following IL-15 stimulation. Sorted CD8$^+$CD44^{hi}CD49d^{lo} T_{VM} cells from 12-week-old

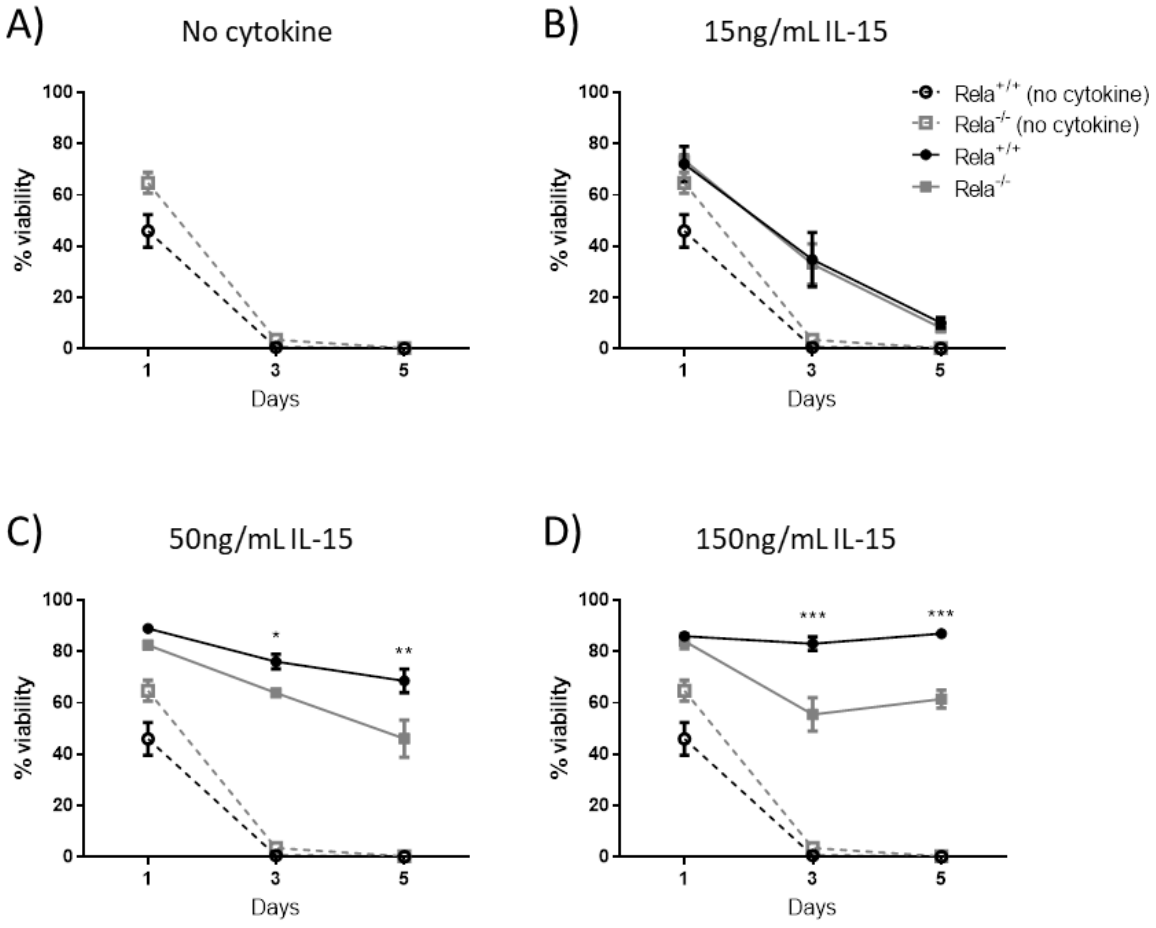


Figure 5.2. *Rela*$^{-/-}$ T_{VM} cells have reduced viability when cultured in various concentrations of IL-15. (**A-D**) $CD8^{+}CD44^{hi}CD49d^{lo}$ T_{VM} cells isolated from 12-week-old *Lck*^{cre}*Rela*$^{wt/wt}$ and *Lck*^{cre}*Rela*$^{fl/fl}$ mice were stimulated *in vitro* with varying concentrations of IL-15 and viability measured after 1, 3 and 5-days by LiveDead aqua staining. (**A**) The frequency of viable *Rela*$^{+/+}$ and *Rela*$^{-/-}$ T_{VM}s in the absence of IL-15 (n=4). (**B**) The frequency of viable *Rela*$^{+/+}$ and *Rela*$^{-/-}$ T_{VM}s stimulated with 15ng/mL IL-5 (n=3). (**C**) The frequency of viable *Rela*$^{+/+}$ and *Rela*$^{-/-}$ T_{VM}s stimulated with 50ng/mL IL-5 (n=8). (**D**) The frequency of viable *Rela*$^{+/+}$ and *Rela*$^{-/-}$ T_{VM}s stimulated with 150ng/mL IL-15 (n=4). All graphs presented as the mean +/- SEM, all IL-15-stimulated graphs compared to same no cytokine control assay to demonstrate survival-promoting effect of IL-15, n numbers represent independent assays taken as average of triplicate repeat wells, statistical significance was determined using Multiple T test. *, p≤0.05; ** p≤0.01; *** p≤0.001. T_{VM}=$CD8^{+}CD44^{hi}CD49d^{lo}$ Virtual Memory cells; CTV=cell trace violet.

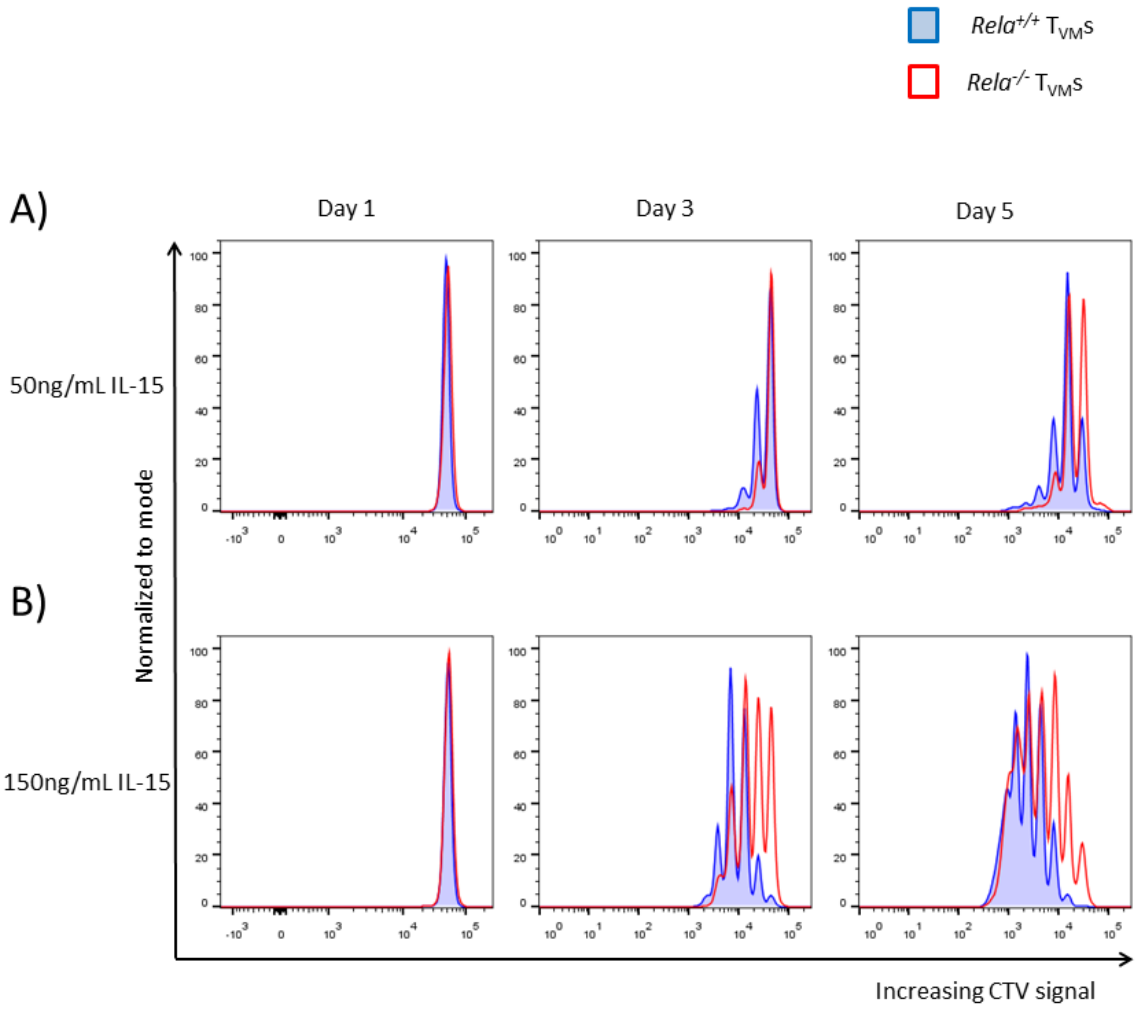


Figure 5.3. *Rela*$^{-/-}$ T_{VM} cells have a delayed proliferative response in higher concentrations of IL-15. (**A-B**) $CD8^{+}CD44^{hi}CD49d^{lo}$ T_{VM} cells isolated from 12-week-old *Lck*^{cre}*Rela*$^{wt/wt}$ and *Lck*^{cre}*Rela*$^{fl/fl}$ mice were stimulated *in vitro* with different concentrations of IL-15 and proliferation measured after 1, 3 and 5-days by CTV dilution. (**A**) Proliferation of *Rela*$^{+/+}$ and *Rela*$^{-/-}$ T_{VM}s stimulated with 50ng/mL IL-15. (**B**) Proliferation of *Rela*$^{+/+}$ and *Rela*$^{-/-}$ T_{VM}s stimulated with 150ng/mL IL-15. T_{VM}=$CD8^{+}CD44^{hi}CD49d^{lo}$ Virtual Memory cells; CTV=cell trace violet.

Lck^{cre}*Rela*$^{wt/wt}$ and *Lck*^{cre}*Rela*$^{fl/fl}$ mice were stimulated with 50ng/mL IL-15 in culture for 10, 30, 60 and 120-minutes, after which the levels of STAT-5 phosphorylation were measured by the flow cytometric analysis of cells subjected to intracellular staining with anti-phospho-STAT5 (p-STAT5) antibodies. A comparison of the p-STAT5 MFI at each time-point for both unstimulated and IL-15 treated cells revealed no significant difference in STAT-5 phosphorylation for *Rela*$^{+/+}$ and *Rela*$^{-/-}$ T_{VM} cells over the 2 hour time-course of IL-15 stimulation (Fig.5.4). This result indicates that the reduced IL-15-dependent survival of RelA-deficient T_{VM} cells does not appear to be due to impaired phosphorylation-induced activation of the STAT-5 transcription factor.

5.2.3 Bcl-2 transgene expression appears to rescue the T_{VM} cell phenotype in Lck^{cre}*Rela*$^{fl/fl}$ *mice*

The T cell-conditional inactivation of RelA in *Lck*^{cre}*Rela*$^{fl/fl}$ mice leads to a selective drop in T_{VM} cell numbers between 6 and 12-weeks of age, with the T_{VM} cell population found to be reduced by approximately 50% thereafter throughout adult life. The reduced RelA-deficient T_{VM} cell population of *Lck*^{cre}*Rela*$^{fl/fl}$ mice also exhibit a reduction in the surface expression levels of CD122 and CD127, coinciding with a decreased survival rate when cultured in the presence of IL-15 or IL-7, two cytokines crucial for driving the generation and homeostatic maintenance of T_{VM} cells (Carrio et al, 2007; Sandau et al, 2007; White et al, 2017). This cytokine regulated homeostatic maintenance of the T_{VM} cell population is determined by the combined rates of cell survival and proliferation. In MP $CD8^+$ T_{VM} cells, IL-15 and IL-7-induced STAT transcription factor activation regulates the transcription of genes encoding molecules that regulate survival and proliferation. The survival of MP $CD8^+$ T cells is regulated by a number of pro-survival factors, including Bcl-xL, Mcl-1 and Bcl-2, the latter of which is induced following IL-15 or IL-7 stimulation (Surh and Sprent, 2009). To determine what contribution a survival defect makes to the reduced numbers of T_{VM} cells in adult *Lck*^{cre}*Rela*$^{fl/fl}$ mice, this strain was intercrossed with the vav-bcl2 strain that expresses high levels of a human *Bcl2* transgene under the transcriptional control of the pan-

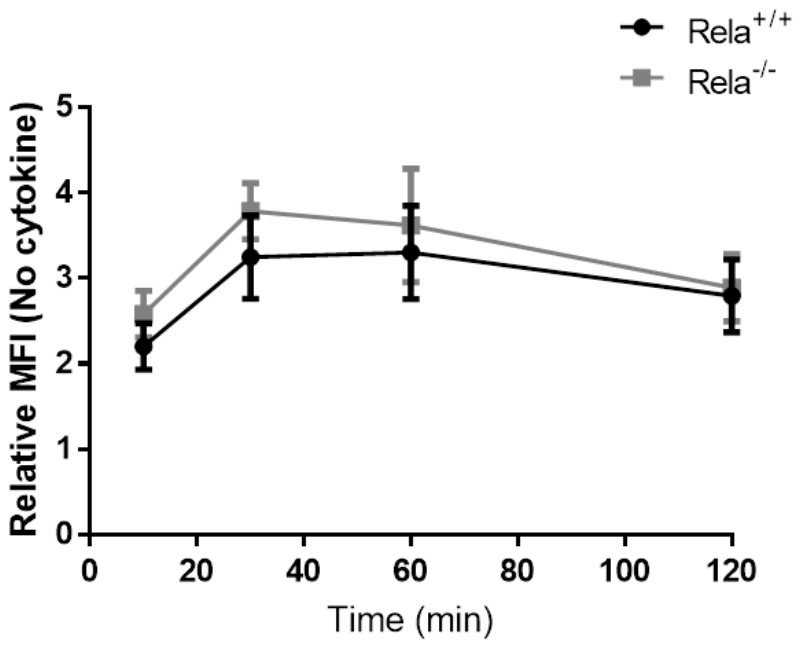


Figure 5.4. STAT-5 phosphorylation following IL-15 stimulation is equivalent in *Rela*$^{+/+}$ and *Rela*$^{-/-}$ T_{VM} cells. $CD8^{+}CD44^{hi}CD49d^{lo}$ T_{VM} cells isolated from 12-week-old *Lck*^{cre}*Rela*$^{wt/wt}$ and *Lck*^{cre}*Rela*$^{fl/fl}$ mice were stimulated for 2-hours *in vitro* with 50ng/mL IL-15 and the levels of phosphorylated STAT-5 measured by flow cytometry (n=4). Graph presented as the mean +/- SEM of the MFI of IL-15-stimulated T_{VM} cells relative to the MFI of unstimulated T_{VM} cells, n numbers represent independent assays taken as average of triplicate repeat wells, statistical significance was determined using Multiple T test. T_{VM}=$CD8^{+}CD44^{hi}CD49d^{lo}$ Virtual Memory cells.

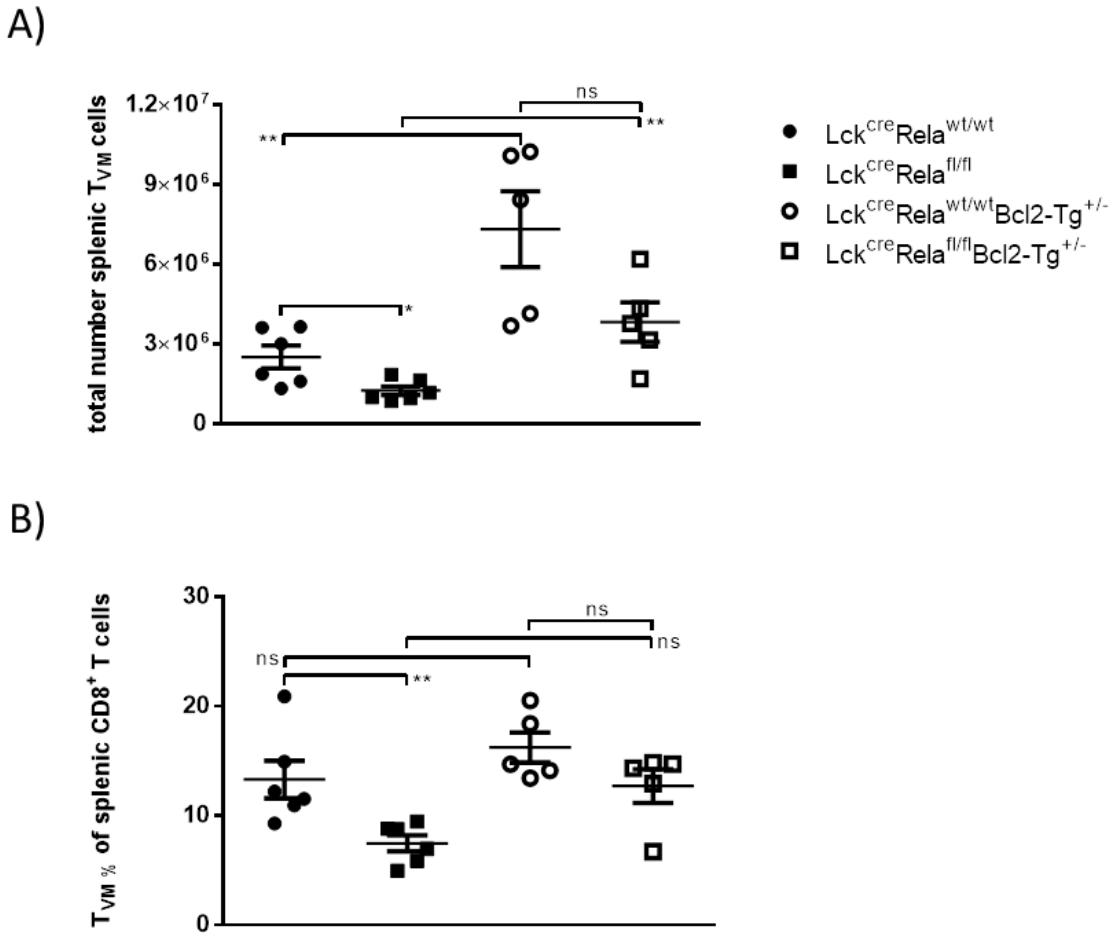


Figure 5.5. ***Rela*$^{-/-}$ T_{VM} cells have reduced viability when cultured in various concentrations of IL-15.** (**A-B**) Flow cytometric analysis of T_{VM} cells from spleen of 12-week-old *Lck*^{cre}*Rela*$^{wt/wt}$, *Lck*^{cre}*Rela*$^{fl/fl}$, *Lck*^{cre}*Rela*$^{wt/wt}$*Bcl2-Tg*$^{+/-}$ and *Lck*^{cre}*Rela*$^{fl/fl}$ *Bcl2-Tg*$^{+/-}$ mice (n=5-6). (**B**) Total number of T_{VM} cells in spleen. (**A**) Percentage of T_{VM} cells of $CD8^+$ T cells from spleen. All graphs presented as the mean +/- SEM, statistical significance was determined using Student's T tests. ns, not significant; *, p≤0.05; ** p≤0.01. T_{VM}=$CD8^+CD44^{hi}CD49d^{lo}$ Virtual Memory cells.

hematopoietic cell restricted mouse *vav2* promoter (Ogilvy et al, 1998; 1999). In all studies using an introduced Bcl-2 transgene, *Lck*^{cre}*Rela*$^{wt/fl}$*Bcl2-Tg*$^{+/-}$ and *Lck*^{cre}*Rela*$^{wt/fl}$ mice were mated to generate litter-matched mice heterozygous for the *Bcl2* transgene (*Bcl2-Tg*$^{+/-}$) and homozygous for the WT (*Rela*$^{wt/wt}$) or floxed (*Rela*$^{fl/fl}$) *Rela* gene. A comparison of *Lck*^{cre}*Rela*$^{wt/wt}$*Bcl2-Tg*$^{+/-}$ and *Lck*^{cre}*Rela*$^{fl/fl}$*Bcl2-Tg*$^{+/-}$ mice was then undertaken using flow cytometry to determine what effect the introduction of a *Bcl2* transgene had on the sizes of the splenic T_{VM} cell populations of these mice, and whether the introduction of this transgene could rescue the drop in T_{VM} cell numbers seen in *Lck*^{cre}*Rela*$^{fl/fl}$ mice. Analysis of 12-week-old *Lck*^{cre}*Rela*$^{wt/wt}$*Bcl2-Tg*$^{+/-}$ and *Lck*^{cre}*Rela*$^{fl/fl}$*Bcl2-Tg*$^{+/-}$ mice revealed a significant increase in the numbers of the T_{VM} cells in both strains when compared to age- matched *Lck*^{cre}*Rela*$^{wt/wt}$ and *Lck*^{cre}*Rela*$^{fl/fl}$ counterparts (Fig5.5A). Moreover, when comparing the sizes of the splenic T_{VM} cell populations of *Lck*^{cre}*Rela*$^{wt/wt}$*Bcl2-Tg*$^{+/-}$ and *Lck*^{cre}*Rela*$^{fl/fl}$*Bcl2-Tg*$^{+/-}$ mice, no significant difference was observed for either the total number of T_{VM} cells (Fig-5.5A), or the proportion of T_{VM} cells within the total $CD8^+$ T cell population (Fig.5.5B). These findings show that the over-expression of a *Bcl2* transgene in the hematopoietic cell compartment could overcome the comparative reduction in the T_{VM} cell population resulting from the loss of RelA.

5.2.4 Bcl-2 transgene expression rescues the survival of RelA-deficient T_{VM} cells at lower doses of IL-15

When cultured in the presence of recombinant IL-15, T_{VM} cells taken from *Lck*^{cre}*Rela*$^{fl/fl}$ mice exhibit a reduction in the rates of survival and proliferation when compared to T_{VM} cells taken from WT littermate controls (*Lck*^{cre}*Rela*$^{wt/wt}$ mice). After both 3 and 5-days, *Rela*$^{-/-}$ T_{VM} cells cultured with either intermediate (50ng/mL) or high (150ng/mL) concentrations of IL-15, displayed significantly lower cell viability than *Rela*$^{+/+}$ T_{VM} cells, with the surviving *Rela*$^{-/-}$ T_{VM} cells found to have undergone fewer rounds of cell division. Whilst this finding suggests that RelA independently promotes both the survival and the proliferation of T_{VM} cells responding to IL-15, another possible explanation for the delayed proliferative

response of *Rela*$^{-/-}$ T_{VM} cells is that RelA is primarily required for maintaining the survival of activated T_{VM} cells following IL-15 stimulation, with proliferating RelA-deficient T_{VM} cells undergoing increased IL-15 activation-induced cell death. To determine whether the T cell-intrinsic over-expression of a Bcl2 transgene could resolve the relative importance the rates of IL-15 regulated survival and proliferation play in the homeostasis of *Rela*$^{-/-}$ T_{VM} cells, these *in vitro* experiments were repeated using T_{VM} cells taken from *Lck*^{cre}*Rela*$^{wt/wt}$*Bcl2-Tg*$^{+/-}$ and *Lck*^{cre}*Rela*$^{fl/fl}$*Bcl2-Tg*$^{+/-}$ mice. T_{VM} cells isolated by FACS from 12-week-old mice were pre-stained with the cell division monitoring dye, Cell Trace Violet (CTV), and then incubated in the absence or presence of intermediate (50ng/mL) or high concentrations (150ng/mL) of recombinant IL-15. After 1, 3 and 5-days incubation, cells were stained with the viability dye, LiveDead Aqua and analyzed by flow cytometry to quantify cell survival and proliferation. At intermediate concentrations of IL-15 (50ng/mL), no difference was observed in the survival of *Rela*$^{+/+}$*Bcl2-Tg*$^{+/-}$ and *Rela*$^{-/-}$*Bcl2-Tg*$^{+/-}$ T_{VM}s after 1, 3 or 5-days incubation (Fig.5.6B). A similar outcome was observed for T_{VM}s of both genotypes cultured in the absence of cytokine (Fig.5.6A). Despite this concentration of IL-15 being unable to further elevate the survival of *Rela*$^{+/+}$*Bcl2-Tg*$^{+/-}$ and *Rela*$^{-/-}$*Bcl2-Tg*$^{+/-}$ T_{VM} cells in culture above that conferred by enforced Bcl2 expression (Fig.5.6C), it still induced the division of these cells. Whilst a modest, yet comparable level of proliferation was observed for *Rela*$^{+/+}$*Bcl2-Tg*$^{+/-}$ and *Rela*$^{-/-}$*Bcl2-Tg*$^{+/-}$ T_{VM} cells after 3-days of cell culture, after 5-days incubation, the division of *Rela*$^{-/-}$*Bcl2-Tg*$^{+/-}$ T_{VM} cells was comparatively slower compared to *Rela*$^{+/+}$*Bcl2-Tg*$^{+/-}$ T_{VM} cells (Fig.3.6D). Given the comparable rates of *Rela*$^{+/+}$*Bcl2-Tg*$^{+/-}$ and *Rela*$^{-/-}$*Bcl2-Tg*$^{+/-}$ T_{VM} cell survival throughout the duration of the assay, this finding confirms that T_{VM} cells lacking RelA have a slower rate of proliferation following stimulation with intermediate concentrations of IL-15 and that RelA serves a survival-independent role in driving the IL-15-induced proliferation of T_{VM} cells.

A similar defect in the IL-15-induced proliferation of *Rela*$^{-/-}$*Bcl2-Tg*$^{+/-}$ T_{VM} cells was also seen at higher doses of IL-15 (150ng/mL). At this concentration of IL-15, the defective proliferative response of *Rela*$^{-/-}$

Bcl2-Tg$^{+/-}$ T_{VM} cells was apparent earlier in the assay, with these cells found to have undergone fewer rounds of cell division after 3-days of cell culture when compared to *Rela*$^{+/+}$*Bcl2-Tg*$^{+/-}$ T_{VM} cells (Fig.5.7C). Moreover, the *Rela*$^{-/-}$*Bcl2-Tg*$^{+/-}$ T_{VM} cell proliferative defect was even greater after 5-days incubation. In addition to demonstrating that high concentrations of IL-15 were unable to rescue the proliferative response of *Rela*$^{-/-}$*Bcl2-Tg*$^{+/-}$ T_{VM} cells, this assay produced some unexpected results when cell survival responses were examined. After 3-days incubation in the presence of this higher concentration of IL-15, not only was the survival rate of *Rela*$^{-/-}$*Bcl2-Tg*$^{+/-}$ T_{VM} cells reduced significantly when compared to *Rela*$^{+/+}$*Bcl2-Tg*$^{+/-}$ T_{VM}s cultured at an equivalent concentration of cytokine (Fig.5.7A), but also significantly reduced when compared to *Rela*$^{-/-}$*Bcl2-Tg*$^{+/-}$ T_{VM} cells cultured in the absence of IL-15 (Fig.5.7.B). This result revealed an IL-15-induced survival defect for *Rela*$^{-/-}$*Bcl2-Tg*$^{+/-}$ T_{VM} cells, demonstrating a susceptibility to undergo activation-induced cell death following stimulation with high concentrations of IL-15 that could not be blocked by Bcl-2 transgene expression. After 5-days incubation in the higher concentration of IL-15, despite increasing the survival rate to levels comparable with *Rela*$^{-/-}$*Bcl2-Tg*$^{+/-}$ T_{VM}s cultured in the absence of IL-15 (Fig.5.7B), the viability of *Rela*$^{-/-}$*Bcl2-Tg*$^{+/-}$ T_{VM} cells was still significantly reduced when compared to *Rela*$^{+/+}$*Bcl2-Tg*$^{+/-}$ T_{VM} cells cultured for 5-days in 150ng/mL IL-15 (Fig.5.7A). These data, together with related experiments described previously (*Chapter 5.2.1*), collectively show that T_{VM} cells lacking RelA have reduced rates of proliferation, as well as survival when activated *in vitro* with various concentrations of IL-15. Moreover, at higher concentrations of IL-15, RelA-deficient T_{VM}s undergo IL-15-mediated activation-induced cell death, a survival defect that could not be overcome by the transgenic over-expression of the pro-survival factor Bcl-2.

5.2.5 Immediate ex vivo analysis of T_{VM} cell proliferation using intracellular Ki-67 stains

Data obtained from *in vitro* IL-15 stimulation assays revealed roles for RelA in regulating both the survival and proliferation of T_{VM} cells responding to IL-15. This discovery prompted a comparison of Ki-67

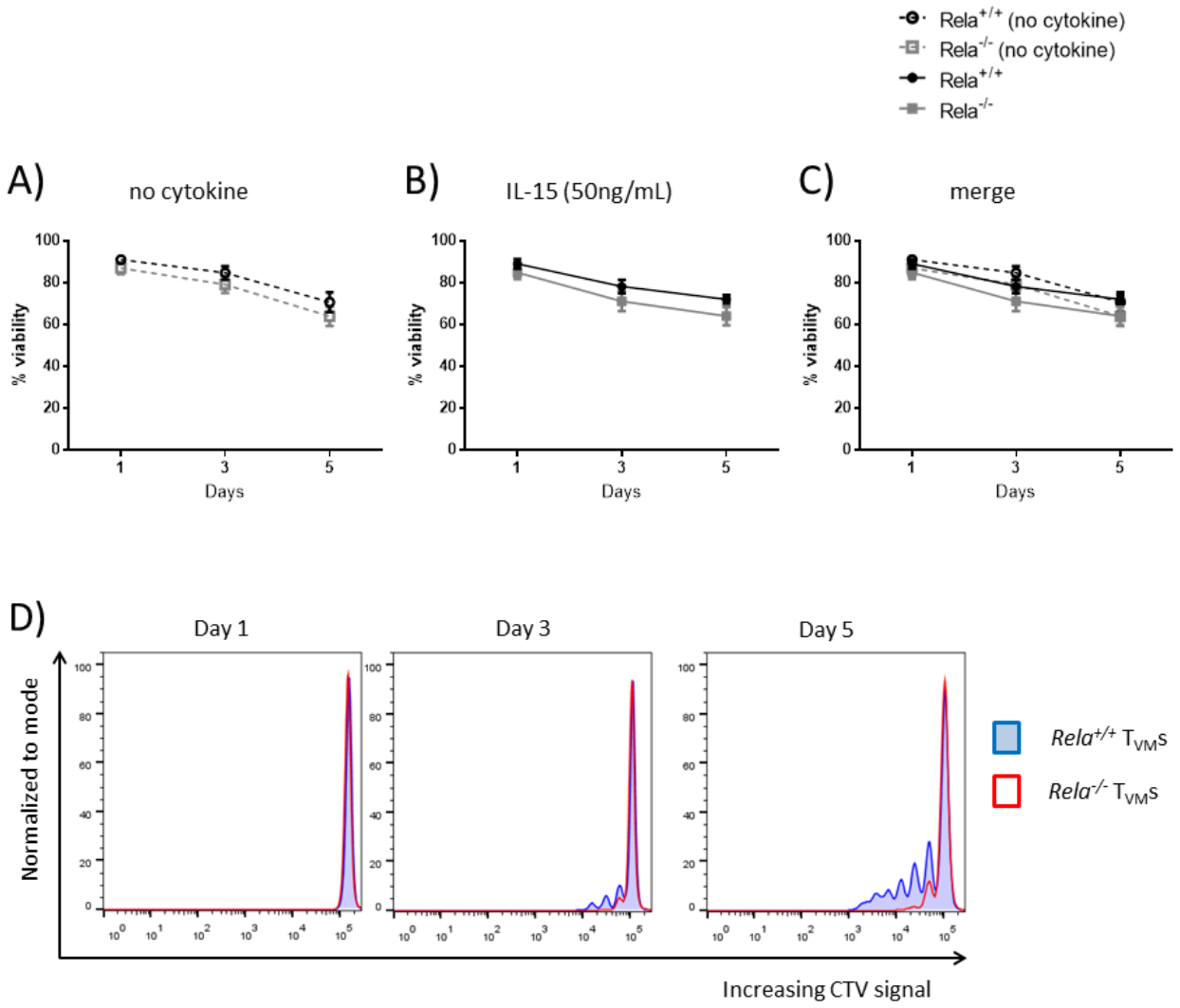


Figure 5.6. The presence of the Bcl-2-Tg rescues the viability of *Rela*$^{-/-}$ T_{VM}s at the intermediate concentration of IL-15. (**A-D**) $CD8^{+}CD44^{hi}CD49d^{lo}$ T_{VM} cells isolated from 12-week-old *Lck*^{cre}*Rela*$^{wt/wt}$*Bcl2-Tg*$^{+/-}$ and *Lck*^{cre}*Rela*$^{fl/fl}$ *Bcl2-Tg*$^{+/-}$ mice were stimulated *in vitro* with 50ng/mL IL-15. After 1, 3 and 5-days incubation viability was measured by LiveDead aqua staining and proliferation measured by CTV dilution (n=5). (**A**) The frequency of viable *Rela*$^{+/+}$*Bcl2-Tg*$^{+/-}$ and *Rela*$^{-/-}$*Bcl2-Tg*$^{+/-}$ T_{VM}s in the absence of IL-15. (**B**) The frequency of viable *Rela*$^{+/+}$*Bcl2-Tg*$^{+/-}$ and *Rela*$^{-/-}$*Bcl2-Tg*$^{+/-}$ T_{VM}s stimulated with 50ng/mL IL-5. (**C**) Comparative overlay of *Rela*$^{+/+}$*Bcl2-Tg*$^{+/-}$ and *Rela*$^{-/-}$*Bcl2-Tg*$^{+/-}$ T_{VM} cell viability in the absence of cytokine and in the presence of 50ng/mL IL-15. (**D**) Proliferation of *Rela*$^{+/+}$*Bcl2-Tg*$^{+/-}$ and *Rela*$^{-/-}$*Bcl2-Tg*$^{+/-}$ T_{VM}s stimulated with 50ng/mL IL-15. All graphs presented as the mean +/- SEM, statistical significance was determined using Multiple T test. *, p≤0.05; ** p≤0.01; *** p≤0.001. T_{VM}=$CD8^{+}CD44^{hi}CD49d^{lo}$ Virtual Memory cells; CTV=cell trace violet; Tg=transgene.

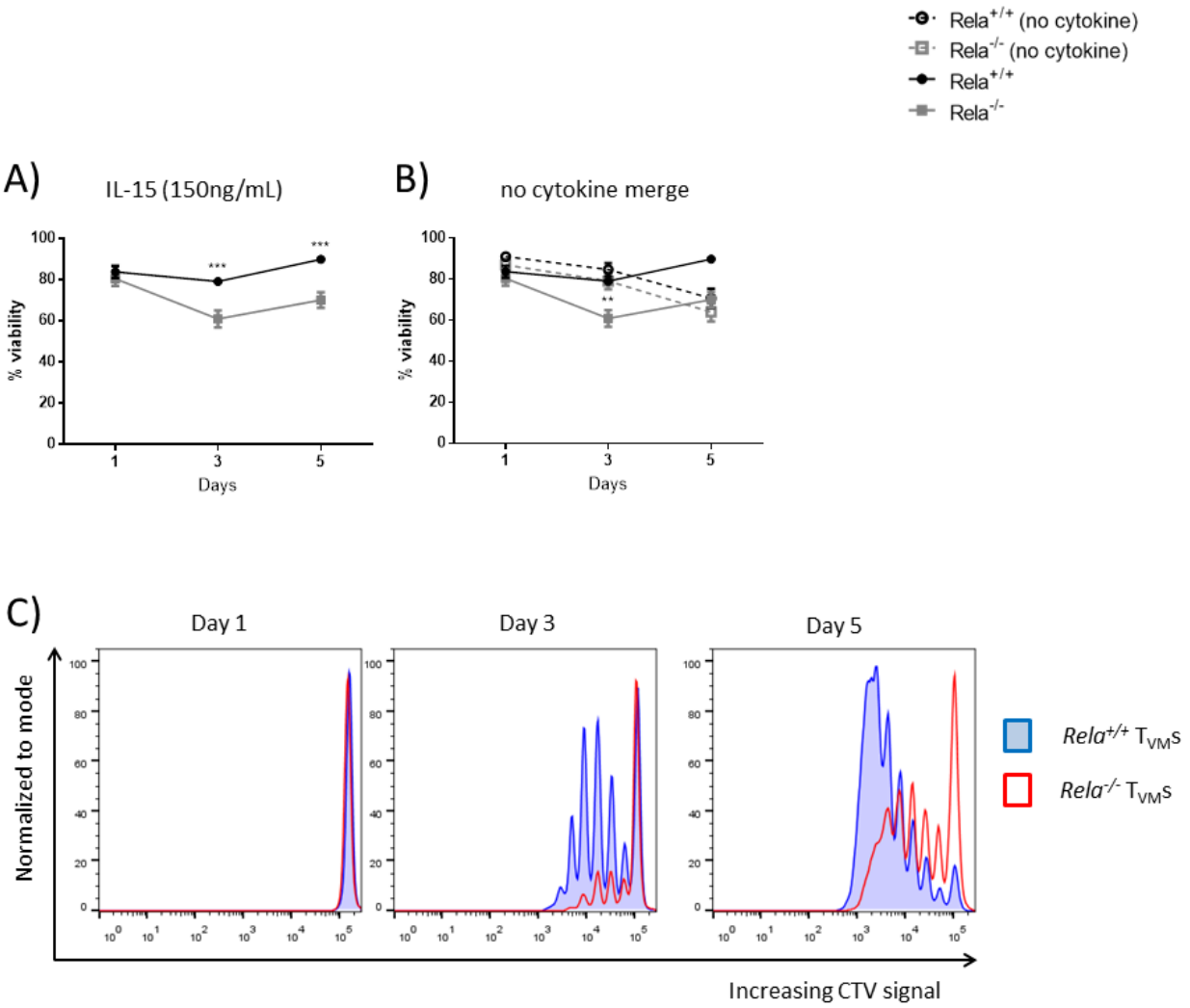


Figure 5.7. *Rela*$^{-/-}$ T_{VM}s display a proliferative lag and are susceptible to activation-induced cell death when stimulated with the higher concentration of IL-15. (**A-C**) $CD8^{+}CD44^{hi}CD49d^{lo}$ T_{VM} cells isolated from 12-week-old *Lck*^{cre}*Rela*$^{wt/wt}$*Bcl2-Tg*$^{+/-}$ and *Lck*^{cre}*Rela*$^{fl/fl}$ *Bcl2-Tg*$^{+/-}$ mice were stimulated *in vitro* with 150ng/mL IL-15. After 1, 3 and 5-days incubation viability was measured by LiveDead aqua staining and proliferation measured by CTV dilution (n=5). (**A**) The frequency of viable *Rela*$^{+/+}$*Bcl2-Tg*$^{+/-}$ and *Rela*$^{-/-}$*Bcl2-Tg*$^{+/-}$ T_{VM}s stimulated with 150ng/mL IL-15. (**B**) Comparative overlay of *Rela*$^{+/+}$*Bcl2-Tg*$^{+/-}$ and *Rela*$^{-/-}$*Bcl2-Tg*$^{+/-}$ T_{VM} cell viability in the absence of cytokine and in the presence of 150ng/mL IL-15. (**C**) Proliferation of *Rela*$^{+/+}$*Bcl2-Tg*$^{+/-}$ and *Rela*$^{-/-}$*Bcl2-Tg*$^{+/-}$ T_{VM}s stimulated with 150ng/mL IL-15. All graphs presented as the mean +/- SEM, statistical significance was determined using Multiple T test. *, p≤0.05; ** p≤0.01; *** p≤0.001. T_{VM}=$CD8^{+}CD44^{hi}CD49d^{lo}$ Virtual Memory cells; CTV=cell trace violet; Tg=transgene.

expression levels in the immediate *ex vivo* T_{VM} cell populations of *Lck*^{cre}*Rela*$^{wt/wt}$ and *Lck*^{cre}*Rela*$^{fl/fl}$ mice to determine whether a steady-state proliferative defect may account in part for the reduction in T_{VM} cell numbers of *Lck*^{cre}*Rela*$^{fl/fl}$ mice. Ki-67 is a nuclear protein marker that is commonly used as indicator of the overall rate of proliferation within a given cell population (Scholzen and Gerdes, 2000). Whilst cells in a resting state (G_0) do not express Ki-67, it is up-regulated following entry into G_1 of the cell cycle and sustained through all stages of cell growth and division (Scholzen and Gerdes, 2000). To determine how the T cell-conditional deletion of RelA impacts the rate of T_{VM} cell homeostatic proliferation under non-lymphopenic conditions, Ki-67 expression levels in splenic T_{VM} cells of 12-week-old *Lck*^{cre}*Rela*$^{wt/wt}$ and *Lck*^{cre}*Rela*$^{fl/fl}$ mice were compared using flow cytometry. This analysis showed statisticall comparable levels of Ki-67 in T_{VM} cells taken from 12-week-old *Lck*^{cre}*Rela*$^{wt/wt}$ and *Lck*^{cre}*Rela*$^{fl/fl}$ mice (Fig.5.8), suggesting that the drop in T_{VM} cell numbers that occurs between 6 and 12-weeks in *Lck*^{cre}*Rela*$^{fl/fl}$ mice is not caused by a reduction in the rate of homeostatic proliferation under steady-state conditions.

5.2.6 RelA-deficient T_{VM} cells fail to homeostatically expand in a chronic lymphopenic Rag1$^{-/-}$ environment

This investigation into the role of RelA in T_{VM} cell development using T cell conditional KO (*Lck*^{cre}*Rela*$^{fl/fl}$) and radio-ablated (*Rela*$^{-/-}$) HSC chimeric mouse models, has shown that RelA is not absolutely essential for generating T_{VM} cells. Instead, data obtained from the analysis of both models of *Rela* gene-inactivation would suggest that T_{VM} cells lacking RelA are in the main impaired in their capacity to undergo homeostatic expansion and/or maintain population homeostasis in response to the γc cytokines IL-7 and IL-15. Whilst further investigation has found that RelA-deficient T_{VM} cells stimulated with recombinant IL-7/15 *in vitro* have significantly reduced rates of proliferation and/or survival, how the loss of RelA impacts the homeostatic expansion of T_{VM} cells *in vivo* is yet to be properly investigated. As a start to addressing this question, the capacity of mature RelA-deficient T_{VM} cells to homeostatically expand *in vivo* was examined by engrafting T_{VM} cells from 12-week-old *Lck*^{cre}*Rela*$^{wt/wt}$ and *Lck*^{cre}*Rela*$^{fl/fl}$

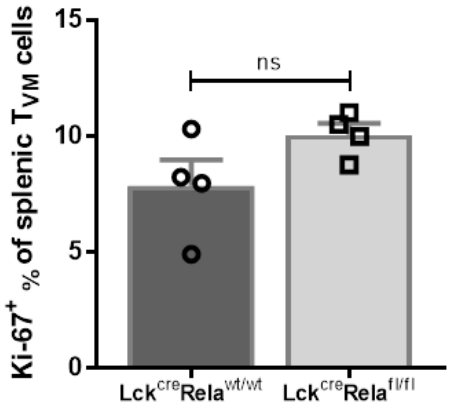


Figure 5.8. The levels of nuclear Ki-67 in T_{VM} cells taken from *Lck^{cre}Rela$^{wt/wt}$* and *Lck^{cre}Rela$^{fl/fl}$* mice is statistically comparable. Flow cytometric analysis of T_{VM} cells from spleens of 12-week-old *Lck^{cre}Rela$^{wt/wt}$* and *Lck^{cre}Rela$^{fl/fl}$* mice measuring nuclear Ki-67 levels (n=4). Whilst the proportion of T_{VM} cells expressing Ki-67 is marginally increased in *Lck^{cre}Rela$^{fl/fl}$* mice, no statistical difference was determined when compared with T_{VM} cells in *Lck^{cre}Rela$^{wt/wt}$* mice. Graph presented as the mean +/- SEM, statistical significance was determined using Student's T test. ns, not significant. T_{VM}=CD8^{+}CD44^{hi}CD49d^{lo} Virtual Memory cells.

mice into congenic C57BL/6 recipient mice homozygous for a null mutation in the gene encoding the recombination activating gene-1 (RAG-1) protein (*Rag1*$^{-/-}$ mice). *Rag1*$^{-/-}$ mice are unable to activate V(D)J recombination during lymphocyte development, arresting lymphocyte differentiation in the BM and thymus resulting in a complete absence of B and T cells. In turn, the lack of B and T cells within various lymphoid organs of *Rag1*$^{-/-}$ mice allows for the investigation of the role RelA serves in the homeostatic expansion of T_{VM} cells without the confounding influence of factors created by the irradiation process. After 7 or 30-days following the transplantation of 2x10^6 *Rela*$^{+/+}$ or *Rela*$^{-/-}$ T_{VM} cells (>95% purity; see *Methods 2.3.4*) into individual *Rag1*$^{-/-}$ recipient mice, these animals were culled and the spleens analysed by flow cytometry to determine cell numbers and the extent of T_{VM} cell expansion. Whilst a considerable number of T_{VM} cells were recovered from the spleen of *Rag1*$^{-/-}$ mice receiving *Rela*$^{+/+}$ T_{VM} cells 7-days following cell transplantation, T_{VM} cells were largely absent at this time point from the spleen of *Rag1*$^{-/-}$ mice receiving *Rela*$^{-/-}$ T_{VM} cells (Fig.5.9A). Whilst the number of T_{VM} cells recovered from *Rag1*$^{-/-}$ mice receiving *Rela*$^{-/-}$ T_{VM}s 30-days after cell transplant was greater than at day 7, these numbers were still significantly reduced when compared to *Rag1*$^{-/-}$ mice receiving *Rela*$^{+/+}$ T_{VM}s 30-days post-transfer. This finding demonstrates that RelA-deficient T_{VM} cells are not only less responsive to homeostatic signals elicited by IL-15 and IL-7, but are also impaired in their capacity to undergo homeostatic expansion within a lymphopenic environment lacking other T cells competing for the γc family cytokines involved in T_{VM} cell homeostasis. Notwithstanding a severe lack of T_{VM} cells, there was not a complete absence of transferred cells in the spleen of *Rag1*$^{-/-}$ mice receiving *Rela*$^{-/-}$ T_{VM} cells. Some transferred T_{VM} cells were also found to have up-regulated CD49d expression, indicating these cells had received sustained TCR signaling, most likely through recognition of cognate antigen from interaction with commensal bacteria. Whilst T_{VM} cells were largely absent 7 days after the transfer of *Rela*$^{-/-}$ T_{VM} cells, significant numbers of CD44^{hi}CD49d^{hi}CD8$^+$ T cells were recovered from the spleen of these *Rag1*$^{-/-}$ mice at this time point (Fig.5.9B), showing comparable numbers to an equivalent CD44^{hi}CD49d^{hi}CD8$^+$ T

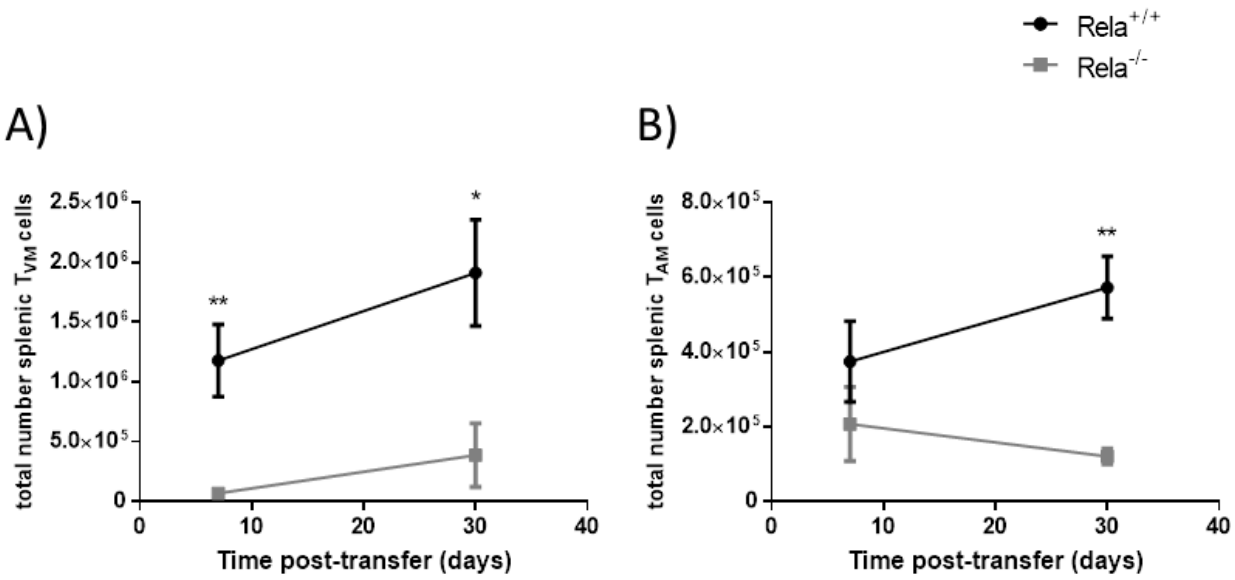


Figure 5.9. *Lck^{cre}Rela$^{fl/fl}$* T_{VM} cells are impaired in their capacity to homeostatically expand when adoptively transferred into *Rag1$^{-/-}$* mice. (**A-B**) Flow cytometric analysis of $CD8^+$ T cells recovered from spleen of *Rag1$^{-/-}$* mice receiving $2x10^6$ FACS sorted T_{VM} cells ($CD8^+CD44^{hi}CD49d^{lo}$) isolated from 12-week-old *Lck^{cre}Rela$^{wt/wt}$* or *Lck^{cre}Rela$^{fl/fl}$* mice (n=5). Analysis performed 7 and 30-days following transfer showing: (**A**) Total number of T_{VM} cells in the spleens of *Rag1$^{-/-}$* recipient mice. (**B**) Total number of T_{AM} cells in the spleens of *Rag1$^{-/-}$* recipient mice. All graphs presented as the mean +/- SEM, statistical significance was determined using Multiple T test. *, p≤0.05; ** p≤0.01. T_{VM}=$CD8^+CD44^{hi}CD49d^{lo}$ Virtual Memory cells; T_{AM}=$CD8^+CD44^{hi}CD49d^{hii}$ conventional antigen-primed memory cells.

cell population in the spleen of *Rag1*$^{-/-}$ mice receiving *Rela*$^{+/+}$ T_{VM}s (Fig.5.9B). This indicates that the early antigen-induced conversion of *Rela*$^{-/-}$ T_{VM} cells in a lymphopenic environment is not impaired by the absence of RelA. Whilst the *Rela*$^{+/+}$ CD44^{hi}CD49d^{hi}CD8^{+} T cell population underwent further expansion in *Rag1*$^{-/-}$ mice between 7 and 30-days, the *Rela*$^{-/-}$ CD44^{hi}CD49d^{hi}CD8^{+} T cell population failed to be maintained, exhibiting significantly reduced cell numbers compared to the *Rela*$^{+/+}$ CD44^{hi}CD49d^{hi}CD8^{+} T cell population 30-days following transfer (Fig.5.9B). This drop in *Rela*$^{-/-}$ T_{VM} cells that have up-regulated CD49d in response to sustained TCR signaling may reflect a role for RelA in promoting the sustained TCR-driven expansion and maintenance responses of T_{VM} cells following antigen-induced activation.

5.2.7 The TCR-driven proliferation of T_{VM} cells in culture is impacted by the loss of RelA

Data obtained from the *Rag1*$^{-/-}$ adoptive transfer experiments indicated there was an issue involving the TCR-driven proliferation of T_{VM} cells lacking RelA. This prompted an investigation into the capacity of T_{VM} cells to proliferate *in vitro* following TCR stimulation. In these experiments, splenic CD8^{+}CD44^{hi}CD49d^{lo} T_{VM} cells were isolated by FACS from 12-week-old *Lck*^{cre}*Rela*$^{wt/wt}$ and *Lck*^{cre}*Rela*$^{fl/fl}$ mice, pre-stained with the cell division monitoring dye CTV and stimulated for 3 days with 1ug/mL plate-bound anti-CD3 antibodies in the absence or presence of recombinant IL-7 (2ng/mL) or IL-15 (50ng/mL). After 1, 2 or 3-days incubation, cells were stained with the viability dye, LiveDead Aqua and analyzed by flow cytometry to determine rates of survival and proliferation. While both *Rela*$^{+/+}$ and *Rela*$^{-/-}$ T_{VM} cells stimulated with plate-bound anti-CD3 in the absence of cytokine showed a comparable decline in cell viability throughout the duration of the assay (Fig.5.10A), in the presence of 2ng/mL IL-7, *Rela*$^{-/-}$ T_{VM} cells stimulated with plate-bound anti-CD3 showed a significant reduction in survival compared to *Rela*$^{+/+}$ T_{VM} cells after 1, 2 and 3-days incubation (Fig.5.10B). Whilst a combination of plate-bound anti-CD3 and 2ng/mL IL-7 was sufficient to induce cell division in both *Rela*$^{+/+}$ and *Rela*$^{-/-}$ T_{VM} cell cultures, *Rela*$^{-/-}$ T_{VM} cells exhibited a proliferative lag after 3-days incubation that was more pronounced after 5-days

incubation (Fig.5.10D). In the presence of 50ng/mL IL-15, *Rela*$^{+/+}$ and *Rela*$^{-/-}$ T_{VM} cells stimulated with plate-bound anti-CD3 showed comparable rates of survival throughout the duration of the assay (Fig.5.11A). Notwithstanding comparable rates of survival, *Rela*$^{-/-}$ T_{VM} cells stimulated with a combination of plate-bound anti-CD3 and 50ng/mL IL-15 showed a delayed rate of proliferation when compared to WT control (*Rela*$^{+/+}$) cultures after 3 and 5-days incubation. These results show that the TCR-driven proliferation of T_{VM} cells is impaired in the absence of RelA.

5.2.8 Comparative RNA-sequencing analysis of gene expression in Rela$^{+/+}$ and Rela$^{-/-}$ T_{VM} cells

To help gain a better understanding of how the genetic inactivation of *Rela* might affect T_{VM} cell homeostasis, RNA sequencing (RNAseq) analysis was undertaken to compare the gene expression profiles of WT and RelA-deficient T_{VM} cells purified from 12-week-old *Lck*^{cre}*Rela*$^{wt/wt}$ and *Lck*^{cre}*Rela*$^{fl/fl}$ mice. Given that only two replicates of each *Rela*$^{+/+}$ and *Rela*$^{-/-}$ T_{VM} cell samples could be obtained, changes in gene expression resulting from the loss of RelA, as detailed below, is provided to give indication only of key findings (Supplementary Table.1). In comparing the gene expression profiles of *Rela*$^{+/+}$ and *Rela*$^{-/-}$ T_{VM} cells, ~80 genes were found to be differentially expressed. Despite an absence of changes in the expression of known NF-κB target genes encoding cell survival or cell cycle proteins, a number of gene expression changes of potential relevance to *Rela*$^{-/-}$ T_{VM} cell homeostasis were noted. Consistent with the CD127 flow cytometry data, *Il7r* transcripts were found to be reduced in *Rela*$^{-/-}$ T_{VM} cells. By contrast, despite the clear reduction in CD122 cell surface expression levels on RelA-deficient T_{VM} cells, no significant difference in the transcription of *Il2rb* was observed. Transcription of the gene encoding the α-chain of the IL-18R (*Il18ra*; CD218a) was also reduced in *Rela*$^{-/-}$ T_{VM} cells, which may infer a decreased responsiveness to inflammatory signals that appear to contribute to T_{VM} cell homeostasis and trigger bystander immune responses (White et al, 2017). T_{VM} cells lacking RelA also showed a reduction in the transcription of genes encoding other NF-κB pathway proteins (*Relb, Nfkb2 and Nfkbia*),

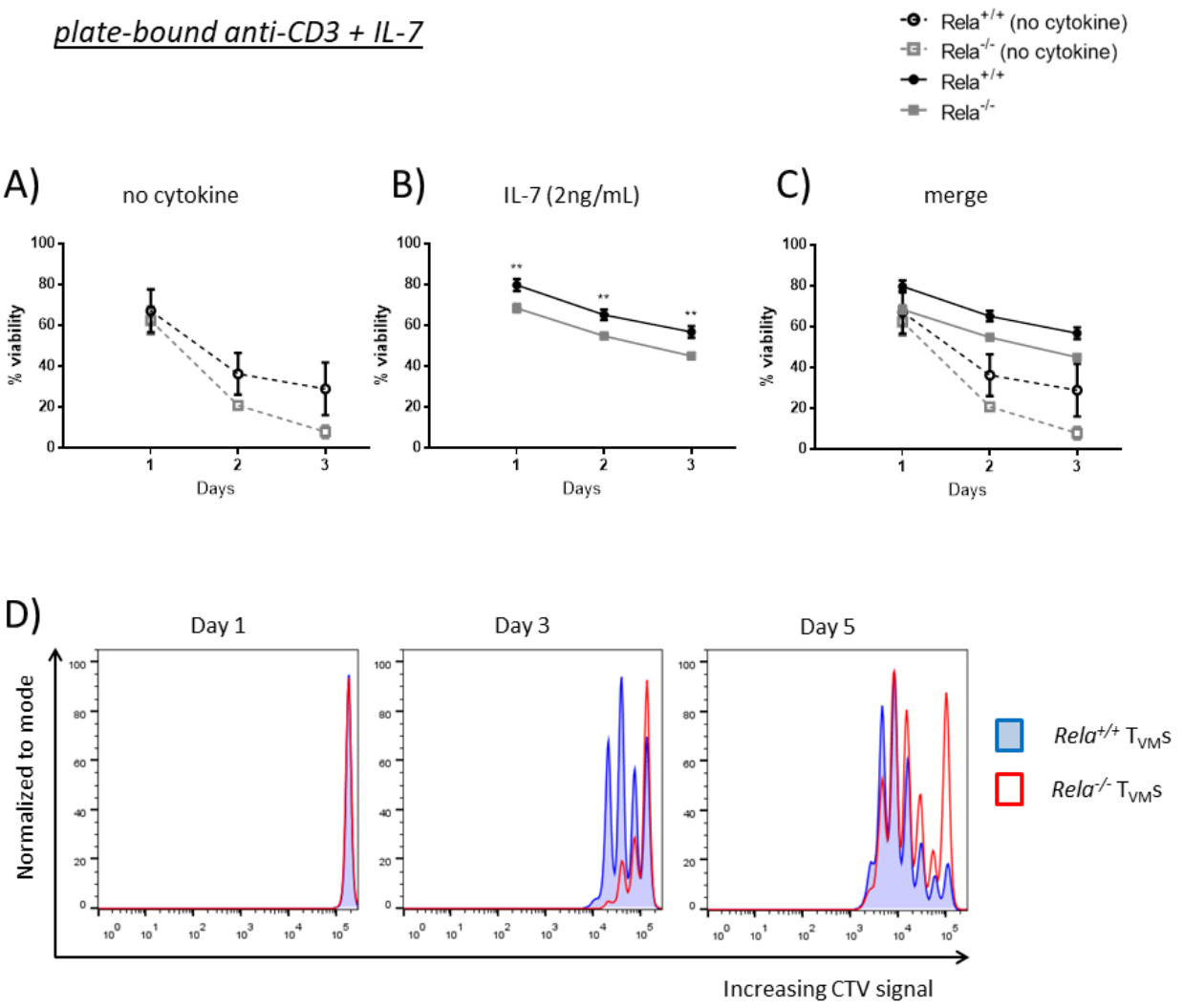


Figure 5.10. The survival and TCR-driven proliferation of *Rela*$^{-/-}$ T_{VM}s is reduced in the presence of IL-7. (**A-D**) $CD8^{+}CD44^{hi}CD49d^{lo}$ T_{VM} cells isolated from 12-week-old *Lck*^{cre}*Rela*$^{wt/wt}$ and *Lck*^{cre}*Rela*$^{fl/fl}$ mice were stimulated *in vitro* with 1ug/mL plate-bound anti-CD3 in the presence or absence of 2ng/mL IL-7. After 1, 2 and 3-days incubation viability was measured by LiveDead aqua staining and proliferation measured by CTV dilution (n=4). (**A**) The frequency of viable *Rela*$^{+/+}$ and *Rela*$^{-/-}$ T_{VM}s stimulated with plate-bound anti-CD3 in the absence of IL-7. (**B**) The frequency of viable *Rela*$^{+/+}$ and *Rela*$^{-/-}$ T_{VM}s stimulated with a combination of plate-bound anti-CD3 and IL-7. (**C**) Comparative overlay of *Rela*$^{+/+}$ and *Rela*$^{-/-}$ T_{VM} cell viability when stimulated with plate-bound anti-CD3 in the absence of cytokine and in the presence of IL-7. (**D**) Proliferation of *Rela*$^{+/+}$ and *Rela*$^{-/-}$ T_{VM}s stimulated with plate-bound anti-CD3 and IL-7. All graphs presented as the mean +/- SEM, statistical significance was determined using Multiple T test. ** $p \leq 0.01$. T_{VM}=$CD8^{+}CD44^{hi}CD49d^{lo}$ Virtual Memory cells; CTV=cell trace violet.

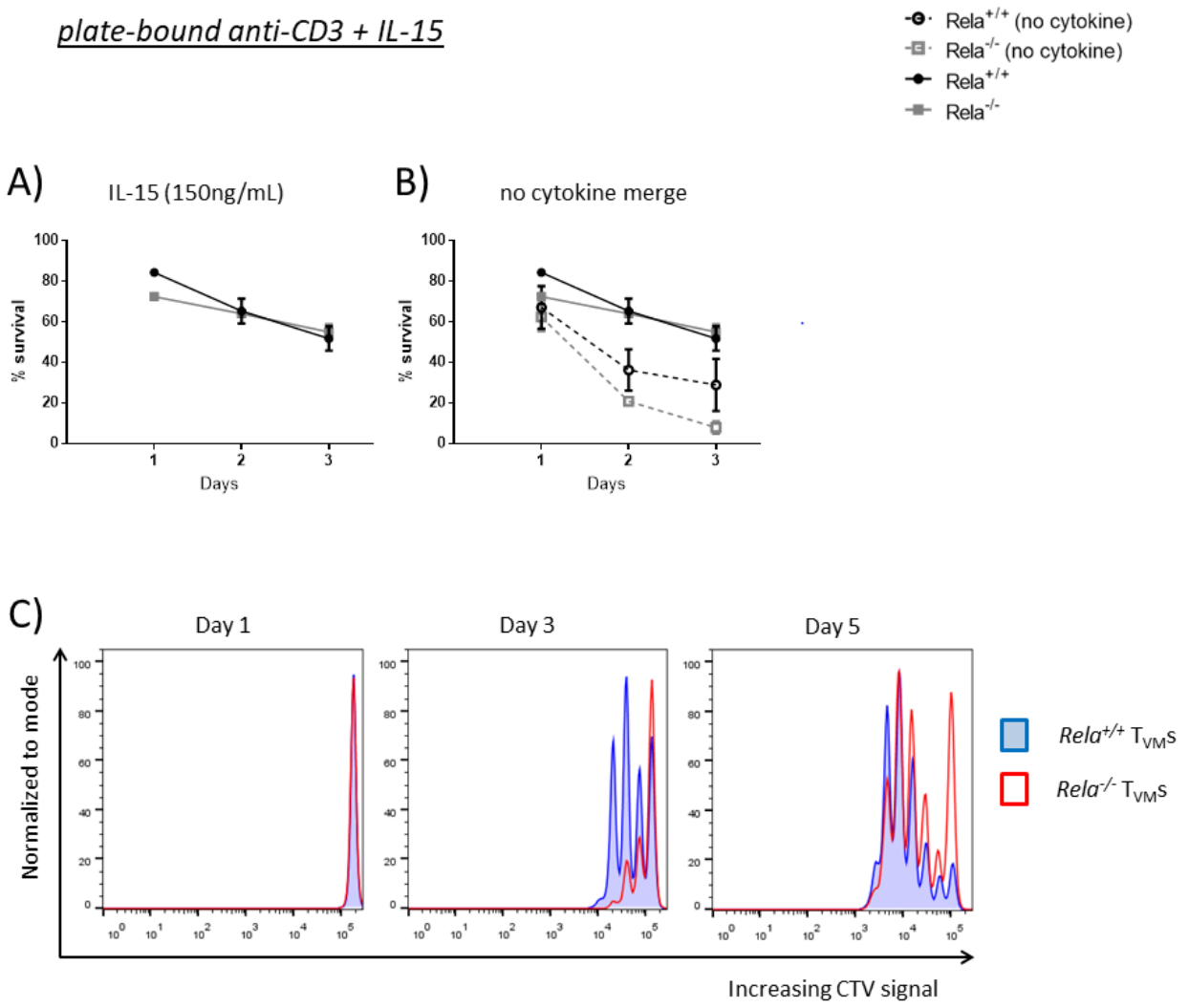


Figure 5.11. The TCR-driven proliferation of *Rela*$^{-/-}$ T_{VM}s is reduced in the presence of IL-15. (**A-C**) $CD8^{+}CD44^{hi}CD49d^{lo}$ T_{VM} cells isolated from 12-week-old *Lck*^{cre}*Rela*$^{wt/wt}$ and *Lck*^{cre}*Rela*$^{fl/fl}$ mice were stimulated *in vitro* with 1ug/mL plate-bound anti-CD3 in the presence or absence of 50ng/mL IL-15. After 1, 2 and 3-days incubation viability was measured by LiveDead aqua staining and proliferation measured by CTV dilution (n=4). (**A**) The frequency of viable *Rela*$^{+/+}$ and *Rela*$^{-/-}$ T_{VM}s stimulated with a combination of plate-bound anti-CD3 and IL-15. (**B**) Comparative overlay of *Rela*$^{+/+}$ and *Rela*$^{-/-}$ T_{VM} cell viability when stimulated with plate-bound anti-CD3 in the absence of cytokine and in the presence of IL-15. (**C**) Proliferation of *Rela*$^{+/+}$ and *Rela*$^{-/-}$ T_{VM}s stimulated with plate-bound anti-CD3 and IL-15. All graphs presented as the mean +/- SEM, statistical significance was determined using Multiple T test. ** $p \leq 0.01$. T_{VM}=$CD8^{+}CD44^{hi}CD49d^{lo}$ Virtual Memory cells; CTV=cell trace violet.

in particular the non-canonical activation pathway. Given the established roles the NF-κB pathway plays in the division and survival of numerous cell types, down-regulation of the non-canonical NF-κB pathway arising from the loss of RelA may contribute to impaired *Rela*$^{-/-}$ T_{VM} cell homeostasis. Of note was the finding that *Rela*$^{-/-}$ T_{VM} cells also showed significant increases in the transcription of genes encoding the immune inhibitory receptors CTLA-4 and LAG-3 (*Ctla4* and *Lag3*). This pattern of changes in inhibitory receptor gene expression is a feature of T cell exhaustion, raising the possibility that the homeostatic defect may exhibit some similarities with T cell exhaustion.

5.2.9 Rela$^{-/-}$ *T_{VM} cells exhibit impaired effector cytokine production in response to IL-2/IL-12/IL-18 cytokine-induced activation*

When compared to their WT counterparts, T_{VM} cells lacking RelA exhibit characteristic signs of T cell exhaustion, such as a reduced capacity for responding to memory T cell homeostatic signals (Akbar and Henson, 2001; Wherry and Kurachi, 2015). This is demonstrated by the marked reduction in the cell surface expression levels of the cytokine receptor subunits CD122 and CD127 and a reduced proliferative and/or survival response *in vitro* when cultured in the presence of IL-7 or IL-15. Furthermore, RelA-deficient T_{VM} cells exhibit an up-regulation of multiple genes encoding for inhibitory receptors and a down-regulation of NK cell receptor markers. Whilst it is currently unclear exactly what mechanisms could result in T_{VM} cells lacking RelA acquiring features of an exhausted phenotype, it was of interest to investigate whether RelA-deficient T_{VM} cells exhibited additional characteristic signs of exhaustion, such as impaired effector cell functions. Therefore, a study was conducted to determine the capacity of RelA-deficient T_{VM} cells to produce effector cytokines upon activation. In this study, T_{VM} cells isolated by FACS from 12-week-old *Lck*^{cre}*Rela*$^{wt/wt}$ and *Lck*^{cre}*Rela*$^{fl/fl}$ mice were incubated in a number culture conditions that included in the absence of cytokine, in the presence of recombinant IL-2, in a combination of recombinant IL-2, IL-12 and IL-18, or IL-2 in combination with plate-bound anti-CD3

antibodies. After 24-hours incubation, cells were co-stained with the viability dye, LiveDead Aqua and antibodies against TNF-α and IFN-γ. This analysis showed no significant difference in the viability of *Rela*$^{+/+}$ and *Rela*$^{-/-}$ T_{VM} cells cultured under each condition (Fig.5.12A), with neither the absence of cytokine, nor IL-2 alone able to induce cultured T_{VM} cells to produce IFN-γ or TNF-α. Whilst stimulation with IL-2 in combination with plate-bound anti-CD3 was able to activate cultured T_{VM} cells to produce both IFN-γ and TNF-α, the proportion of cells producing IFN-γ (Fig.5.12B), TNF-α (Fig.5.12C) or a combination of both IFN-γ and TNF-α (Fig.5.12D) was essentially comparable between *Rela*$^{+/+}$ and *Rela*$^{-/-}$ T_{VM} cells. The most striking difference in effector cytokine output was observed for T_{VM} cells co-stimulated with IL-2 and IL-18, where a significantly smaller proportion of cultured *Rela*$^{-/-}$ T_{VM} cells produced IFN-γ (Fig.5.12B), or a combination of IFN-γ and TNF-α (Fig.5.12D). In addition to showing that T_{VM} cells lacking RelA are compromised in their ability to produce effector cytokines following bystander activation by IL-12 and IL-18, this result also shows that these cells exhibit yet another feature of T cell exhaustion.

5.3 Discussion

5.3.1 RelA regulates survival responses of the T_{VM} cell population and influences T_{VM} cell proliferation in response to IL-15

This investigation into the role NF-κB transcription factor family members play in T_{VM} cells has identified a crucial role for RelA in maintaining the steady state size of the T_{VM} cell population. *Rela*$^{-/-}$ HSC chimeras and *Lck*^{cre}*Rela*$^{fl/fl}$ conditional knockout (cKO) mice, two distinct models of *Rela* gene-inactivation, both exhibit a significant reduction in T_{VM} cell numbers. The reduced RelA-deficient T_{VM} cell populations present in these models also display a down-regulation in the surface expression levels of a number of molecules encoding components of the receptors for various common γ-chain cytokines, most notably

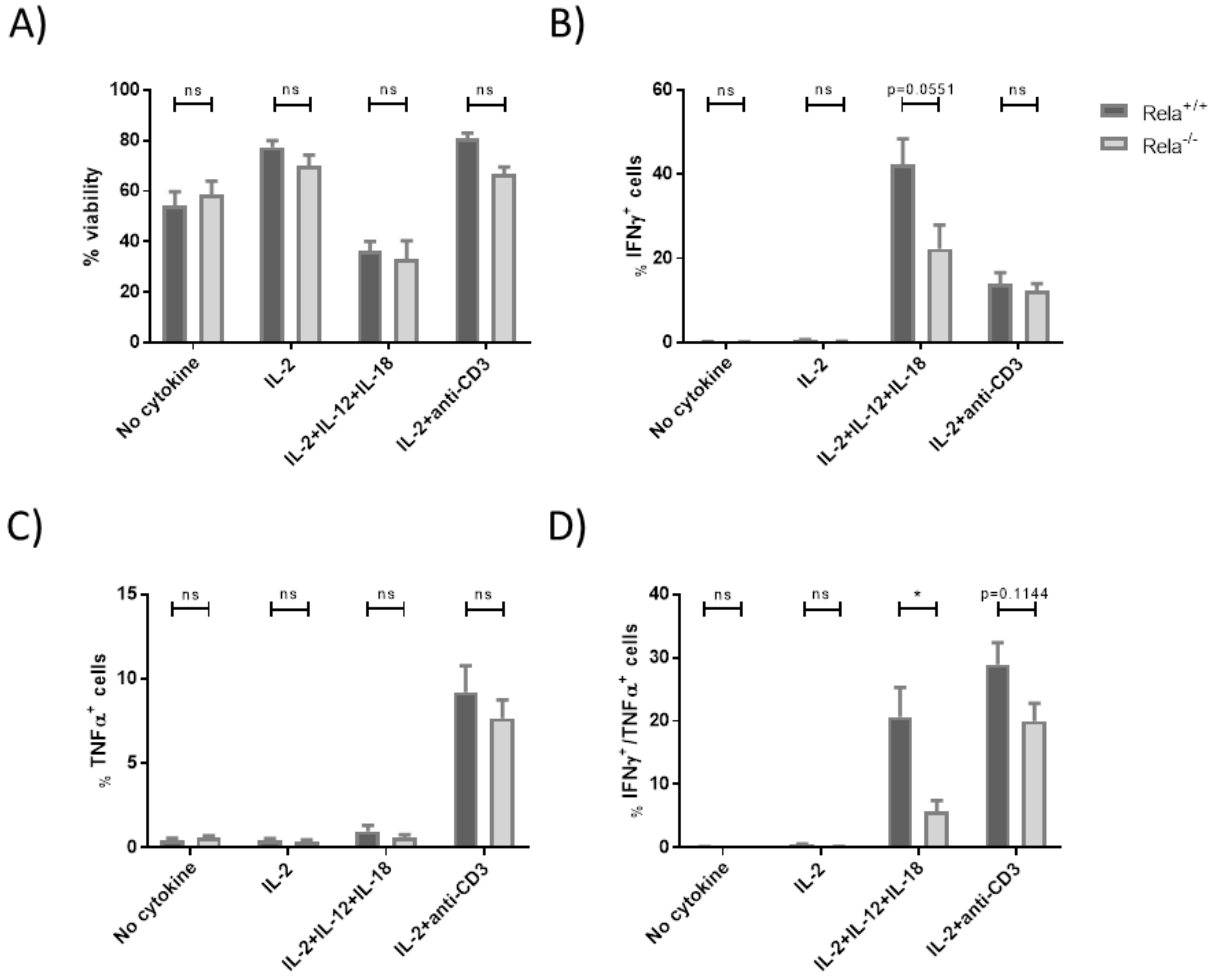


Figure 5.12. *Rela*$^{-/-}$ T_{VM} cells exhibit impaired effector cytokine production in response to IL-2/IL-12/IL-18 cytokine-induced activation. (**A-D**) $CD8^{+}CD44^{hi}CD49d^{lo}$ T_{VM} cells isolated from 12-week-old *Lck*^{cre}*Rela*$^{wt/wt}$ and *Lck*^{cre}*Rela*$^{fl/fl}$ mice were stimulated *in vitro* in the absence of cytokine, in the presence of recombinant IL-2, in a combination of recombinant IL-2, IL-12 and IL-18, or IL-2 in combination with plate-bound anti-CD3 antibodies. After 1-day incubation viability was measured by LiveDead aqua staining and effector cytokine production measured using intracellular antibodies against TNF-α and IFN-γ (n=4) using flow cytometry. (**A**) The frequency of viable *Rela*$^{+/+}$ and *Rela*$^{-/--}$ T_{VM}s under each culture condition. (**B**) The frequency of viable *Rela*$^{+/+}$ and *Rela*$^{-/-}$ T_{VM}s producing IFN-γ. (**C**) The frequency of viable *Rela*$^{+/+}$ and *Rela*$^{-/-}$ T_{VM}s producing TNF-α. **B**) The frequency of viable *Rela*$^{+/+}$ and *Rela*$^{-/-}$ T_{VM}s producing a IFN-γ and TNF-α. All graphs presented as the mean +/- SEM, statistical significance was determined using Student's T tests. ns, not significant; * p≤0.05. T_{VM}=$CD8^{+}CD44^{hi}CD49d^{lo}$ Virtual Memory cells.

CD122, an essential component of the receptors for IL-2 and IL-15. The importance of IL-15 in the homeostatic maintenance of the T_{VM} cell population has been well characterized (White et al, 2016), and data obtained from the investigation of both models indicates that RelA functions in part to maintain T_{VM} cell numbers by enhancing responsiveness to IL-15. An analysis of the T_{VM} cell populations in *Lck*^{cre}*Rela*$^{wt/wt}$ and *Lck*^{cre}*Rela*$^{fl/fl}$ mice of different ages reveals that a strong correlation exists between changes in CD122 surface expression levels on RelA-deficient T_{VM} cells and a failure to maintain T_{VM} cell numbers. Prior to the drop in cell numbers that occurs between 6 and 12-weeks of age, T_{VM} cells taken from 3-week-old *Lck*^{cre}*Rela*$^{wt/wt}$ and *Lck*^{cre}*Rela*$^{fl/fl}$ mice exhibit equivalent levels of CD122 expression (Supplementary Fig.4). A further investigation of RelA-deficient T_{VM} cells confirmed that the reduction in CD122 surface expression levels coincides with a decreased responsiveness to IL-15 in culture (Fig.5.2 & 5.3). T_{VM} cells lacking RelA had reduced cell viability and a diminished proliferative response following stimulation with various concentrations of IL-15, including an increased susceptibility to activation-induced cell death when stimulated with higher concentrations of IL-15. This finding is in contrast to what is known about the effect of IL-15 on WT $CD8^+$ T cells, which has been shown to counteract the activation-induced death of $CD8^+$ T cells stimulated with high levels of IL-2 (Marks-Konczalik et al, 2000).

Whilst a reduction in CD122 expression levels on RelA-deficient T_{VM} cells coincides with reduced survival and proliferative responses to IL-15 *in vitro*, it remains unclear how the loss of RelA in T_{VM} cells brings about a change in CD122 expression levels and to what extent this change accounts for the dysfunction in T_{VM} cell maintenance seen in *Rela*$^{-/-}$ HSC chimeras and *Lck*^{cre}*Rela*$^{fl/fl}$ mice. The regulation of CD122 expression on MP $CD8^+$ T cells is most commonly associated with the T-box transcription factor Eomes. In a perpetual forward feedback loop, IL-15-mediated signalling through CD122 activates Eomes, which promotes the up-regulation of CD122 on the cell surface to further enhance IL-15 responsiveness (Boyman et al, 2007). However, T_{VM} cells taken from *Lck*^{cre}*Rela*$^{fl/fl}$ mice express normal levels of Eomes, suggesting that RelA does not regulate CD122 surface expression levels and the maintenance of T_{VM} cell

numbers by controlling Eomes expression (*Chapter 4.2.5*). Alternatively, the reduction in CD122 cell surface expression and the impaired IL-15 responsiveness of T_{VM} cells lacking RelA might have been attributed to a defect in intracellular signaling downstream of the IL-15R, such as reduced STAT-5 activation. Notably in the context of this study, the gene encoding CD122 is amongst the known transcriptional targets of STAT-5 (Basham et al, 2008), having been shown to increase the cell surface expression of CD122, which in turn leads to enhanced IL-15 responsiveness (Judge et al, 2002; Kennedy et al, 2002). In response to IL-15, the tyrosine kinases JAK1 and JAK3, non-covalently associated with the intracellular region of CD122 and CD132 respectively, phosphorylate and consequently activate one another before subsequently phosphorylating STAT-5. Phosphorylated STAT-5 then forms homodimers or heterodimers with other STAT family members before translocating into the nucleus to promote gene transcription. Accordingly, the level of phosphorylated STAT5 is routinely used to gauge the effectiveness of IL-15 induced intracellular signaling. Despite reduced cell surface expression of both the β-chain (CD122) and γ-chain (CD132) on *Rela*$^{-/-}$ T_{VM} cells, when stimulated with an intermediate dose of IL-15 (50ng/mL), a concentration sufficient to reveal a difference in the rates of survival and proliferation of cultured *Rela*$^{+/+}$ and *Rela*$^{-/-}$ T_{VM} cells, RelA-deficient T_{VM} cells showed no difference in the level of STAT-5 phosphorylation over the course of a 2-hour assay (Fig.5.4). Whilst this study showed that the defects observed in T_{VM} cells lacking RelA could not be attributed to a reduction in the IL-15-induced activation of STAT-5, it remains a possibility that specific STAT-5 target genes also require RelA to be fully expressed and/or maintain normal expression levels. Among the many genes known to be regulated by RelA and/or STAT-5, those impacting cell survival or proliferation emerge as the candidates most likely to be responsible for the impaired homeostatic maintenance of *Rela*$^{-/-}$ T_{VM} cells. Regarding cell survival, both STAT-5 and RelA are known to regulate multiple members of the Bcl-2 family of pro-survival factors, including Mcl-1, Bcl-xL and Bcl-2 (Lee et al, 1999; Braun et al, 2013). Indeed, the impaired maintenance of RelA-deficient T_{VM} cells did appear to involve a defect in cell survival, given that the

over-expression of a transgene encoding the pro-survival factor Bcl-2 was able to rescue the T_{VM} cell deficit in *Lck*^{cre}*Rela*$^{fl/fl}$ mice (Fig.5.5). Moreover, Bcl-2 transgene expression in *Rela*$^{+/+}$ and *Rela*$^{-/-}$ T_{VM} cells also resolved the difference in the rates of survival following stimulation with an intermediate dose of IL-15 (Fig.5.6). Collectively, these finding indicate that RelA plays a role in maintaining T_{VM} cell numbers by assisting in the induction and/or regulated expression of cellular pro-survival pathways. However, none of the known Bcl-2 pro-survival family members were amongst the differentially regulated genes detected in *Rela*$^{-/-}$ T_{VM} cells. In the case of Bcl-2, this conclusion was corroborated by the finding that expression of this pro-survival factor measured by intracellular Bcl-2 stains of immediate 'ex-vivo' T_{VM} cells isolated from 12 wk old *Lck*^{cre}*Rela*$^{wt/wt}$ and *Lck*^{cre}*Rela*$^{fl/fl}$ mice was equivalent (data not shown). Nevertheless, the RelA regulated expression of factors contributing to T_{VM} cell survival that modulate the core pro-survival pathway in ways that are yet to be understood, remains a plausible explanation for these findings. Alternatively, the time points in which pro-survival gene and protein expression were examined may have failed to capture the critical window in which altered expression of these molecules impacted a significant part of the *Rela*$^{-/-}$ T_{VM} cell population. Future studies that look at mice of varying ages will address this issue. Despite *Rela*$^{-/-}$*Bcl2-Tg*$^{+/-}$ T_{VM} cells stimulated with an intermediate dose of IL-15 exhibiting comparable rates of survival, CTV analysis revealed they undergo a slower rate of proliferation when compared to *Rela*$^{+/+}$*Bcl2-Tg*$^{+/-}$ T_{VM} cells (Fig.5.6). This result pointed to the possibility that RelA may also contribute to T_{VM} cell homeostasis by also-driving proliferation in response to IL-15. However, the relative importance that RelA regulated proliferation and survival might play in IL-15 dependent T_{VM} cell homeostasis is likely to be complex given the finding that *Rela*$^{-/-}$*Bcl2-Tg*$^{+/-}$ T_{VM} cells stimulated with a high concentration of IL-15 (150ng/mL) were susceptible to activation-induced cell death that was not overcome by Bcl-2 transgene expression (Fig.5.7). These findings point to RelA serving distinct IL-15 concentration dependent roles in T_{VM} cell survival and division.

To gain a better understanding of RelA's contribution to T_{VM} cell division, the proliferative rates of immediate *ex vivo* T_{VM} cells isolated from 12-week old $Lck^{cre}Rela^{wt/wt}$ and $Lck^{cre}Rela^{fl/fl}$ mice were examined by analyzing the levels of nuclear Ki-67, a protein marker expressed in dividing cells (Scholzen and Gerdes, 2000). This analysis showed statistically comparable Ki-67 expression levels between the two genotypes (Fig.5.8). While this is consistent with RelA regulated cell division not playing a major role in T_{VM} cell homeostasis, like the caveat pertaining to RelA regulated pro-survival gene expression, the critical age associated window during which RelA does have a key role in T_{VM} cell proliferation, may have been missed. On balance, the data as it currently stands indicates that the main role RelA serves in controlling T_{VM} cell homeostasis involves regulating cell survival that is dependent on IL-15 signaling.

5.3.2 RelA-deficient T_{VM} cells are impaired in their ability to expand within a chronic lymphopenic environment

Whilst *in vitro* cytokine stimulation assays confirmed that RelA-deficient T_{VM} cells are less responsive to the homeostatic cytokines IL-7 and IL-15, the discovery that a proportion of RelA-deficient T_{VM} cells were susceptible to cytokine-induced cell death when stimulated with a high concentration of IL-15, suggests a more complex dysfunction in T_{VM} cells lacking RelA. To further investigate the mechanisms underlying these defects, T_{VM} cells isolated from $Lck^{cre}Rela^{wt/wt}$ and $Lck^{cre}Rela^{fl/fl}$ mice were transferred into syngeneic Rag-1-deficient hosts ($Rag1^{-/-}$ mice) to determine the capacity of $Rela^{-/-}$ T_{VM} cells to undergo HP in response to chronic lymphopenia. Due to a lack of endogenous T cells in $Rag1^{-/-}$ mice competing for stimuli such as IL-7 and Il-15 that control T_{VM} cell homeostasis, the lymphopenic environment in these mice provide optimal conditions to drive MP $CD8^+$ T cells such as T_{VM}s to undergo rapid cell division in order to fill lympho-deficient immune compartments (Surh and Sprent, 2008). Notwithstanding the increased availability of IL-7 and IL-15 in $Rag1^{-/-}$ mice, T_{VM} cells deficient for RelA were found to be impaired in their capacity to undergo lymphopenia-induced HP (Fig.5.9). Only very

small numbers of T_{VM} cells were recovered from the spleen of *Rag1*$^{-/-}$ mice receiving *Rela*$^{-/-}$ T_{VM} cells 7-days post-cell transfer. In contrast, *Rela*$^{+/+}$ T_{VM}s transplanted into *Rag1*$^{-/-}$ mice were present in considerable numbers in the spleen after 7-days, and underwent further significant expansion between the day 7 and 30-day post-transplant time points. Whilst *Rela*$^{-/-}$ T_{VM} cell population also underwent some expansion between 7 and 30-days, at the end of the study, significantly fewer cells were recovered from the spleens of *Rag1*$^{-/-}$ mice receiving *Rela*$^{-/-}$ T_{VM} cells when compared to the *Rela*$^{+/+}$ T_{VM} cell recipients.

Given the competitive disadvantage of RelA-deficient T_{VM} cells expressing reduced cell surface levels of CD122 is eliminated in *Rag1*$^{-/-}$ mice, when combined with the ready availability of IL-7 and IL-15, these findings confirm that *Rela*$^{-/-}$ T_{VM} cells have a reduced intrinsic capacity to undergo cytokine driven lymphopenia-induced HP. While the sparse numbers of *Rela*$^{-/-}$ T_{VM} cells recovered from the spleens of *Rag1*$^{-/-}$ mice at day 7 in particular raises the possibility that these cells could have a trafficking defect to the spleen, the normal cell surface expression of L-selectin (CD62L), the lymphocyte homing receptor for secondary lymphoid organs (data not shown), suggests that this is unlikely. Notwithstanding some role for impaired IL-15 driven cell division during the rapid cell expansion that characterizes lymphopenia-induced T cell HP, another possibility is that RelA serves an important survival role in T_{VM} cells undergoing lymphopenia-induced proliferation. This would be consistent with the findings showing that RelA-deficient T_{VM} cells exhibit impaired IL-7 and IL-15-dependent survival responses. Moreover, the results obtained from the *in vitro* IL-15 stimulation assays using high concentrations of IL-15 raise the intriguing possibility that the increased availability of IL-15 in *Rag1*$^{-/-}$ mice may have detrimental effects on RelA-deficient T_{VM} cells undergoing HP. T_{VM} cells lacking RelA were found to be susceptible to activation-induced cell death in culture following stimulation with high concentrations of IL-15 (Fig.5.7). Consequently, a similar mechanism might account for the loss of RelA-deficient T_{VM} cells transferred into *Rag1*$^{-/-}$ mice, where increased levels of IL-15 induce *Rela*$^{-/-}$ T_{VM}s to undergo cytokine-induced cell death. Similarly, the greater availability of IL-7 in *Rag1*$^{-/-}$ mice might also contribute to the reduced capacity of

Rela$^{-/-}$ T_{VM}s to undergo lymphopenia-induced expansion. High levels of IL-7 induce naïve CD8$^+$ T cells to proliferate, differentiate into MP cells and produce IFN-γ (Kimura et al, 2013). While this production of IFN-γ can trigger cytokine-induced cell death of proliferating naïve CD8$^+$ T cells, it is counteracted by tonic TCR signals that intermittently interrupts IL-7 signaling in a process known as co-receptor tuning (Park et al, 2007; Kimura et al, 2013). Although the HP dependency of MP CD8 T cells shifts from TCR to IL-15 signaling, the reduced IL-15-dependent survival of RelA-deficient T_{VM} cells may indirectly make these cells more susceptible to IFN-γ-triggered cell death in response to high levels of IL-7. Whilst T_{VM} cells lacking RelA were found to be susceptible to cytokine-induced cell death at high concentrations of IL-15, how they respond to high concentrations of IL-7 will need to be investigated to determine the validity of this hypothesis. Indeed, the reduced survival of RelA-deficient T_{VM} cells cultured in the presence of IL-7 provides support for such a model. Nevertheless, the adoptive transfer experiments do not rule out that the loss of T_{VM} cell numbers in *RelA*$^{-/-}$ HSC chimeras and *Lck*^{cre}*Rela*$^{fl/fl}$ mice is due to a combination of cytokine-induced cell death and an impaired capacity to undergo cell division, two potentially independent defects.

5.3.3 T_{VM} cells lacking RelA exhibit some features reminiscent of an exhausted phenotype

The discovery that RelA-deficient T_{VM} cells are impaired in their capacity to undergo HP in a chronic lymphopenic environment and are susceptible to cytokine-induced cell death when cultured in high concentrations of IL-15, indicates that the impaired homeostatic maintenance of T_{VM} cells in *RelA*$^{-/-}$ HSC chimeras and *Lck*^{cre}*Rela*$^{fl/fl}$ cKO mice is a complex phenomenon. Despite a significant reduction in CD122 cell surface expression levels (*Chapter 4.2.3*), this investigation has shown that the extent of phosphorylation-induced activation of STAT-5 in response to intermediate concentrations of IL-15 appears to be normal in *Rela*$^{-/-}$ T_{VM} cells (Fig.5.4). Moreover, the expression of the key MP CD8$^+$ T cell transcription factor Eomes, like STAT-5, was unchanged in RelA-deficient T_{VM} cells (*Chapter 4.2.5*).

Nevertheless, RelA may well promote T_{VM} cell homeostasis either by assisting in controlling the expression of Eomes and STAT-5 target genes, or regulating the expression of other molecules independently of these key $CD8^+$ MP associated transcription factors. To gain a better understand of the changes in gene transcription that arise from the T cell-conditional inactivation of RelA, RNA sequencing analysis (RNAseq) was performed to compare the gene expression profiles of *Rela*$^{+/+}$ and *Rela*$^{-/-}$ T_{VM} cells. This analysis revealed a relatively small number of genes (~80 genes) that were differentially expressed (DE) between these two populations. Given the small number of DE genes, programs designed to detect changes that alter specific physiological pathways proved to be of little value. In addition to a number of known NF-κB target genes, including those encoding canonical and non-canonical NF-κB pathway factors such as RelB, NF-κB2 and IκB-α, RelA-deficient T_{VM} cells also differentially expressed genes relevant to T_{VM} cell homeostasis. Reduced transcription of *Il7r* in *Rela*$^{-/-}$ T_{VM} cells is indicative of a decreased responsiveness to homeostatic signals provided by IL-7 and is consistent with flow cytometric and *in vitro* IL-7 stimulation data (Fig.5.1). Of note was the finding that in spite of the significant reduction in the surface expression levels of CD122 on T_{VM} cells in *Rela*$^{-/-}$ HSC chimeras and *Lck*^{cre}*Rela*$^{fl/fl}$ mice, RNAseq analysis revealed no significant difference in *Il2rb* transcript levels in *Rela*$^{-/-}$ T_{VM} cells. This could be explained by the reduction of CD122 on a proportion of T_{VM} cells in 12-week-old *Lck*^{cre}*Rela*$^{fl/fl}$ mice being insufficient to reflect an overall reduction in *Il2rb* transcript levels within the entire T_{VM} cell population that was sampled in this analysis, or that this reduction in CD122 levels was due to post-transcriptional changes in *Rela*$^{-/-}$ T_{VM} cells. It is worth noting that the proportion of T_{VM} cells expressing reduced cell surface levels of CD122 increases in *Lck*^{cre}*Rela*$^{fl/fl}$ mice with advancing age. A reduction in the surface expression levels of CD127 and CD122, combined with the decreased responsiveness of the cultured RelA-deficient T_{VM} cell population to IL-7 and IL-15 are established features linked with an exhausted T cell phenotype. T cell exhaustion is also characterized by a reduction in the expression of memory cell surface markers, increased expression of multiple inhibitory receptors and a reduction in the expression

of NK cell-specific surface markers (Wherry and Kurachi, 2015). As discussed at length, the RelA-deficient T_{VM} cell population in *Lck*^{cre}*Rela*$^{fl/fl}$ mice expressed significantly lower levels of both CD127 and CD122 and exhibited a drop in the number of T_{VM} cells expressing the NK cell-specific marker NKG2D (*Chapter 4.2.3 & 4.2.2*, respectively). Moreover, RNAseq analysis revealed that RelA-deficient T_{VM} cells possess significantly higher levels of transcripts for the genes encoding the inhibitory receptors CTLA-4 and LAG-3 (*Ctla4* and *Lag,* respectively). These inhibitory receptors are normally expressed following T cell activation and function to curtail effector T cell responses by inhibiting further stimulation of T cells via the TCR or other co-stimulatory molecules (Sharma et al, 2015; He et al, 2016). These data are consistent with features of an exhausted-like phenotype in the RelA-deficient T_{VM} cell population of *Lck*^{cre}*Rela*$^{fl/fl}$ mice. T cell exhaustion is also characterized by a decreased proliferative capacity, reduced responsiveness to cytokines that maintain MP $CD8^+$ T cell homeostasis, including IL-7 and IL-15, and the loss of various effector functions (Akbar et al, 2011). T_{VM} cells lacking RelA possess a reduced capacity for responding to memory T cell homeostatic signals, as well as impaired proliferative responses when stimulated with either IL-15, or the TCR agonist, plate-bound anti-CD3 (Fig.5.3 & Fig.5.10/11, respectively). Whilst a reduction in the TCR-driven proliferation of T_{VM} cells lacking RelA could potentially be accommodated by exhaustion, this defect may well be independent of an exhaustion phenotype given the well characterized roles served by RelA in regulating survival and proliferative responses downstream of TCR signaling (Kingeter et al, 2010; Gerondakis et al, 2014). Also consistent with an exhausted phenotype, *Rela*$^{-/-}$ T_{VM} cells exhibit a loss of certain effector functions. The functional capacity of T_{VM} cells to mediate bystander protective immunity in response to inflammation seemed to be impaired in the absence of RelA. T_{VM} cells respond to inflammatory signals by producing multiple inflammatory cytokines (Lee et al, 2013). When T_{VM} cells lacking RelA were stimulated *in vitro* with a combination of IL-2, or IL-12 and IL-18, the proportion of cells found to produce IFN-γ, or a combination of IFN-γ and TNF-α was significantly reduced when compared to WT T_{VM} cells (Fig.5.12). Normally, T_{VM}

cells express high cell surface levels of the IL-18R α-chain (CD218a), thereby allowing a heightened responsiveness to the inflammatory milieu that is believed to contribute to the maintenance of T_{VM} cell homeostasis and mediate bystander immunity (White et al, 2016; White et al, 2017). The reduced transcript levels of the gene encoding the α-chain of the IL-18R (CD218a; *Il18ra*) expressed in *Rela*$^{-/-}$ T_{VM} cells may well contribute to the reduced production of IFN-γ and TNF-α in cultures stimulated with IL-18. Whilst RelA may directly regulate T_{VM} cell effector function by promoting the IL-2/IL-12/IL-18-induced production of IFN-γ and TNF-α, particularly given the genes for both of these cytokines are known NF-κB targets (Gerondakis et al, 2005), it also remains a possibility that this functional defect is an indirect consequence of a state of terminal differentiation associated with T cell exhaustion. However, given T cell exhaustion typically arises from chronic infection following persistent activation through repetitive TCR stimulation and T_{VM} cells in *Lck*^{cre}*Rela*$^{fl/fl}$ mice maintain low expression of CD49d, this suggests if the impaired homeostasis of *Rela*$^{-/-}$ T_{VM} cells is in part due to exhaustion, then it is likely to involve a different mechanism. One other possibility worthy of consideration could involve the signals proposed by Quinn and colleagues (2018), that they suggest drive acquisition of a senescent phenotype in the T_{VM} cell population of aged mice. In this study, it was shown that T_{VM} cells in aged hosts acquired a senescent phenotype through a lifetime of cytokine stimulation. Despite T_{VM} cells not having experienced TCR signaling of sufficient strength to promote the up-regulation of CD49d, these cells nonetheless exist in a semi-activated state as a result of intermittent cytokine stimulation. Excessive cytokine-driven cell proliferation throughout a lifetime induced T_{VM} cells in aged host to acquire a terminal differentiation state of cellular senescence, resulting in a reduced proliferative potential. The reduced IL-15 driven proliferation of RelA-deficient T_{VM} cells might also reflect aspects of a quasi-senescent state. How a cytokine induced state of exhaustion or senescence can be reconciled with the drop in T_{VM} cell numbers in a defined window of ontogeny in *Lck*^{cre}*Rela*$^{fl/fl}$ mice between 6 and 12 weeks of age, is unclear. Certainly the heightened susceptibility to IL-15-induced cell death might contribute to the inability of the

RelA-deficient T_{VM} cell population of *Lck*^{cre}*Rela*$^{fl/fl}$ mice to recover from the drop in cell numbers. A better understanding of how the interplay between cytokine driven T_{VM} cell division and survival that could help explain the phenotype of Rela$^{-/-}$ T_{VM} cells requires a more detailed age-dependent analysis of these parameters. Regardless of the role impaired cell division and cell survival play in the *Rela*$^{-/-}$ T_{VM} cell homeostatic defect, importantly the data presented in this thesis clearly points to a heterogeneity of *Rela* dependence within the T_{VM} cell population. In fact, evidence exists to indicate there are two major subsets within the T_{VM} cell population that arise from distinct progenitor cell populations (Smith et al., 2018). The possibility that these T_{VM} cell subsets have a differential dependency on RelA represents an intriguing alternate explanation for why there is a ~50% drop in the T_{VM} cell numbers of adult *Lck*^{cre}*Rela*$^{fl/fl}$ mice, and subsequent sustained defects in the T_{VM} cell population of post-12-week old *Lck*^{cre}*Rela*$^{fl/fl}$ mice. In a preliminary comparative analysis of our RNAseq data with the RNA signatures of the two distinct T_{VM} cell populations (Smith et al, 2018), provides evidence that there may be a preferential loss of the initial wave of T_{VM} cells that develop from neonatal HSCs arising in the foetal liver. The hypothesis that the first developmental wave of T_{VM} cells has a greater dependency on RelA is currently the subject of further analysis and experimentation.

Chapter 6:

Overarching discussion and summary

This project set out to investigate the roles of the NF-κB family of transcription factors in $CD8^+$ Virtual Memory (T_{VM}) cells, a population of memory phenotype (MP) $CD8^+$ T cells that develop independently of antigen-induced stimulation and instead arise through a process of cytokine-driven homeostatic proliferation (HP) (Kieper et al, 1999; Goldrath et al, 2002; Boyman et al, 2009; Haluszczak et al, 2009). Originating from a pool of naïve $CD8^+$ T cell precursors during neonatal development, T_{VM} cells comprise ~10-20% of the total $CD8^+$ T cell compartment of adult mice and continue to accumulate with advancing age to eventually dominate the MP $CD8^+$ T cell compartment of older mice (Akue et al, 2012; Chiu et al, 2013). T_{VM} cells are capable of mediating both antigen-specific and bystander immune responses and likely play an important role in immunity at all ages (Lee et al, 2013; White et al, 2016). Despite the undoubted importance of these $CD8^+$ T cells, the genetic programs and by inference the transcription factors that influence T_{VM} cell development and function in the main remain unknown. The evidence that implicated the NF-κB family of transcription factors in T_{VM} cell biology was founded in studies showing that the NF-κB pathway influences the size of the MP $CD8^+$ T cell population (Hettmann et al, 2003; Schmidt-Supprian et al 2004; Gugasyan et al, 2012). Specifically, mice lacking the canonical NF-κB family member NF-κB1 (*Nfkb1*$^{-/-}$ mice), develop increased numbers of MP $CD8^+$ T cells in the thymus and peripheral immune organs, characterized by high cell surface expression of the IL-15R β-chain (CD122) and an up-regulation of the memory T cell-associated transcription factor, Eomes (Gugasyan et al, 2012). Further investigation of *Nfkb1*$^{-/-}$ mice undertaken in my research project revealed that the majority of the expanded MP $CD8^+$ T cell population expressed low levels of CD49d, a phenotype indicative of these cells having developed independently of antigen-induced activation (*Chapter 3.2.3*). These properties are consistent with this expanded MP population being T_{VM} cells. *Nfkb1*$^{-/-}$ mice also have significantly increased numbers of $CD8^+CD44^{hi}CD49d^{lo}$ cells that express the NK cell marker, NKG2D, a cell surface receptor proposed to distinguish T_{VM} cells from another antigen-inexperienced MP $CD8^+$ T cell populations (White et al, 2017). This later finding further reinforces the idea that the MP $CD8^+$

phenotype in *Nfkb1*$^{-/-}$ mice is due in part to an increase in T_{VM} cell numbers. In contrast to the expansion of MP CD8$^+$ T cells in mice deficient for NF-κB1, pan-inhibition of the canonical NF-κB pathway in T cells of mIκB-α transgenic and *Ikkβ*$^{-/-}$ mice resulted in a significant reduction of MP CD8$^+$ T cell numbers in the various lymphoid organs of both murine models (Hettmann et al, 2003; Schmidt-Supprian et al 2004). This difference to the *Nfkb1*$^{-/-}$ mouse phenotype most likely reflects the broader inhibition of the NF-κB pathway in mIκB-α transgenic and *Ikkβ*$^{-/-}$ mice, where the activation of multiple NF-κB family members (NF-κB1, RelA and cRel) is blocked. Given that the MP CD8$^+$ T cell population in adult mice mIκB-α and *Ikkβ*$^{-/-}$ mice consists mostly of cells with a CD49d^{lo} T_{VM} phenotype (Halusczak et al, 2009), the deficiency of MP CD8$^+$ T cells in both mIκB-α and *Ikkβ*$^{-/-}$ mice must at the very least be partly attributed to a reduction in T_{VM} cell numbers. Taken together, these studies provide strong evidence for other canonical NF-κB family members in addition to NF-κB1 controlling T_{VM} cell numbers, thereby instigating my hypothesis that cRel and/or RelA play an opposing regulatory role to NF-κB1 in regulating T_{VM} cell homeostasis. Investigating NF-κB DNA binding activity in CD8$^+$ T cell subsets taken from 8-12-week-old C57BL/6 mice using Electrophoretic Mobility Shift Assays (EMSA) established that NF-κB1 homodimers and NF-κB1/RelA heterodimers were present in the nucleus of immediate *ex vivo* T_{VM} cells (*Chapter 3.2.1*). This finding prompted my investigation to focus on the canonical NF-κB family member, RelA. The initial investigation into the role of RelA in T_{VM} cells, using radiation chimera mice reconstituted with hematopoietic stem cells (HSCs) derived from the foetal livers of E13.5 *Rela*$^{+/+}$ or *Rela*$^{-/-}$ mouse embryos, revealed the presence of a minor population of *Rela*$^{-/-}$ HSC-derived T_{VM} cells in *Rela*$^{-/-}$ HSC chimeras 12-weeks post-reconstitution. While establishing that the differentiation of T_{VM} cells from naïve CD8$^+$ T cell precursors could occur independently of RelA, the significantly reduced number of donor HSC-derived T_{VM} cells in chimeric mice receiving *Rela*$^{-/-}$ HSCs when compared to mice receiving *Rela*$^{+/+}$ HSCs, confirmed that RelA must make a significant contribution to T_{VM} cell biology (*Chapter 3.2.5*). Importantly, significant numbers of donor HSC-derived naïve CD8$^+$ T cells in *Rela*$^{+/+}$ and *Rela*$^{-/-}$ HSC chimeras at this

time point showed that the deficiency of *Rela*$^{-/-}$ T_{VM}s in *Rela*$^{-/-}$ HSC chimeras was unlikely to be caused by a lack of precursor cells. One obvious explanation for this finding is that RelA promotes the generation of T_{VM} cells from naïve CD8^{+} T cell precursors by serving as part of a transcriptional program that drives the efficient differentiation of naïve CD8^{+} T cells into T_{VM} cells. However, the lack of RelA-containing NF-κB dimers in the nucleus of immediate *ex vivo* naïve CD8^{+} T cells and the properties of donor HSC-derived T_{VM} cells in *Rela*$^{-/-}$ HSC chimera mice, specifically reduced expression of CD122 and a failure to maintain RelA-deficient T_{VM} cells in these mice, instead pointed to an alternative model in which RelA plays a post-developmental role in T_{VM} cells. While we are unable to definitively rule out any possible involvement of RelA in driving the generation of T_{VM} cells from naïve CD8^{+} precursors, the collective findings presented in this thesis instead provide compelling evidence that RelA primarily controls lymphopenia-induced expansion under certain circumstances, plus the homeostatic maintenance of the existing T_{VM} cell population by regulating IL-15-induced survival and proliferative responses. Much like conventional memory CD8^{+} T cells, the homeostatic maintenance of the T_{VM} cell population is highly dependent on signals provided by IL-15 (Judge et al, 2002; Rubinstein et al, 2008; Sosinowski et al, 2013; White et al, 2016). Survival and proliferative responses controlled by IL-15 are induced in MP CD8^{+} T cells by two mechanisms. One involves binding of soluble IL-15 to the high-affinity IL-15 receptor (IL-15R), which comprises the IL-15R α-chain (CD215) in complex with CD122 and the common-γ-chain (γc; CD132). Signaling also occurs via the trans-presentation of IL-15 bound to CD215 expressed on the surface of monocytes and DCs to the low-affinity IL-15R consisting of CD122 and CD132 (Surh and Sprent, 2008; Castro et al, 2011; Sosinowski et al, 2013). Of these two modes of IL-15 signaling, studies using CD215-deficient mice show that MP CD8^{+} T cells have a greater dependency on IL-15 *trans*-presented via CD215, than on soluble IL-15 bound by the high-affinity IL-15R (Berkett et al, 2002). Whilst CD215 expression on other hematopoietic cells, most likely monocytes and DCs, is indispensable for maintaining MP CD8^{+} T cell numbers, this particular component of the IL-15R lacks an intracellular

signaling domain and its expression on $CD8^+$ T cells is not essential for MP $CD8^+$ T cell homeostasis (Berkett et al, 2002). By contrast, mice with T cells lacking CD122 are severely deficient in MP $CD8^+$ T cells, a phenotype analogous to that of IL-15-deficient mice, demonstrating the importance of this receptor component in conveying survival and proliferative responses elicited by IL-15 (Judge et al, 2002; Kennedy et al, 2002). Given the dependency of MP $CD8^+$ T cell homeostasis on IL-15 signaling, a plausible explanation for the progressive loss of *Rela*$^{-/-}$ donor HSC-derived T_{VM} cells in *Rela*$^{-/-}$ HSC chimeras is provided by the observation that a significant proportion of the *Rela*$^{-/-}$ T_{VM} cell population in these chimeric mice express significantly reduced levels of CD122 when compared to the endogenous *Rela*$^{+/+}$ T_{VM} cell population that survive radio-ablation and subsequently re-expand in these mice (*Chapter 3.2.6*). MP $CD8^+$ T cells expressing reduced levels of CD122 have previously been shown to respond poorly to IL-15 stimulation both *in vitro* and *in vivo* (Judge et al, 2002), offering further support for the proposal that the failure to maintain *Rela*$^{-/-}$ T_{VM} cells numbers in *Rela*$^{-/-}$ HSC chimeras occurs, in part, due to changes in CD122 surface expression levels on *Rela*$^{-/-}$ T_{VM} cells, which in turn lead to these cells being outcompeted by residual *Rela*$^{+/+}$ T_{VM} cells for IL-15 homeostatic signals. Therefore, this proposition would suggest that RelA contributes to T_{VM} cell homeostasis by either directly or indirectly regulating CD122 expression levels in order to maintain IL-15 responsiveness. In support of this theory, further investigation into the role RelA serves in T_{VM} cells obtained using a different model of *Rela* gene-inactivation produced results similar to those acquired from experiments using *Rela*$^{-/-}$ HSC chimeras. Utilizing Cre-loxP-dependent gene targeting to achieve the T cell-conditional inactivation of RelA, *Lck*^{cre}*Rela*$^{fl/fl}$ conditional knockout (cKO) mice develop a MP $CD8^+$ T cell phenotype comparable to those of adult mIκB-α and *Ikkβ*$^{-/-}$ mice (Hettmann et al, 2003; Schmidt-Supprian et al, 2004). A detailed age dependent analysis of the $CD8^+$ T cell populations in *Lck*^{cre}*Rela*$^{fl/fl}$ mice revealed the unexpected finding that between 6 and 12-weeks of age, there is a select reduction of approximately 50% in the number of antigen-inexperienced ($CD49d^{lo}$) MP $CD8^+$ T cells in these mice when compared to WT littermate controls

(*Lck*^{cre}*Rela*$^{wt/wt}$ mice). Notably, this deficit is sustained thereafter throughout adult life and into old age (*Chapter 4.2.1*). Moreover, this change also encompasses a significant loss of the CD49d^{lo} MP CD8^{+} T cell population expressing the NK cell surface marker, NKG2D, which confirmed that the conditional inactivation of RelA in T-lineage cells did indeed impact the T_{VM} cell population (*Chapter 4.2.2*). The ensuing investigation of the persisting T_{VM} cell population in *Lck*^{cre}*Rela*$^{fl/fl}$ mice revealed that a proportion of these cells express reduced levels of CD122; thereby confirming previous findings obtained from the *Rela*$^{-/-}$ HSC chimera mouse model. *Rela*$^{-/-}$ T_{VM} cells from the conditional T cell knockouts also displayed a reduction in the surface expression levels of CD127, the α-chain of the IL-7R (*Chapter 4.2.3*). CD127 is ubiquitously expressed on all major T cell subsets and plays an indispensable role conveying homeostatic signals elicited by the homeostatic γc cytokine, IL-7 (Schluns et al, 2000; Carrio et al, 2007; Boyman et al, 2009). Further investigation of T_{VM} cells isolated from *Lck*^{cre}*Rela*$^{fl/fl}$ mice confirmed that these changes in CD122 and CD127 cell surface expression levels on RelA-deficient T_{VM} cells coincide with a reduced viability of these cells cultured in the presence of either IL-15 or IL-7 (*Chapter 5.2.1*). Given these cytokines are known to be crucial in influencing the T_{VM} cell population (Schluns et al, 2000; Judge et al, 2002; Boyman et al, 2009; Castro et al, 2011; White et al, 2016), the findings in *Lck*^{cre}*Rela*$^{fl/fl}$ mice strongly suggest RelA serves a key role regulating T_{VM} cell homeostasis by controlling the appropriate expression levels of CD122 and CD127. Whilst these changes in CD122 and CD127 levels were also observed on *Rela*$^{-/-}$ T_{AM} cells, TCR-induced survival responses, or perhaps an increase in the expression of other IL-15R subunits (*Chapter 4.2.3*), compensates for these changes to maintain T_{AM} cell numbers (Dubois et al, 2002; Liu et al, 2002; Sato et al, 2007; *Chapter 4.2.3*). Of these two receptor subunits, the evidence suggests that the change in CD122 levels is the major determinant responsible for the reduction in T_{VM} cell numbers in *Lck*^{cre}*Rela*$^{fl/fl}$ mice. Prior to the drop in T_{VM} cell numbers that occurs in *Lck*^{cre}*Rela*$^{fl/fl}$ mice after 6-weeks of age, the T_{VM} cell populations in 3-week-old *Lck*^{cre}*Rela*$^{wt/wt}$ and *Lck*^{cre}*Rela*$^{fl/fl}$ mice are comparable in size and express equivalent levels of CD122 (*Chapter 4.2.1;*

Supplementary Fig.4). The reduction in CD122 expression levels is then first observed on T_{VM} cells in 6-week old *Lck*^{cre}*Rela*$^{fl/fl}$ mice (*Chapter 4.2.3*), coinciding with the age at which the T_{VM} cell population starts to decline (*Chapter 4.2.1*). In comparison, CD127 expression is altered on all CD8$^+$ T cell subsets examined in *Lck*^{cre}*Rela*$^{fl/fl}$ mice, being consistently reduced on naïve, T_{AM} and T_{VM} CD8$^+$ T cells at all ages, when compared to the equivalent CD8$^+$ T cell population in WT littermate controls (*Lck*^{cre}*Rela*$^{wt/wt}$ mice) (*Chapter 4.2.3*). The impact these changes in CD127 expression levels have on each of the CD8$^+$ subsets is currently unclear, particularly given that the sizes of the naïve and T_{AM} CD8$^+$ T cell populations are largely comparable between *Lck*^{cre}*Rela*$^{wt/wt}$ and *Lck*^{cre}*Rela*$^{fl/fl}$ mice of all ages (*Chapter 4.2.1*). Whilst RelA-deficient T_{VM} cells expressing reduced levels of CD127 exhibit impaired IL-7-induced survival responses *in vitro*, how the reduced expression of CD127 impacts the other CD8$^+$ T cell subsets, certainly requires further investigation to determine what influence, if any, this change has on the overall homeostasis of the CD8$^+$ T cell compartment in *Lck*^{cre}*Rela*$^{fl/fl}$ mice. The consistency across all CD8$^+$ subsets in *Lck*^{cre}*Rela*$^{fl/fl}$ mice regarding changes in CD127 expression is indicative of a defect arising early in T cell development and one which is maintained thereafter. NF-κB activity during the double negative (DN) stage of thymocyte development appears to enhance CD127 surface expression levels as a result of RelA/p50 heterodimers binding to an evolutionarily conserved region of the locus located 3.6 kb upstream of the gene coding for CD127 (*Il7r*). This in turn is thought to result in the co-operative interaction with other transcription factors, namely Foxo1 and Ets1, to enhance *Il7r* transcription (Kerdiles et al, 2009; Ouyang et al, 2009; Miller et al, 2014).

Whilst a role for RelA-containing NF-κB dimers in promoting CD127 expression on T cells has previously been identified (Miller et al, 2014), how the lack of RelA in T_{VM} cells brings about a change in CD122 expression remains unresolved. Possible explanations include establishing an appropriate *il2rb* transcriptional landscape in T_{VM} cell precursors prior to, or during T_{VM} cell development, or alternatively controlling CD122 expression following T_{VM} cell differentiation. Changes in CD122 expression could

potentially occur as a result of a defect arising in naïve $CD8^+$ T cells. T_{VM} cells are generated from a pool of naïve $CD8^+$ T cells that bear TCRs with a higher affinity for self-pMHC-I complexes and express marginally higher levels of CD122 (White et al, 2016). These naïve $CD8^+$ T cells preferentially acquire a T_{VM} cell phenotype in response to IL-15 exposure in the periphery following emigration from the thymus. Given that NF-κB promotes the survival of developing $CD8^+$ thymocytes undergoing positive selection (Voll et al, 2000; Jimi et al, 2008; Gerondakis et al, 2010; 2014), it was possible that the inactivation of RelA in the early T cell precursors of $Lck^{cre}Rela^{fl/fl}$ mice following the induction of *Lck-Cre* might negatively impact selection in the thymus, leading to a loss of those naïve $CD8^+$ T cells bearing TCRs with higher affinity for self-pMHC-I and expressing higher levels of CD122. However, the T_{VM} cell populations in $Lck^{cre}Rela^{wt/wt}$ and $Lck^{cre}Rela^{fl/fl}$ mice express comparable levels of CD5 (*Chapter 4.2.4*), a cell surface marker expressed on T cells, the levels of which directly correlate with the strength of TCR signals received during development (Azzam et al, 1998). This suggests that the loss of RelA is unlikely to compromise the generation of those naïve $CD8^+$ T cells that preferentially acquire a T_{VM} cell phenotype following emigration from the thymus. In fact, naïve $CD8^+$ T cells in $Lck^{cre}Rela^{fl/fl}$ mice, both prior to and following the drop in T_{VM} cells numbers that occurs between 6 and 12-weeks of age, express significantly higher levels of CD122 when compared to naïve $CD8^+$ T cells in WT littermate controls (*Chapter 4.2.3*).

Alternatively, RelA regulates T_{VM} cell homeostasis during, or following the differentiation process. For example, one plausible model that was entertained to account for the reduced CD122 cell surface expression levels on RelA-deficient T_{VM} cells, was that RelA assists in the induction of Eomes, a transcription factor closely aligned with the regulation of CD122 expression on MP $CD8^+$ T cells (Pearce et al, 2003; Intlekofer et al, 2005; Boyman et al, 2007; Martinet et al, 2015). However, T_{VM} cells taken from $Lck^{cre}Rela^{fl/fl}$ mice express normal levels of Eomes, indicating that RelA does not appear to regulate CD122 cell surface expression levels by assisting in the induction of Eomes (*Chapter 4.2.5*). Given that T_{VM} cell development is largely driven by IL-15 (White et al, 2016), the deficiency of T_{VM} cells in

Lck^{cre}*Rela*$^{fl/fl}$ mice, coupled with the reduced expression of CD122 on a significant proportion of the residual T_{VM} cell population in this model, might instead involve impaired signaling downstream of the IL-15R. In response to IL-15, the tyrosine kinases JAK1 and JAK3 that non-covalently associate with the intracellular region of CD122 and CD132 respectively activate one another before subsequently activating STAT-5 (Nosaka et al, 1999; Rawlings et al, 2004). Translocation of activated STAT-5 into the nucleus then results in the induction of numerous target genes, including those encoding factors that regulate cell survival and proliferation (Liu et al, 2002). STAT-5 also promotes the transcription of *Il2rb* to increase CD122 expression levels on the cell surface (Basham et al, 2008). Therefore, a disruption in the regulation of STAT-5 target genes could account for the T_{VM} cell phenotype observed in both models of *Rela* gene-inactivation. STAT-5 transcription factors are not only activated in response to IL-15, but also by other γc cytokines, most notably IL-7 (Lin et al, 1995; Pallard et al, 1999; Mazzucchelli and Durum, 2007). For example, naïve $CD8^+$ T cells responding to high levels of IL-7 activate STAT-5 and acquire a MP through proliferation that involves an up-regulation of numerous memory cell markers, including CD122 (Lin et al, 1995; Cho et al, 2000; Goldrath et al, 2000; Schluns et al, 2000; Kieper et al, 2002). Whilst the contribution of STAT-5 to T_{VM} cell differentiation is unclear, it is probable that IL-7-induced STAT-5 activation contributes to the up-regulation of CD122 on naïve $CD8^+$ T cells undergoing cytokine-induced HP (Basham et al, 2008). Therefore, a disruption in IL-15, or IL-7-induced STAT-5 activation might account for the reduced surface expression levels of CD122 and impaired homeostatic maintenance of RelA-deficient T_{VM} cells in *Lck*^{cre}*Rela*$^{fl/fl}$ mice. Given that CD127 is reduced on naive $CD8^+$ T cells in *Lck*^{cre}*Rela*$^{fl/fl}$ mice, one possibility explored early in my analysis was that newly generated naïve $CD8^+$ T cells responding to elevated levels of IL-7 in the lymphopenic environment of neonatal *Lck*^{cre}*Rela*$^{fl/fl}$ mice fail to induce appropriate levels of STAT-5, in turn resulting in the incomplete up-regulation of CD122 upon differentiating into a MP cell. However, this was deemed unlikely given that the T_{VM} cell populations in 3-week-old *Lck*^{cre}*Rela*$^{wt/wt}$ and *Lck*^{cre}*Rela*$^{fl/fl}$ mice, an age when lymphopenia associated T_{VM}

cell generation was largely complete, express equivalent levels of CD122. This also confirms that naïve $CD8^+$ T cells lacking RelA are capable of up-regulating CD122 to normal levels upon differentiating to a T_{VM} cell. Instead, the reduced expression levels of CD122 on RelA-deficient T_{VM} cells in *Lck*^{cre}*Rela*$^{fl/fl}$ mice that first arise at 6-weeks of age, indicate that T_{VM} cells lacking RelA undergo a post-developmental change in CD122 expression. Whilst the mechanism that brings about this change in CD122 regulation remains to be determined, data obtained from *in vitro* cytokine stimulation assays confirmed that the reduction in CD122 expression on *Rela*$^{-/-}$ T_{VM} cells coincides with a decreased responsiveness to IL-15. When compared with *Rela*$^{+/+}$ T_{VM} cell cultures, *Rela*$^{-/-}$ T_{VM} cells had reduced cell viability and a delayed proliferative response following stimulation with various concentrations of IL-15 (*Chapter 5.2.1*). The IL-15-dependent survival and proliferation of MP $CD8^+$ T cells is largely regulated by STAT-5, which in turn regulates target genes involved in controlling MP $CD8^+$ T cell homeostasis. These genes include *Bcl2* and *Bcl2l1* that code for the pro-survival factors Bcl-2 and Bcl-xL respectively, as well as *cdk4*, *sipa1l3* and *c-myc*; genes encoding proteins that promote cell cycle progression and division (Liu et al, 2002). This raised the possibility that the reduced expression of CD122 on the surface of RelA-deficient T_{VM} cells led to lower levels of activated STAT-5 following IL-15 stimulation, in turn leading to the impaired homeostatic maintenance of T_{VM} cells. However, *Rela*$^{+/+}$ and *Rela*$^{-/-}$ T_{VM} cells cultured in the presence of an intermediate concentration of IL-15 (50ng/mL) showed no difference in the levels of phosphorylated STAT-5 examined over the course of a 2-hour assay. Whilst this concentration of IL-15 was sufficient to incite a difference in the rates of RelA-deficient T_{VM} cell survival and proliferation, the design limitations of the STAT-5 activation assay, which include the use of a single concentration of IL-15 over a short duration, are acknowledged. Given the important role STAT-5 transcription factors play in driving the IL-15-induced survival and proliferation of MP $CD8^+$ T cells, how the reduction in CD122 surface expression levels on *Rela*$^{-/-}$ T_{VM} cells affects the downstream activation of STAT-5 following prolonged exposure to physiological concentrations of IL-15 certainly requires a more thorough investigation to determine

what role, if any RelA serves in IL-15-induced STAT-5 signaling. Alternatively, given that RelA and STAT-5 share certain common target genes (Gerondakis et al, 1999; Kelly et al, 2003; Gerondakis et al, 2006; Basham et al, 2008), it remains a possibility that even if IL-15-induced STAT-5 activation is normal in *Rela*$^{-/-}$ T_{VM} cells, RelA is needed to cooperatively achieve full expression and/or maintain the normal expression levels of some target genes that regulate survival and proliferative responses in T_{VM} cells. While this study was unable to establish a disruption in the IL-15-induced activation of STAT-5 with the survival and proliferative defects observed in RelA-deficient T_{VM} cells taken from *Lck*^{cre}*Rela*$^{fl/fl}$ mice, it was subsequently shown that a survival defect did contribute to the reduced number of T_{VM} cells in *Lck*^{cre}*Rela*$^{fl/fl}$ mice. Introducing a human Bcl-2 transgene under the transcriptional control of the pan-hematopoietic cell restricted *vav2* promoter onto our *Lck*^{cre}*Rela*$^{fl/fl}$ strain was able to rescue the deficit in T_{VM} cell numbers in these mice, confirming that the impaired homeostatic maintenance of RelA-deficient T_{VM} cells can be attributed, at the very least in part, to a defect in cell survival (*Chapter 5.2.3*). Whilst expression of the Bcl-2 transgene was able to resolve the difference in survival rates between *Rela*$^{+/+}$ and *Rela*$^{-/-}$ T_{VM} cells stimulated in culture with an intermediate dose of IL-15, CTV analysis revealed that *Rela*$^{+/+}$*Bcl2-Tg*$^{+/-}$ T_{VM} cells still maintained a slower rate of proliferation compared to *Rela*$^{+/+}$*Bcl2-Tg*$^{+/-}$ T_{VM} cells. This finding indicates that RelA could also potentially contribute to T_{VM} cell homeostasis by helping drive proliferation in response to IL-15 (*Chapter 5.2.4*). Whilst the findings presented in this thesis establish that RelA contributes to IL-15 concentration-dependent T_{VM} cell survival and division, the mechanism(s) by which RelA regulates these functions are likely to be complex, particularly given the finding that T_{VM} cells lacking RelA are susceptible to Bcl-2-independent, activation-induced cell death when stimulated with higher concentrations of IL-15 (*Chapter 5.2.1 & 5.2.4*). Notwithstanding the clear IL-15-mediated proliferative defect in cultured *Rela*$^{-/-}$ T_{VM} cells, no significant difference in Ki-67 levels were detected in immediate *ex vivo* T_{VM} cells taken from *Lck*^{cre}*Rela*$^{wt/wt}$ and *Lck*^{cre}*Rela*$^{fl/fl}$ mice, a finding that indicates an IL-15-mediated proliferative defect does not appear to contribute significantly to the

impaired homeostatic maintenance of T_{VM} cells in *Lck*^{cre}*Rela*$^{fl/fl}$ mice (*Chapter 5.2.5*). This proliferative difference between *Rela*$^{-/-}$ T_{VM} cells *in vivo* with those cultured in the presence of IL-15 can be reconciled if there are additional signals *in vivo* that overcome the IL-15-dependent proliferative defect of cultured cells. The susceptibility of cultured *Rela*$^{-/-}$ T_{VM} cells to undergo activation-induced cell death in response to higher concentrations of IL-15 may help to explain the sparsity of *Rela*$^{-/-}$ T_{VM} cells in *Rela*$^{-/-}$ HSC chimera mice and the failure of the T_{VM} cell population to recover in *Lck*^{cre}*Rela*$^{fl/fl}$ mice. When T_{VM} cells isolated from *Lck*^{cre}*Rela*$^{wt/wt}$ and *Lck*^{cre}*Rela*$^{fl/fl}$ mice were transferred into syngeneic Rag-1-deficient hosts (*Rag1*$^{-/-}$ mice), *Rela*$^{-/-}$ T_{VM} cells were found to be significantly impaired in their capacity to undergo lymphopenia-induced HP when compared to *Rela*$^{+/+}$ T_{VM} cells. In principle, the increased availability of IL-7 and IL-15 in *Rag1*$^{-/-}$ mice should provide optimal conditions that drive T_{VM} cells into rapid cell division as these cells attempt to fill the deficient immune compartment. Instead, a lack of *Rela*$^{-/-}$ T_{VM} cell expansion in *Rag1*$^{-/-}$ mice raises the possibility that higher levels of the homeostatic cytokines IL-7 and IL-15 likely to be encountered in *Rag1*$^{-/-}$ mice might mimic what occurs in *Rela*$^{-/-}$ T_{VM} cells exposed to high concentrations of IL-15 in culture. Notwithstanding the susceptibility of *Rela*$^{-/-}$ T_{VM} cells to death when exposed to high levels of IL-15 *in vitro,* what effect an increased availability of IL-7 has on these cells under these circumstances will need to be investigated. Regardless, the inability of *Rela*$^{-/-}$ T_{VM} cells to undergo HP in a chronic lymphopenic *Rag1*$^{-/-}$ environment with an ample availability of IL-7 and IL-15, when combined with the other data presented in this thesis, appears to point to a complex dysfunction in T_{VM} cells lacking RelA.

To gain a better understanding of this dysfunction, RNA sequencing analysis (RNAseq) was performed on T_{VM} cells taken from 12 wk old *Lck*^{cre}*Rela*$^{wt/wt}$ and *Lck*^{cre}*Rela*$^{fl/fl}$ mice to determine the changes in gene expression associated with the loss of T_{VM} cells that accompany the T cell-conditional inactivation of RelA. This analysis revealed that only a relatively small number of genes (~80) were differentially expressed (DE) between *Rela*$^{+/+}$ and *Rela*$^{-/-}$ T_{VM} cells (*Chapter 5.2.6*). Amongst these DE genes were those

that code for other NF-κB pathway proteins, specifically IκB-α and the non-canonical NF-κB family members, RelB and NF-κB2 (*Nfkbia*, *Relb* and *Nfkb2*, respectively). The reduced transcription of *Nfkbia* is not unexpected, given this gene is a well characterized direct target of RelA (Gilmore, 2006), while the reduced number of *Relb* and *Nfkb2* transcripts in *Rela*$^{-/-}$ T_{VM} cells suggests cross-talk occurs between RelA and the non-canonical NF- κB pathway. A future study needs to determine what contribution, if any, impaired regulation of the non-canonical NF- κB activation pathway makes to the T_{VM} cell phenotype observed in *Lck*^{cre}*Rela*$^{fl/fl}$ mice. While a preliminary investigation of mice lacking NF-κB2 (*Nfkb2*$^{-/-}$) showed this strain has normal numbers of T_{VM} cells (data not shown), implying the impact of reduced NF-κB2 expression is likely to be minimal, the role of reduced RelB expression, either alone or in combination with NF-κB2 remains unclear. RNAseq analysis also revealed *Rela*$^{-/-}$ T_{VM} cells possess fewer *Il7r* transcripts, confirming the flow cytometry data showing these cells express reduced levels of CD127 (*Chapter 4.2.3*). Other notable changes in *Rela*$^{-/-}$ T_{VM} cells include an increase in transcription of the genes encoding the inhibitory receptors CTLA-4 and LAG-3 (*Ctla4* and *Lag,* respectively). An increase in the expression of multiple inhibitory receptors, together with a reduction in the surface expression levels of CD127 and CD122 and decreased responsiveness to IL-7 and IL-15, are all established properties associated with an exhausted T cell phenotype (Rudd et al, 2018). T cell exhaustion is also characterized by a decreased proliferative capacity and the loss of various effector functions (Akbar et al, 2011). *Rela*$^{-/-}$ T_{VM} cells activated *in vitro* definitely exhibit a proliferative lag in response to moderate to high levels of IL-15, or plate-bound anti-CD3 antibodies (*Chapter 5.2.1; 5.2.7*), while the production of IFN-γ and TNF-α by these cells is impaired when stimulated with a combination of IL-2, IL-12 and IL-18 (*Chapter 5.2.9*). While a direct role for NF-κB in driving the expression of the genes encoding IFN-γ and TNF-α (Gerondakis et al, 2005), as well as regulating T cell survival and proliferation following TCR signaling is consistent with previous findings (Kingeter et al, 2010; Gerondakis et al, 2014), an alternative explanation is that these characteristics of RelA-deficient T_{VM} cells may be an indirect consequence of a

state of terminal differentiation associated with T cell exhaustion. How a state of T_{VM} cell exhaustion can be reconciled with the drop in T_{VM} cell numbers that occurs between 6 and 12-weeks of age is unclear. One possibility is that a state of exhaustion arises in the surviving T_{VM} cell population following the decline in T_{VM} cell numbers that begins to occur in *Lck*^{cre}*Rela*$^{fl/fl}$ mice around 6-weeks of age. A new set of findings concerning the development of T_{VM} cells may help to shed light on this issue. It has recently been proposed that the T_{VM} cell population is divided into two subsets that develop from distinct T cell precursor populations (Smith et al, 2018). T_{VM} cells were found to arise from naïve CD8$^+$ T cells that develop in the thymus in two distinct waves; those initially derived from neonatal HSCs derived from the foetal liver, followed by precursors that arise from adult HSCs present in the BM (Wong et al, 2016). These two T cell precursor populations, are metabolically and epigenetically distinct, so by inference the T_{VM} cells that develop from them are therefore likely to differ in their reliance on particular signaling networks for their development, differentiation and maintenance. This raises the intriguing possibility that these two T_{VM} cell populations display a disparate dependency on RelA activity. Using time-stamping methodology involving the inducible expression of either a red or yellow fluorescent protein (RFP and YFP respectively), it was shown that naïve CD8$^+$ T cells that develop from foetal liver-derived HSCs acquire a T_{VM} cell phenotype at a much greater frequency than naïve CD8$^+$ T cells that develop from adult BM HSCs (Smith et al, 2018). Upon comparing the gene expression profiles of T_{VM} cells arising from neonatal or adult HSCs, approximately 300 genes were found to be DE between these two sub-populations. When a preliminary comparison of this RNAseq data with that obtained from our own RNAseq analysis of *Rela*$^{+/+}$ and *Rela*$^{-/-}$ T_{VM} cells was undertaken, a trend in the altered gene expression emerged that points to a preferential loss of the neonatal HSC-derived T_{VM} cell population in *Lck*^{cre}*Rela*$^{fl/fl}$ mice. While this finding is currently the subject of an ongoing investigation that is beyond the scope of the study outlined in this thesis, it is tempting to speculate that a specific, or even preferential loss of the neonatal HSC-derived T_{VM} cell population could explain the persistent reduction in T_{VM} cell numbers

in *Lck*^{cre}*Rela*$^{fl/fl}$ mice (*Chapter 4.2.1*). Indeed, the observation that *Rela*$^{-/-}$ T_{VM} cells taken from *Rela*$^{-/-}$ HSC chimeras, or older (> 6 weeks) *Lck*^{cre}*Rela*$^{fl/fl}$ mice display a biphasic CD122 expression profile is certainly consistent with the *Rela*$^{-/-}$ T_{VM} population comprising two distinct cellular subsets. A model where there is a preferential loss of a RelA-dependent T_{VM} cell subset may also help to explain the acquisition of an exhausted-like phenotype in the residual *Rela*$^{-/-}$ T_{VM} cell population. The reduction in T_{VM} cell numbers resulting from the selective loss of the RelA-dependent T_{VM} cell subset should increase the availability of the key homeostatic cytokines. This increased availability of IL-7 and IL-15 could provide a heightened level of stimulation to the residual T_{VM} cells, in turn inducing a faster rate of cell division to compensate for the drop in T_{VM} cell numbers in an attempt to maintain homeostasis of the overall T cell compartment. Given that the T_{VM} cell population in *Lck*^{cre}*Rela*$^{fl/fl}$ mice fails to recover after the initial decline in numbers that occurs between 6 and 12-weeks of age, in such a model the remaining T_{VM} cells would likely experience persistently higher levels of cytokine stimulation throughout most of adult life. Just like T_{VM} cells in aged WT mice that have been shown to acquire a senescent phenotype over a lifetime of repetitive cytokine stimulation (Quinn et al, 2018) despite having never experienced a level of TCR signaling of sufficient strength to induce cellular activation that would result in the up-regulation of CD49d, T_{VM} cells in *Lck*^{cre}*Rela*$^{fl/fl}$ mice may develop a state of terminal differentiation similar to T cell exhaustion as a result of persistently high levels of cytokine stimulation. Although the Ki-67 stains of T_{VM} cells taken from 12-week-old *Lck*^{cre}*Rela*$^{wt/wt}$ and *Lck*^{cre}*Rela*$^{fl/fl}$ mice were comparable, it may be that the enhanced proliferation of *Rela*$^{-/-}$ T_{VM} cells that promotes T cell exhaustion occurs at an earlier juncture. Accordingly, Ki-67 stains of ex-vivo T_{VM} cells needs to be performed on *Lck*^{cre}*Rela*$^{wt/wt}$ and *Lck*^{cre}*Rela*$^{fl/fl}$ mice of varying ages that span 3 to 12 weeks of age.

Finally, the key question still remains as to what signals are driving the activation and nuclear translocation of RelA-containing NF-κB heterodimers in T_{VM} cells. Given the consistently low expression of CD49d on the overall T_{VM} cell population, it is unlikely these complexes are activated by signals

transduced via the TCR. This conclusion is indirectly supported by our EMSA data showing RelA-containing NF-κB heterodimers are expressed in the nucleus of T_{VM} cells, but not antigen-activated CD49d^{hi} T_{AM} cells (*Chapter 3.2.1*). Low level tonic TCR signaling is also unlikely to activate NF-κB in T_{VM} cells given the lack of RelA-containing NF-κB heterodimers in the nucleus of naïve CD8$^+$ T cells, a population that is subject to constant tonic TCR signals. This conclusion is also supported by our data obtained using Nur77^{GFP} reporter mice that showed the levels of tonic TCR signaling received by T_{VM} cells in the periphery is equivalent to those experienced by naïve CD8$^+$ T cells (*Chapter 3.2.2*). Another possibility yet to be considered is the role NKG2D might play in the induction of RelA in T_{VM} cells. NKG2D is an activating receptor that recognizes a number of structurally distinct MHC-I-like ligands expressed by infected and cancerous cells, including members of the RAE-1 and H60 protein families (Raulet, 2003; Whitman and Barber, 2015). NKG2D ligands are also expressed on DCs, a finding suggestive of a potential role in naïve CD8$^+$ T cell priming (Ebihara et al, 2007). Of relevance to my study is the observation that NKG2D assists in the induction of NF-κB in CD8$^+$ T cells activated by antigen-induced stimulation (Whitman and Barber, 2015) and that NKG2D expression is selectively induced on certain CD8$^+$ T cell subsets by specific activation signals (Wensveen et al, 2018; Perez et al, 2019), including IL-15 (Roberts et al, 2001). Specifically, NKG2D acts as a co-stimulatory receptor on activated CD8$^+$ T cells by regulating signaling through other receptors, specifically the TCR and IL-15R, to enhance CD8$^+$ T cell effector function and promote memory cell formation (Wensveen et al, 2013; Whitman and Barber, 2015; Perez et al, 2019). In the case of TCR signaling, NKG2D ligands that bind to effector CD8$^+$ T cells potentiate TCR signaling by driving the activation and nuclear translocation of RelA-NF-κB1 heterodimers downstream of the PI3K/AKT signaling pathway (Lanier et al, 2009; Whitman and Barber, 2015). NF-κB induction from the simultaneous stimulation of the TCR and NKG2D receptors also enhances effector functions of antigen-primed CD8$^+$ T cells by increasing expression of the genes encoding IFN-γ, TNF-α and Fas ligand (FasL), while simultaneously decreasing expression of the genes

encoding the anti-inflammatory cytokines IL-10 and CCL2 (Whitman and Barber, 2015). Of potential relevance to this study, NKG2D also potentiates IL-15R dependent PI3K/AKT signaling in effector $CD8^+$ T cells to promote the survival of memory precursor cells via the increased expression of the pro-survival factor Mcl-1 (Wensveen et al, 2013; Perez et al, 2019). Whilst the gene encoding Mcl-1 (*Mcl1)* is not a known target of NF-κB, a similar mechanism may account for the impaired homeostatic maintenance of the MP $CD8^+$ T cell population in $Lck^{cre}Rela^{fl/fl}$ mice, in which synergistic NKG2D/IL-15R-induced activation of RelA-containing NF-κB complexes downstream of PI3K and AKT signaling intermediates promotes the expression of other pro-survival genes. A key link in this model is the need to formally establish whether or not NKG2D/IL-15R co-stimulation of $CD8^+$ T cells is capable of inducing the activation of NF-κB and if this occurs via the PI3K/AKT pathway.

Whilst it has been proposed that NKG2D can distinguish T_{VM} cells from other $CD49d^{lo}$ MP $CD8^+$ T cell populations, particularly thymic-derived innate memory (T_{IM}) $CD8^+$ T cells (White et al, 2017), my investigation of NKG2D expression in C57BL/6 mice between 6 and 24-weeks of age showed that the majority of $CD49d^{lo}$ MP $CD8^+$ T cells were $NKG2D^-$ (*Chapter 4.2.2*). Coupled with previous reports that $CD49d^{lo}$ MP $CD8^+$ T_{IM} cells only make up a minor percentage of the MP $CD8^+$ T cell population of C57BL/6 mice (Weinrich et al, 2010), these data provide evidence for an alternative hypothesis, in which the T_{VM} cell population in mice comprises both $NKG2D^+$ and $NKG2D^-$ subsets. Given the role NKG2D plays in enhancing antigen non-specific cytotoxicity immunity, I propose that NKG2D defines a more mature, or activated subset of T_{VM} cells with enhanced effector capacity. Furthermore, given the additional role of NKG2D in promoting the survival of memory precursors from a pool of effector $CD8^+$ T cells, it is conceivable that the $NKG2D^+$ T_{VM} cell subset possess a greater dependency on RelA activation for survival following synergistic NKG2D/IL-15R stimulation. Herein I propose a model, in which T_{VM} cells encountering high levels of IL-15 induce expression of NKG2D on the cell surface. $NKG2D^+$ T_{VM} cells coming in contact with activated DCs receive two signals; a primary IL-15R signal generated by the *trans*-

presentation of IL-15 bound to CD215, and an NKG2D co-stimulatory signal produced by ligands also expressed on DCs. These signals converge, enhancing PI3K and AKT activation that in turn promotes the nuclear translocation of RelA-containing dimers that participate in controlling genes that regulate T_{VM} cell homeostasis. In the absence of RelA, signals transduced through NKG2D and the IL-15R fail to induce expression of target genes that control survival, thereby resulting in the death of the NKG2D$^+$ T_{VM} cell population. This however, is not a unifying model that can explain all of the defects observed for *Rela*$^{-/-}$ T_{VM} cells, particularly given that the reduction in NKG2D$^+$ T_{VM} cell numbers only accounts for a minor fraction of the overall loss of T_{VM} cells in *Lck*^{cre}*Rela*$^{fl/fl}$ mice, with the majority of cells that are lost coming from NKG2D$^-$ T_{VM} cell population. This indicates that multiple signals almost certainly contribute to RelA activation in the T_{VM} cell population, with specific signals potentially activating RelA in the different T_{VM} cell subsets. Given the complex dysfunction of T_{VM} cells lacking RelA, it is likely that multiple intracellular signaling pathways that impinge on NF-κB are affected. Whilst we can exclude impaired TCR and CD28-induced activation of RelA contributing to the dysfunction of *Rela*$^{-/-}$ T_{VM} cells, a multitude of other surface receptors known to induce the activation of RelA-containing NF-κB complexes will need to be investigated. In particular, numerous members of the TNFR family members that are expressed on T_{VM} cells (K. Quinn, personal communication), including CD27, OX-40 and 4-1BB, the later having also been shown to be important in promoting CD8$^+$ T cell survival (Lee et al, 2002). The data obtained from this research project also indicates that the T_{VM} cell population comprises distinct subsets that have a differing dependency on RelA. This question is currently being addressed using RelA-GFP reporter mice to determine the frequency of immediate *ex vivo* T_{VM} cells that express activated RelA.

In summary, the findings presented in this thesis show that the canonical NF-κB transcription factors NF-κB1 and RelA serve distinct roles regulating T_{VM} cell homeostasis. Further examination of the expanded MP $CD8^+$ T cell population in *Nfkb1*$^{-/-}$ mice performed by us and our collaborators identify a T_{VM} cell-extrinsic role for NF- κB1 as a suppressor of inflammation. In the absence of NF-κB1, increased levels of numerous inflammatory cytokines drive the expansion of various MP $CD8^+$ T cell subsets, including T_{VM} cells. In contrast, findings presented here show that RelA serves a number of cell-intrinsic roles in T_{VM} cells at multiple stages of development. In addition to most likely assisting in the up-regulation of CD127 at the DN stage of thymocyte development and promoting the survival of $CD8^+$ T cell undergoing selection (Voll et al, 2000; Miller et al, 2014), I propose a novel role for RelA in regulating the homeostatic maintenance of the T_{VM} cell population by controlling expression of CD122, a crucial component of the receptor complex that dictates $CD8^+$ MP T cell responsiveness to IL-15, a cytokine crucial in controlling the development, homeostatic maintenance and function of T_{VM} cells (White et al, 2016; 2017). Whilst the precise mechanisms by which RelA controls CD122 expression and regulates IL-15-induced survival and proliferative responses are yet to be determined, my data outlined in this thesis establishes that RelA serves multiple roles in T_{VM} cells. It remains for the signaling pathways that potentiate RelA-activation in T_{VM} cells to be identified and how this controls T_{VM} cell homeostasis and what distinct roles RelA-dependent and independent T_{VM} cell subsets serve in the biology of this poorly understood MP $CD8^+$ T cell population. Given the burgeoning interest in the key roles these cells almost certainly play in the immunity of neonates and the elderly, I am confident that that the findings presented in this thesis will open up new and important directions of investigation of the role RelA plays into how T_{VM} cell development, function and homeostasis is regulated.

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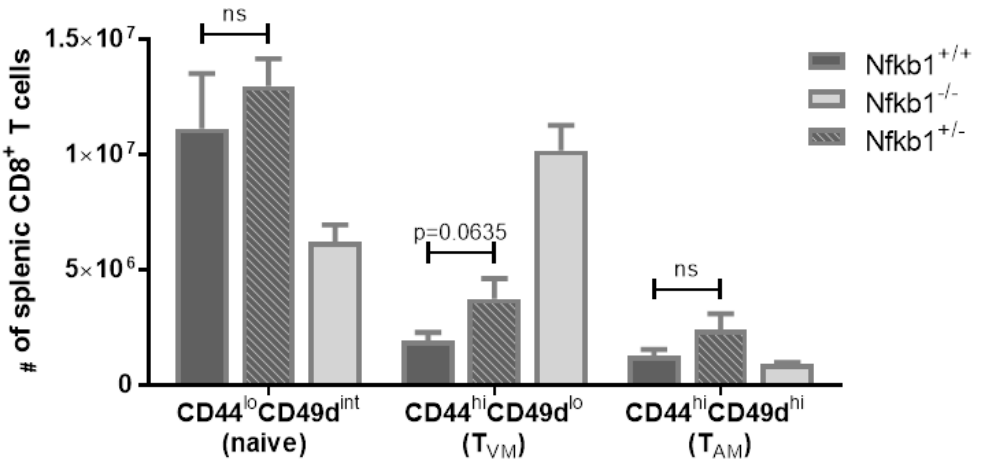
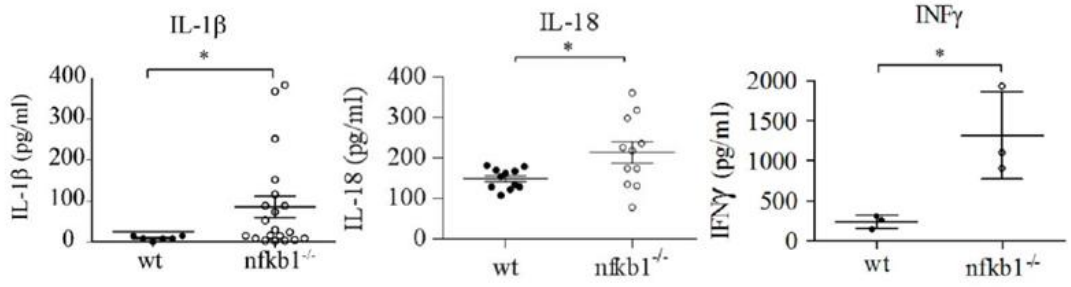
Supplementary Figures

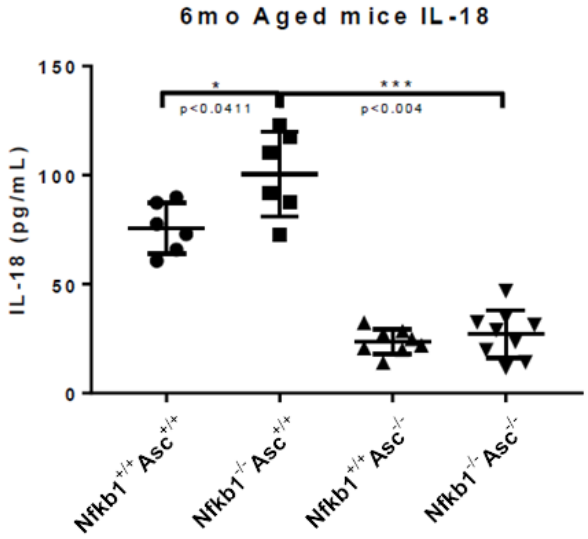


Supplementary Fig. 1. *Nfkb1*$^{+/-}$ mice have statistically comparable CD8^{+} T cell populations to age-matched WT control mice. Flow cytometric analysis of naïve (CD44^{lo}CD49d^{int}), T_{VM} (CD44^{hi}CD49d^{lo}) and T_{AM} (CD44^{hi}CD49d^{hi}) CD8^{+} T cell subsets from spleen of 6-mo-old *Nfkb1*$^{+/-}$, *Nfkb1*$^{-/-}$ and *Nfkb1*$^{+/+}$ (WT) control mice (n=4-5). Total number of CD8^{+} T cell subsets. Graphs presented as the mean +/- SEM, statistical significance was determined using Student's t tests. *, p≤0.05; **, p≤0.01. pLN=peripheral lymph nodes; BM=bone marrow; naïve=CD8^{+}CD44^{lo}CD49d^{int} T_{VM}=CD8^{+}CD44^{hi}CD49d^{lo} Virtual Memory cells; T_{AM}=CD8^{+}CD44^{hi}CD49d^{hi} conventional antigen-primed memory cells; WT=wild type.

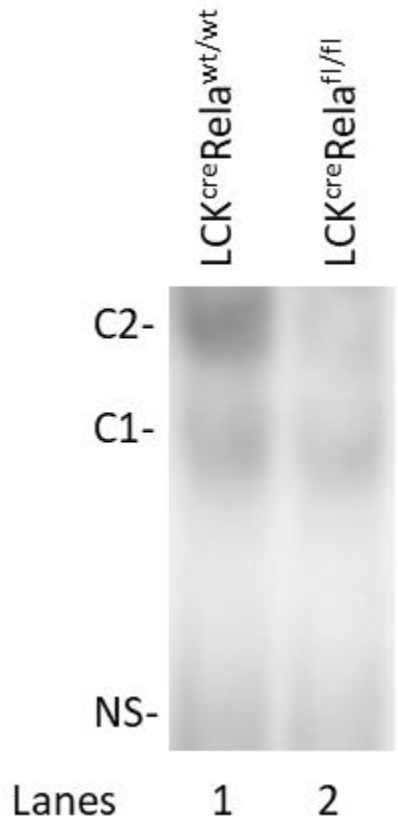
A)



B)

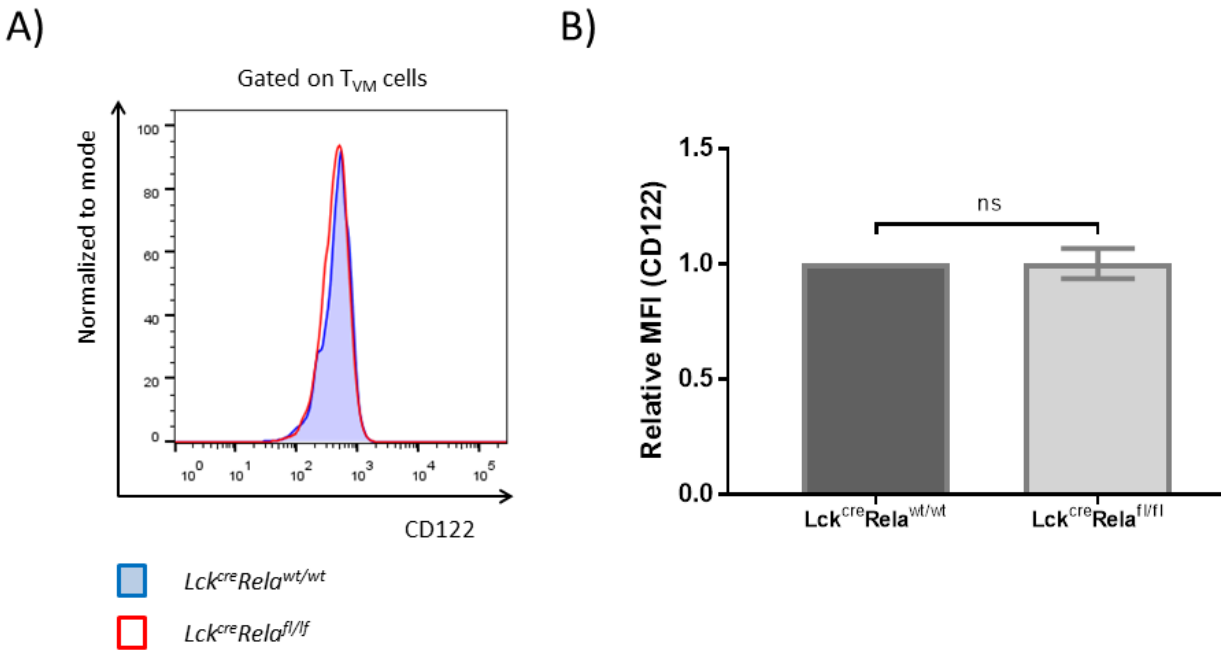


Supplementary Figure 2. (**A-B**) IL-1β, IL-18 and IFN-γ cytokine levels were quantified in serum taken from *Nfkb1*$^{-/-}$ *Asc*$^{+/+}$, *Nfkb1*$^{+/+}$*Asc*$^{-/-}$, *Nfkb1*$^{-/-}$*Asc*$^{-/-}$ and *Nfkb1*$^{+/+}$*Asc*$^{+/+}$ (WT) control mice via ELISA. (**A**) IL-1β, IL-18 and IFN-γ cytokine levels are elevated in *Nfkb1*$^{-/-}$ mice. (**B**) *Nfkb1*$^{+/+}$*Asc*$^{-/-}$ and *Nfkb1*$^{-/-}$*Asc*$^{-/-}$ mice had similar IL18 serum levels that were significantly lower than those of WT controls (*Nfkb1*$^{+/+}$*Asc*$^{+/+}$) and much lower than the IL-18 levels detected in *Nfkb1*$^{-/-}$*Asc*$^{+/+}$ mice. *, p≤0.05 (Ashley Mansel, personal communication).



Supplementary Figure 3. T_{VM} cells in *Lck^{cre}Rela$^{fl/fl}$* mice lack RelA-containing NF-κB complexes. NF-κB activity was analysed in T_{VM} $CD8^+$ T cells of 12-week-old *Lck^{cre}Rela$^{wt/wt}$* (lane 1) and *Lck^{cre}Rela$^{fl/fl}$* (lane 2) mice by EMSA. T_{VM} cells taken from *Lck^{cre}Rela$^{wt/wt}$* and *Lck^{cre}Rela$^{fl/fl}$* mice both express a common nuclear NF-κB complex (C1). Whereas T_{VM} cells taken from *Lck^{cre}Rela$^{wt/wt}$* mice express a unique nuclear NF-κB complex (C2), consisting of a heterodimer of p50-RelA, this C2 complex is absent from the nuclear extract of T_{VM} cells taken from *Lck^{cre}Rela$^{fl/fl}$* mice due to the genetic inactivation of RelA. C1 & C2=NF-κB/DNA complexes; T_{VM}=$CD8^+CD44^{hi}CD49d^{lo}$ Virtual Memory cells; NS=non-specific complex.

During the course of this study, the laboratory had ongoing difficulty getting reliable RelA Western blots to work. As an alternative to verifying the efficiency of RelA deletion in the purified $CD8^{+}CD44^{hi}CD49d^{lo}$ memory T cell population, sorted samples of greater than 95% purity were prepared from 12 week old $Lck^{cre}Rela^{wt/wt}$ and $Lck^{cre}Rela^{fl/fl}$ mice as described in the Methods section and nuclear extracts then subjected to EMSA analysis. As outlined in Fig 3.1 of this thesis, $CD8^{+}CD44^{hi}CD49d^{lo}$ T cells, which we have designated T_{VM} cells, as per the general consensus in the field, express two nuclear NF-κB complexes, C1 and C2. C1 is an NF-κB1 homodimer, whereas C2 is an NF-κB1/RelA heterodimer. As demonstrated in the new additional gel shift figure (Supplementary Fig.3), in $Lck^{cre}Rela^{fl/fl}$ T_{VM}s (lane 2), C1 remains intact, whereas C2 is now absent. This finding is consistent with the selective loss of RelA in these cells. Moreover, the similar behavior of the CD8 T_{VM} cells in both $Lck^{cre}Rela^{fl/fl}$ mice and in the fetal liver hemopoietic stem cells chimeras generated from non-conditional E12 *Rela* KO embryos, this reinforces the conclusion drawn from the new gel shift data showing the inactivation of RelA in in conditionally targeted T_{VM} cells is a highly efficient process. These conclusions are also consistent with a significant body of published data showing that the Lck^{cre} transgene is highly efficient at deleting numerous genes in T cells from the earliest stages of development in all T cell lineages, while the floxed *Rela* mice (Agul et al, 2007) have also been used to successfully delete numerous genes with high efficiency in various tissues, as dictated by a range of specific Cre-deleter mice. These include the efficient deletion of RelA in Tregs (Messina et al, 2016) and total $CD4^{+}$ T cells (Oh et al, 2017) using $Foxp3^{cre}$ and $CD4^{cre}$ transgenes respectively. Collectively, the new gel shift data, the corroborative chimera data and the body of information in the literature on the floxed *Rela* mice and Lck^{cre} transgenic strain makes a convincing case for RelA being efficiently deleted in $CD8^{+}CD44^{hi}CD49d^{lo}$ cells isolated from $Lck^{cre}Rela^{fl/fl}$ mice. Finally, while acknowledging that providing the requested Western blot data would be desirable, with the closure of my supervisor's laboratory at the end of 2018, the $Lck^{cre}Rela^{fl/fl}$ mice are no longer available.



Supplementary Figure 4. CD122 expression levels are equivalent on T_{VM} cells in 3-week-old *Lck^{cre}Rela$^{wt/wt}$* and *Lck^{cre}Rela$^{fl/fl}$* mice. (**A-B**) Flow cytometric analysis of T_{VM} cells from spleens of 3-week-old *Lck^{cre}Rela$^{wt/wt}$* and *Lck^{cre}Rela$^{fl/fl}$* mice (n=6) measuring CD122 surface expression levels. (**A**) Representative histogram of CD122 expression levels on T_{VM} cells. (**B**) MFI of CD122 on splenic T_{VM} cells in *Lck^{cre}Rela$^{fl/fl}$* relative to MFI of CD122 on T_{VM} cells in age-matched WT control (*Lck^{cre}Rela$^{wt/wt}$*) mice. Relative MFI graph presented as the mean +/- SEM. ns, not significant. T_{VM}=CD8^{+}CD44^{hi}CD49d^{lo} Virtual Memory cells; MFI=median fluorescent intensity; WT=wild type.

Gene	*Rela*$^{-/-}$ differential expression ratio (normalized to a 0 score for *Rela*$^{+/+}$ expression levels)	p value	FDR
Il7r (CD127)	-1.38156	8.63E-05	0.033493
Relb (RelB)	-1.4458	5.15E-06	0.00987
Nfkb2 (NF-κB2)	-1.03846	3.14E-05	0.021208
Nfkbia (IκB-α)	-0.64505	0.000106	0.033726
Ctla4 (CTLA-4)	1.951794	0.000133	0.036323
Lag3 (Lag-3)	2.663629	8.96E-05	0.033493
Il18ra (CD218a)	-0.80454	4.75E-05	0.024832

Supplementary Table 1. RNAseq analysis shows differential gene expression between *Rela*$^{+/+}$ and *Rela*$^{-/-}$ T_{VM} cells. RNA sequencing (RNAseq) analysis was undertaken to compare the gene expression profiles of WT and RelA-deficient T_{VM} cells purified from 12-week-old *Lck*^{cre}*Rela*$^{wt/wt}$ and *Lck*^{cre}*Rela*$^{fl/fl}$ mice. Given that only two replicates of each *Rela*$^{+/+}$ and *Rela*$^{-/-}$ T_{VM} cell samples could be obtained, changes in gene expression resulting from the loss of RelA, detailed above, is provided to give indication only of key findings. T_{VM}=CD8^{+}CD44^{hi}CD49d^{lo} Virtual Memory cells; FDR=false discovery rate.