

**ROLE OF T CELL RECEPTOR SIGNALING IN**

**CD8 T CELL MEMORY**

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Doctor of Philosophy

By

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**Role of T Cell Receptor Signaling in CD8 T Cell Memory**

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## **DEDICATION**

This is dedicated to my mother, Wendy Schuett. Without her love, confidence, and support for my explorations of the world and the small things in it, this work would have never been started or completed.

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## LIST OF ABBREVIATIONS

ACT	Adoptive cell transfer
Ag	Antigen
APC	Antigen presenting cell
APL	Altered peptide ligand
Bcl-10	B cell lymphoma protein-10
Bcl-2	B cell lymphoma protein-2
Bcl-6	B cell lymphopma protein-6
CA	Constitutive active
Ca <sup>2+</sup>	Calcium
CARMA1	Caspase-recruitment domain-containing membrane-associated guanylate kinase protein-1
CART	Conserved antigen receptor transmembrane
CBM complex	CARMA1-Bcl10-MALT1 complex
CTL	Cytotoxic T lymphocytes
CTLA-4	Cytotoxic T lymphocyte antigen-4
DAG	Daicylglycerol
DC	Dendritic cell
DUB	Deubiquitinating enzyme
DN	Dominant negative
Eomes	Eomesodermin
ERK	Extracelular signal-regulated kinase
EV	Empty vector
Gads	GRB2-related adaptor protein-2
GLK	Germinal center kinase-like kinase
GRB2	Growth factor receptor-bound protein 2
IFN $\gamma$	Interferon- $\gamma$
IKK complex	I $\kappa$ B kinase complex
IKK $\alpha$	I $\kappa$ B kinase- $\alpha$
IKK $\beta$	I $\kappa$ B kinase- $\beta$
IKK $\gamma$ /NEMO	I $\kappa$ B kinase- $\gamma$
IL-12	Interleukin-12
IL-15	Interleukin-15
IL-2	Interleukin-2
IL-7	Interleukin-7
IL-7R	IL-7 receptor
IP <sub>3</sub>	Inositol 1,4,5-triphosphate
IS	Immunological synapse
ITAM	Immunoreceptor tyrosine-based activation motifs
ITK	Inducible T cell kinase
I $\kappa$ B $\alpha$	Inhibitor of $\kappa$ B- $\alpha$
JNK	c-Jun amino-terminal kinase
kDa	Kilodaltons
LAT	Linker of activated T cells

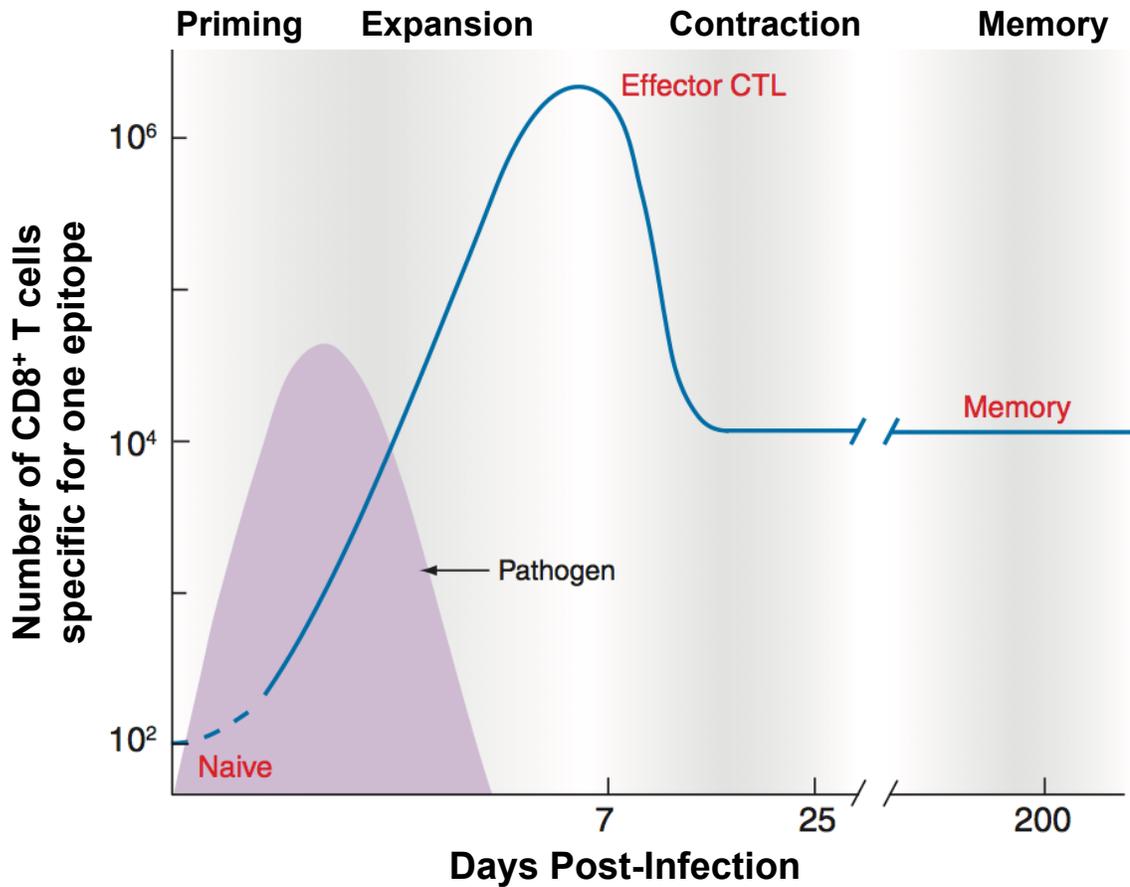
LCK	Lymphocyte-specific protein tyrosine kinase
LCMV	<i>Lymphocytic choriomeningitis virus</i>
LM	<i>Listeria monocytogenes</i>
LM-OVA	Recombinant <i>Listeria monocytogenes</i> expressing OVA peptide
LM-Q4H7	Recombinant <i>Listeria monocytogenes</i> expressing Q4H7 peptide
MALT1	Mucosa-associated-lymphoid-tissue lymphoma-translocation gene-1
MAPK	Mitogen-activated protein kinase
MHC	Major histocompatibility complex
mTOR	Mammalian target of Rapamycin
MUT	TCR $\beta$ transmembrane domain mutant
N4	see OVA
NF- $\kappa$ B	Nuclear factor $\kappa$ B
NFAT	Nuclear factor of activated T cells
Ng	Nanogram
OVA	SIINFEKL peptide of ovalbumin protein
p38	Protein of 38kDa
PDK1	Phosphoinositide dependent kinase-1
PI3K	Phosphoinositide 3-kinase
PIP <sub>2</sub>	Phosphatidylinositol 4,5-biphosphate
PKC $\theta$	Protein kinase C- $\theta$
PLC $\gamma$ 1	Phospholypase C- $\gamma$ 1
pMHC	peptide-bound major histocompatibility complex
Rag	Recombination-activating gene
RIP	Rat insulin promoter
RV	Retrovirus
SLP-76	SH2 domain-containing leukocyte protein of 76kDa
SOS	Son of sevenless
TCF-1	T cell factor-1
TCR	T cell receptor
TCR $\beta$ TMD	T cell receptor $\beta$ chain transmembrane domain
TGF $\beta$	Transforming growth factor beta
TMD	Transmembrane domain
TNFR	TNF receptor
TNF $\alpha$	Tumor necrosis factor alpha
TRAF6	TNFR-associated factor 6
WT	Wildtype
ZAP-70	Zeta chain-associated protein kinase 70
$\beta$ TMD	T cell receptor $\beta$ chain transmembrane domain
$\beta$ TMDmut	TCR $\beta$ transmembrane domain mutant
$\mu$ g	Microgram
$\mu$ M	Micromolar

# **I: Introduction**

Karin M. Knudson

## CD8 T Cells

CD8 T lymphocytes are an essential component of the adaptive immune system. They protect against intracellular pathogens that cause acute and chronic infection and clearance of tumors. During a typical acute infection, the CD8 T cell response can be divided into 4 phases: priming, expansion, contraction, and memory (Figure 1-1) (Williams and Bevan, 2007). During priming, pathogen-specific naïve T cells integrate the following signals to become activated and differentiate: T cell receptor (TCR) interaction with antigenic peptide bound to molecules of major histocompatibility complex class I (pMHC), costimulation, and inflammation. These signals are typically provided by encounter with activated dendritic cells (DCs) or other antigen presenting cells (APCs). Once stimulated, these T cells undergo clonal proliferation and develop into effector T cells, termed the expansion phase. Effector T cells produce cytotoxic molecules such as perforin and Granzyme B and cytokines IFN $\gamma$  and TNF $\alpha$  to mediate infected host cell killing and pathogen clearance. Once the pathogen is eliminated, the majority of effector T cells (short-lived) undergo apoptosis during contraction. However, the expansion phase also yields cells that will eventually become the memory T cell pool (long-lived). While it is not a definitive marker, the expression of IL-7 receptor (IL-7R) has been widely used to differentiate those cells with greater potential to undergo contraction (low IL-7R) or contribute to the memory pool (high IL-7R) (Kaech et al., 2003). Memory T cells are long-lived and characterized by rapid proliferation and effector function upon re-stimulation (Kedl and Mescher, 1998; Le Campion et al., 2002;



**Figure 1-1. A typical CD8 T cell response.** A typical mouse contains between 15-200 naive T cells specific for a given epitope (Blattman et al., 2002; Moon et al., 2007). After infection with a pathogen, such as *L. monocytogenes*, naive CD8 T cells become primed and undergo a massive clonal expansion phase characterized by differentiation to gain effector function and clear the pathogen (generation of effector cytotoxic T lymphocytes or CTL). Once the pathogen is eliminated, the majority of T cells undergo programmed cell death termed contraction. The remaining T cells become the memory CD8 T cell pool. Figure adapted from (Williams and Bevan, 2007).

Min et al., 2003; Rogers et al., 2000; Zimmermann et al., 1999). Furthermore, after infection, the memory population contains an increased number of pathogen-specific T cells as compared to the naïve pool (Bradley et al., 1993). These characteristics make memory T cells able to provide better responses than their naïve counterparts. Memory CD8 T cell generation is essential for optimal, long-term immunity.

### **Models of Memory CD8 T Cell Differentiation**

Activation of naïve CD8 T cells during infection generates a heterogeneous population of T cells with the potential to enter the effector or memory T cell pool. The factors that influence effector versus memory differentiation have been greatly investigated over the years. There are multiple modes that seek to explain the fate decision that determines whether a T cell will live or die during an immune response.

#### *Single Precursor Model*

One of the simplest explanations for the generation of effector and memory T cells during infection is that these two cell types are generated from different precursors. Thus, a naïve T cell's fate is pre-determined, possibly during its development in the thymus. Studies using adoptive transfer of a single transgenic CD8 T cell and cellular barcoding of transgenic T cells, however, demonstrated that a single naïve T cell could generate both effector and memory T cell pools (Gerlach et al., 2010; Stemberger et al., 2007). These results

supported a naïve multipotent or “one cell, multiple fates” model of effector and memory T cell differentiation. Interestingly, recent studies using polyclonal T cell populations demonstrated that the process of T cell development, or thymic selection, does indeed affect peripheral T cell responses (Fulton et al., 2014; Tubo et al., 2013). Specifically, expression of the negative regulator CD5, which correlates with the strength of self-recognition in the thymus and periphery (Azzam et al., 1998; Smith et al., 2001; Tarakhovsky et al., 1995), affects gene expression of naïve T cells and their response during infection (Fulton et al., 2014; Tubo et al., 2013). Thus, it is possible that the phenotypic diversity of T cells during an immune response is dependent on their developmental conditions.

#### *Asymmetric Cell Division Model*

An extension of the “one cell, multiple fates” model is based on recent studies investigating asymmetric cell division in early T cell responses (Chang et al., 2011; Chang et al., 2007). These reports showed upon naïve T cell stimulation, asymmetric cell division led to differential segregation of receptors and signaling molecules between the two resulting daughter cells. These two daughter cells had a differential capacity to develop into effector or memory T cells (Chang et al., 2007). This model provides an explanation for how a single T cell can give rise to both effector and memory lineages during infection. However, it does not take into account that both effector and memory T cells can express effector-associated molecules during the primary immune response

(Bannard et al., 2009; Harrington et al., 2008; Maris et al., 2003). Furthermore, it does not consider other signals received, especially those encountered after priming, by a T cell during its differentiation.

### *Decreasing Potential Model*

The decreasing potential model takes into account that T cells are amenable to receive different amounts of antigenic, costimulatory and inflammatory signals during the immune response. It states that as a T cell accumulates these signals, it becomes more differentiated. This differentiation is characterized by maintenance of effector function but decreased proliferative and survival potential. Thus, T cells that receive sustained stimulatory signals, or a greater duration of signaling, are more likely to undergo contraction while those that receive more transient signals favor memory generation. In support of this, studies have shown that truncating antigenic pMHC-TCR signaling or attenuating inflammation, either by early use of antibiotics or DC vaccination, increases the rate of memory CD8 T cell conversion (Badovinac et al., 2005; Badovinac et al., 2004). It was also described that T cells primed late in the response, and thus had less exposure to pMHC-TCR and inflammatory signals, were skewed toward a memory fate (D'Souza and Hedrick, 2006). Conversely, increased inflammation drives terminal differentiation of CD8 T cells, and chronic antigen exposure promotes T cell exhaustion and death (Angelosanto and Wherry, 2010; Cui et al., 2009; Joshi et al., 2007). To account for the generation of both effector and memory T cells during infection, the decreasing potential model describes a

continuum or linear explanation of T cell differentiation based on the quantity of total signaling a T cell receives.

### *Signal Quality Model*

The signal quality model, like the decreasing potential model, states that the cumulative strength of signals between TCR, costimulation, and inflammation program T cell fate. However, it also takes into account that the quality of different TCR signals, not only the duration or strength, affects effector and memory differentiation. Due to this distinction, the signal quality model specifies that T cell fate is determined during priming. Supporting this, studies demonstrated that signals provided in the first 1-3 days were sufficient to induce full effector and memory differentiation (Kaech and Ahmed, 2001; van Stipdonk et al., 2003). In addition, some transcription factors are required for either effector or memory generation, but not vice versa, indicating that distinct signals may determine their expression (Ichii et al., 2002; Kallies et al., 2009; Rao et al., 2010; Takemoto et al., 2006). It has also been shown that alteration of TCR proximal signaling events differentially regulates CD8 T cell fate (Nayar et al., 2012; Smith-Garvin et al., 2010; Teixeira et al., 2009).

While there is still debate on which model of T cell differentiation best describes effector and memory generation during immune responses, it is important to remember that these models are not mutually exclusive. Most likely, the differentiation of effector and memory T cells is determined by the combined elements of multiple models.

## Memory CD8 T Cell Programming

During activation of T cells, a transcriptional program is induced which promotes effector and memory differentiation (Belz and Kallies, 2010; Kaech and Cui, 2012). The transcription factors involved in these processes have been extensively studied.

T-box transcription factors T-bet and Eomesodermin (Eomes) play important roles in CD8 T cell effector and memory differentiation and function. T-bet and Eomes are expressed in both activated effector and memory CD8 T cells (Paley et al., 2013; Pipkin et al., 2010). Genetic deficiency in T-bet or Eomes alone does not greatly affect CD8 T cell effector differentiation (Pearce et al., 2003; Szabo et al., 2002). However, compound genetic deficiency prevents normal effector function of CD8 T cells, characterized by less Granzyme B, perforin, and IFN $\gamma$  production (Intlekofer et al., 2005). While T-bet and Eomes have reciprocal and cooperative function in effector T cells, they are regulated differently (Rao et al., 2010; Takemoto et al., 2006). This suggests the potential for independent functions. IL-12 inversely regulates T-bet and Eomes levels, with IL-12 promoting T-bet but inhibiting Eomes expression (Takemoto et al., 2006). mTOR signaling also differentially regulates these transcription factors by inducing T-bet and repressing Eomes expression (Rao et al., 2010). Since high levels of inflammation and mTOR signaling have been shown to favor effector versus memory generation (Araki et al., 2009; Cui et al., 2009; Joshi et al., 2007), this suggests that T-bet and Eomes have divergent roles in effector and memory

development. Indeed, T-bet-deficient CD8 T cells are skewed toward a IL-7R<sup>hi</sup> memory precursor phenotype during infection (Joshi et al., 2007). Conversely, increased Eomes expression promotes greater memory differentiation and maintenance (Rao et al., 2010). Due to these studies, T-bet and Eomes have been labeled as master regulators of effector and memory differentiation, respectively.

Blimp-1 and Bcl-6 have also contribute to control the balance between effector and memory generation. While Blimp-1 is expressed in both effector and memory CD8 T cells, it mainly regulates effector differentiation and function (Kallies et al., 2009; Martins et al., 2006; Rutishauser et al., 2009). Furthermore, Blimp-1-deficient CD8 T cells are diverted toward a memory phenotype after infection (Kallies et al., 2009). On the other hand, Bcl-6 expression is uniquely required for differentiation of memory CD8 T cells (Ichii et al., 2002). Bcl-6 over-expression leads to increased memory generation (Ichii et al., 2004). Bcl-6 also negatively regulates effector function by suppressing Granzyme B expression (Yoshida et al., 2006). Interestingly, Blimp-1 and Bcl-6 are known to directly regulate each other as well as some T-box transcription factor expression. Blimp-1-deficient T cells express less T-bet and more Bcl-6 and Eomes (Rutishauser et al., 2009). Importantly, the balance of these transcription factors is important for the effector/memory fate decision.

### **Memory CD8 T Cell Survival**

Memory CD8 T cells, in contrast to effector cells, are characterized by their longevity. Incredibly, T cell responses against smallpox can be observed up to 75 years after vaccination (Hammarlund et al., 2003). This demonstrates that the antigen-specific memory pool can be maintained throughout the life of the host (Hammarlund et al., 2003). Interestingly, the requirements for survival of naïve and memory T cells are different (Sprent, 2008). While interaction with self-pMHC and responsiveness to the pro-survival cytokine IL-7 is necessary for naïve T cell survival (Polic et al., 2001; Schluns et al., 2000; Tanchot et al., 1997), memory CD8 T cells require different signals. In particular, memory CD8 T cells do not need to encounter self-pMHC to survive (Jabbari and Harty, 2005; Leignadier et al., 2008; Murali-Krishna et al., 1999; Polic et al., 2001). Additionally, while IL-7 does support the survival of memory T cells, IL-15 is also important. IL-15 instills a survival signal as well as promotes homeostatic turnover, enabling maintenance of the memory pool (Becker et al., 2002; Berard et al., 2003). While the extrinsic factors required for memory CD8 T cell survival are known, the signaling pathways that support memory maintenance are less defined.

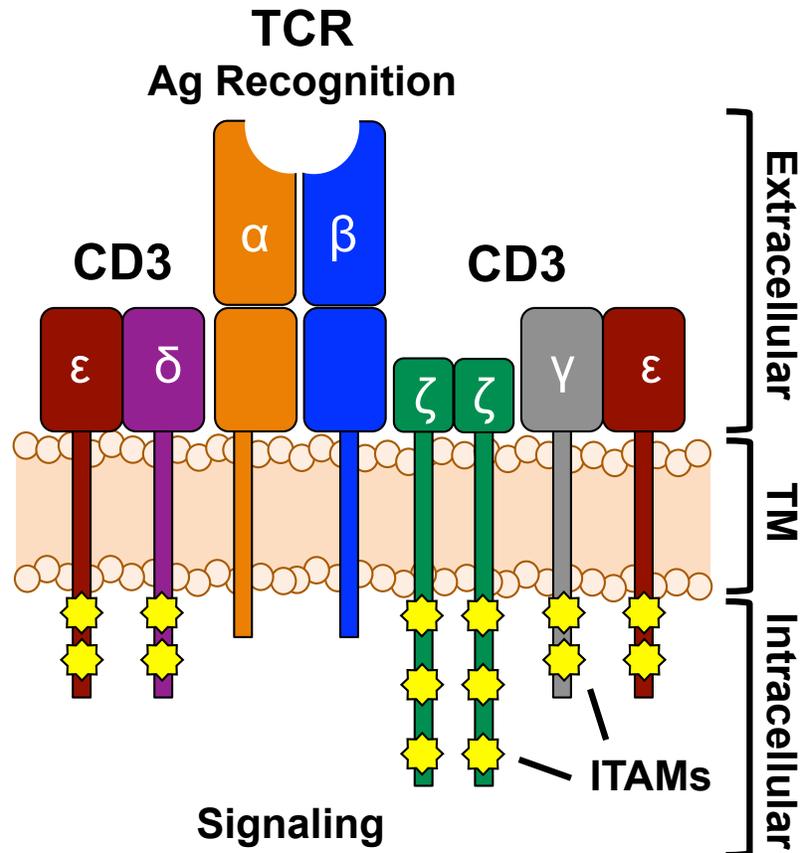
### **T Cell Receptor (TCR)**

Due to the fact that the TCR governs the specificity of the T cell response, and that both naïve and memory T cells require TCR signals to become activated (memory T cells are less dependent on costimulation and inflammation than

naïve T cells to respond (Daniels and Teixeira, 2010)), TCR signaling and its role in programming CD8 T cell responses will be focused on here.

The T cell receptor is unique to T cells and allows for specific recognition of peptides bound in MHC molecules. The TCR/CD3 complex is composed of the TCR $\alpha\beta$  heterodimer which is noncovalently associated to an invariant, multimeric CD3 complex consisting of dimers of CD3 chains  $\gamma\epsilon$ ,  $\delta\epsilon$ , and  $\zeta\zeta$  (Smith-Garvin et al., 2009). Proper assembly and expression of the TCR on the T cell surface is required for T cell activation. The TCR $\alpha\beta$  contains extracellular, transmembrane, and intracellular domains (Figure 1-2).

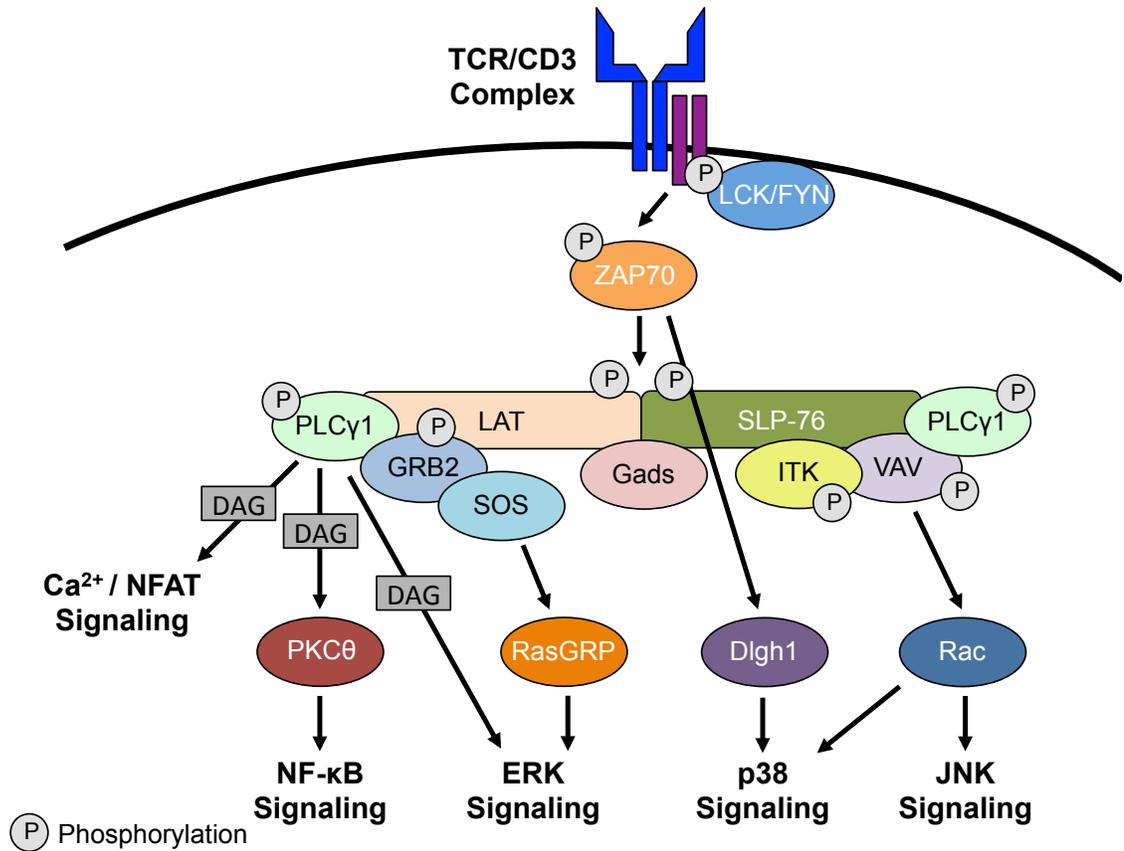
The extracellular domain of the TCR is comprised of the  $\alpha\beta$  heterodimer that contains variable and constant regions. The TCR $\alpha\beta$  variable and constant regions are subjected to recombination and editing of germline-encoded segments during T cell development (Starr et al., 2003). This allows for the generation of a diverse pool of TCRs to recognize a wide array of foreign antigens. It is estimated that the peripheral T cell repertoire of mice and humans contains  $2 \times 10^6$  and  $2 \times 10^8$  unique TCRs, respectively (Casrouge et al., 2000; Robins et al., 2009). The  $\alpha\beta$  chains of the TCR, while they specifically bind antigen, have very short cytoplasmic tails and no intrinsic signaling capabilities (Bäckström et al., 1997). In fact, complete mutation of the TCR  $\alpha$  and  $\beta$  cytoplasmic tails does not affect T cell responses (Teixeiro et al., 2004). The associated CD3 chains are required for signal transduction upon TCR-pMHC interaction (Irving and Weiss, 1991). The CD3 chains have long cytoplasmic tails with multiple immunoreceptor tyrosine-based activation motifs (ITAMs). ITAMs



**Figure 1-2. The TCR/CD3 Complex.** The T cell receptor contains 3 distinct domains. The extracellular domain consists of the TCR $\alpha\beta$  variable and constant regions. This domain is required for antigen (Ag) recognition through interaction with pMHC. The transmembrane domain (TMD) is necessary for appropriate assembly of the TCR/CD3 complex, recruitment to the membrane, and proper TCR signal transduction. The intracellular domains of the TCR $\alpha\beta$  chains do not have intrinsic signaling capability, and the associated CD3 chains are required for signal transduction. The cytoplasmic tails of the CD3 chains contain ITAMs that become phosphorylated upon antigen encounter. This triggers the recruitment of signaling molecules and downstream activation of multiple signaling pathways.

are conserved regions that contain tyrosines phosphorylated upon immunoreceptor engagement and serve as docking sites for interactions with other proteins (Irving and Weiss, 1991; Iwashima et al., 1994). Following ligation of antigenic pMHC by the  $\alpha\beta$ TCR, the Src protein tyrosine kinase LCK is carried by the CD8 coreceptor to the TCR and phosphorylates the CD3 ITAMs. This facilitates recruitment of ZAP-70 to the phosphorylated CD3 ITAMs. ZAP-70 is then phosphorylated by LCK and also in trans by other ITAM-bound active ZAP-70 molecules to induce activation. ZAP-70 then phosphorylates adapter proteins LAT and SLP-76 (Chan et al., 1991). These two scaffolds assemble a multimolecular complex, or the TCR signalosome, that recruits effector molecules required for the activation of multiple signaling pathways, including calcium ( $\text{Ca}^{2+}$ ), SAPK/JNK, p38, MAPK/ERK, NFAT, and NF- $\kappa$ B signaling (Figure 1-3).

Interaction between the highly conserved charged residues in the transmembrane domains (TMD) of the TCR $\alpha\beta$  and CD3 chains are important for mature TCR/CD3 complex assembly and expression in the membrane (Arnaud et al., 1996; John et al., 1989; Manolios et al., 1994; Tan et al., 1991). In addition to the role it plays in proper TCR arrangement, the transmembrane domain has evolutionarily conserved regions important in TCR signal transduction (Bäckström et al., 1997; Fuller-Espie et al., 1998). The TCR $\beta$  transmembrane ( $\beta$ TMD) domain contains a conserved antigen receptor transmembrane (CART) motif (Campbell et al., 1994). Interestingly, the TCR $\beta$  CART domain contains an ITAM-like motif similar to what is found in the CD3 chains (Rodríguez-Tarduchy et al., 1996). Mutation of the tyrosine in the TCR $\beta$  CART motif in CD4 T cells



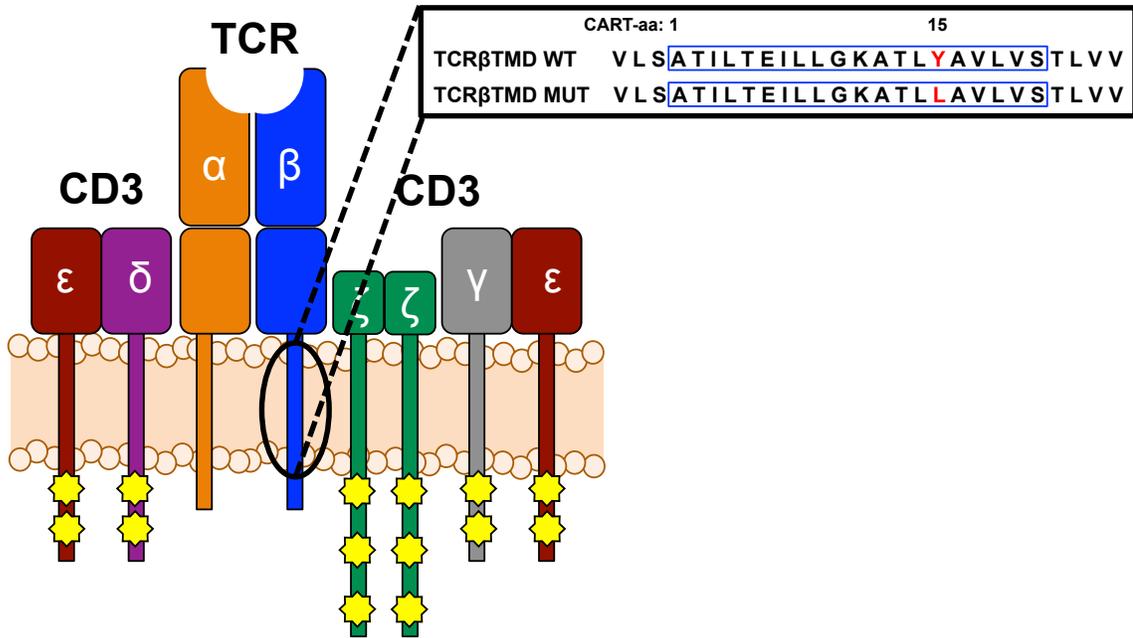
**Figure 1-3. TCR signaling.** Upon TCR engagement with pMHC, LCK phosphorylates the CD3 ITAMS. This induces recruitment and activation of ZAP-70. Active ZAP-70 phosphorylates the adaptor proteins LAT and SLP-76 (constitutively bound to Gads) promoting the formation of a complex containing PLCγ1, VAV, GRB2/SOS, and ITK. The formation of this multimolecular complex promotes activation of downstream signaling pathways  $\text{Ca}^{2+}$ , NFAT, NF-κB, ERK, JNK, and p38.

differentially affects IL-2 production and apoptosis (Rodríguez-Tarduchy et al., 1996; Teixeira et al., 2004). This study suggests a role for the TCR $\beta$ TMD in the regulation of T cell responses. The role of the transmembrane domain in CD8 T cell responses and TCR signaling will be addressed below.

### **$\beta$ TMDmut T Cells**

To study T cell activation and responses, many researchers utilize TCR transgenic mice that contain a single clonotypic T cell population (Pape et al., 1997). While this does not replicate the TCR diversity present in a normal T cell pool, it does allow for the complete analysis of the T cell response to a given, known antigen (Pape et al., 1997). One of the most popular transgenic models for studying CD8 T cell responses, and the one utilized in the studies presented here, is the OT-I TCR transgenic (Hogquist et al., 1994). The OT-I TCR recognizes the SIINFEKL peptide of the chicken ovalbumin protein, or OVA, presented in MHC class I molecules (Hogquist et al., 1994). In addition to the cognate antigen OVA, there is also a well-defined panel of altered peptide ligands (APLs) with varying affinity for the OT-I TCR that can be utilized for T cell stimulation (described later, Table 1-1) (Alam et al., 1999; Daniels et al., 2006; Hogquist et al., 1994).

In 2009, Teixeira et al. demonstrated, using a novel CD8 T cell model, that TCR signals differentially regulate CD8 T cell fate during infection. In order to study the role that TCR signals play in CD8 T cell activation and responses, a substitution mutation of the tyrosine in the TCR $\beta$  CART motif, to affect the ITAM-

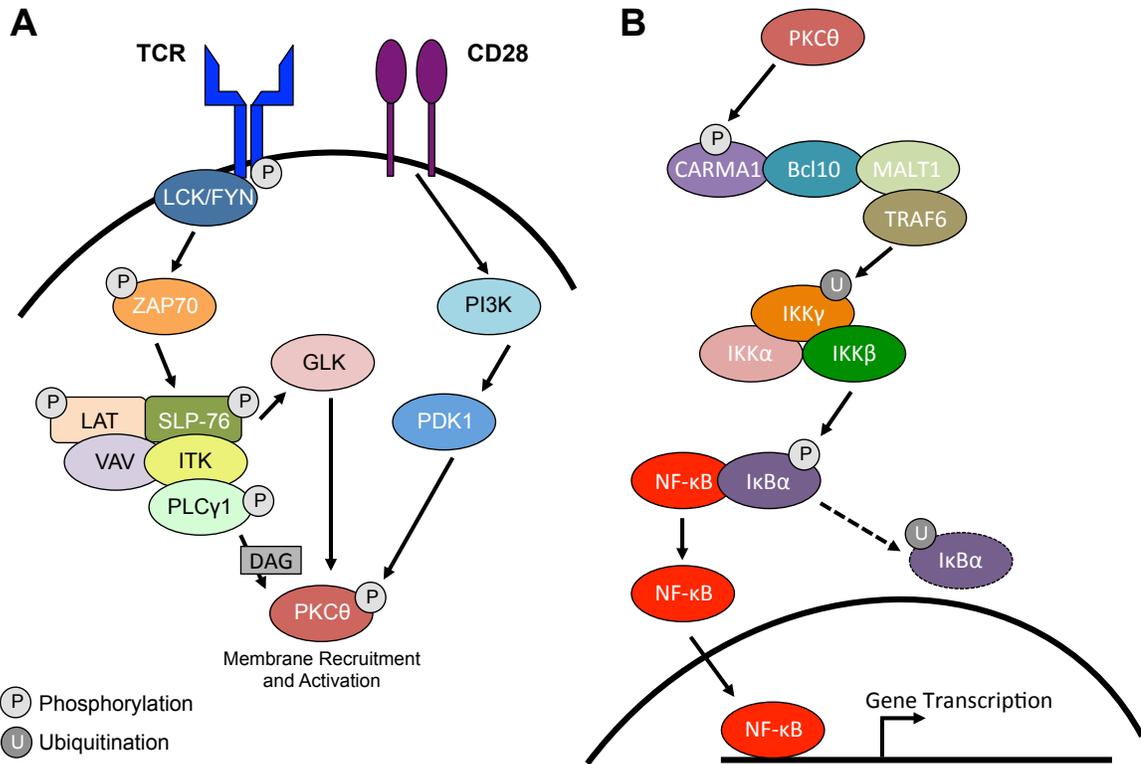


**Figure 1-4. βTMDmut TCR.** βTMDmut CD8 T cells express an OT-I TCR that contains a point mutation (Y→L) in the CART motif of the TCRβ transmembrane domain (βTMD). Assembly and membrane localization of the βTMDmut TCR is normal. Furthermore, this mutation does not affect antigen recognition or TCR-dependent activation of ERK, JNK, mTOR, Wnt, NFAT, or calcium signaling. Activation of NF-κB signaling, specifically, is impaired in βTMDmut CD8 T cells. Figure adapted from (Teixeiro et al., 2009).

like motif, was introduced into an OT-I TCR (Figure 1-4) (Hogquist et al., 1994), termed the TCR $\beta$ TMD mutant,  $\beta$ TMDmut, or MUT here (Teixeiro et al., 2009).  $\beta$ TMDmut CD8 T cells underwent normal thymic selection and populated the peripheral T cell compartment similarly to wildtype (WT) OT-I T cells. Importantly, the mutation in the  $\beta$ TMD did not affect the ability of the MUT T cells to interact with pMHC and become activated upon cognate antigen, OVA, encounter (downregulation of the TCR and upregulation of CD69 expression comparable to WT OT-I counterparts) (Teixeiro et al., 2009). In response to infection with cognate antigen, in the form of challenge with a recombinant *Listeria monocytogenes* strain that expresses (LM-OVA), MUT T cells were activated, obtained an effector T cell phenotype, and expanded comparably to WT CD8 T cells (Teixeiro et al., 2009). Thus, activation and effector differentiation of CD8 T cells were not affected by changing the TCR signal. Strikingly,  $\beta$ TMDmut T cells did not generate a memory pool. The Teixeira et al. study distinctly demonstrates the ability of the TCR signal to discern effector and memory fates of CD8 T cells.

### **NF- $\kappa$ B Signaling and CD8 T Cells**

How did a single mutation in the  $\beta$ TMD of the TCR change the fate of CD8 T cells? Only the TCR was changed in the  $\beta$ TMDmut model. The TCR/CD3 complex is required for activation of signaling pathways necessary for T cell differentiation upon pMHC-TCR encounter. Thus, the obvious possibility is that TCR signaling was altered in MUT T cells. Surprisingly,  $\beta$ TMDmut CD8 T cells had normal induction of many signaling pathways induced upon TCR stimulation,



**Figure 1-5. TCR-dependent activation of NF-κB signaling.** TCR engagement induces the assembly of the TCR signalosome, consisting of LAT, SLP-76, VAV, ITK, and PLCγ1 (among described in Figure 1-3). When activated, PLCγ1 facilitates the generation of IP<sub>3</sub> and DAG from PIP<sub>2</sub>. DAG, along with CD28-dependent activation of PI3K and PDK1, induces activation of PKCθ and recruitment to the membrane. SLP-76 also mediates activation of GLK, which promotes PKCθ activation. (B) Once PKCθ translocates to the membrane, it phosphorylates CARMA1 to mediate the recruitment of Bcl10 and MALT1 to form a stable CBM complex. The CBM complex, in collaboration with TRAF6, promotes activation of the IKK complex consisting of IKKγ (NEMO), IKKα, and IKKβ. IKKβ then phosphorylates a negative regulator of NF-κB called IκBα, leading to its ubiquitination and degradation. NF-κB subunits are then free to translocate to the nucleus and bind promoter targets. Figure adapted from (Smith-Garvin et al., 2009; Vallabhapurapu and Karin, 2009).

including  $\text{Ca}^{2+}$  signaling and ERK, JNK, and NFAT signaling (Teixeiro et al., 2009). MUT T cells only had a defect in TCR-dependent activation of NF- $\kappa$ B signaling, as described below (Teixeiro et al., 2009). As the TCR primarily activates canonical NF- $\kappa$ B signaling, how pMHC-TCR encounter specifically activates the canonical NF- $\kappa$ B signaling cascade will be explained here (Figure 1-5).

In addition to other signaling molecules (Figure 1-3), upon pMHC-TCR engagement, the LAT/SLP-76 scaffold recruits VAV, ITK, and PLC $\gamma$ 1. ITK, which is activated by VAV, phosphorylates PLC $\gamma$ 1. PLC $\gamma$ 1 plays an essential role in TCR signal transduction by hydrolyzing the membrane lipid PIP $_2$  to secondary messengers IP $_3$  and DAG. DAG is important for the activation and membrane recruitment of PKC $\theta$ , which specifically connects the TCR signalosome to NF- $\kappa$ B (Coudronniere et al., 2000). PKC $\theta$  translocation is also dependent on CD28-dependent PI3K-mediated activation of PDK1, a kinase that directly phosphorylates PKC $\theta$ . Membrane recruitment of PKC $\theta$  allows for its phosphorylation of membrane-associated CARMA1. This induces the recruitment of Bcl10 and MALT1 to form a stable CARMA1-Bcl10-MALT1 (CBM) complex. The active CBM complex then induces activation of the IKK complex. The IKK complex contains three members: IKK $\gamma$  (NEMO), IKK $\beta$ , and IKK $\alpha$ . IKK $\gamma$  is ubiquitinated, possibly by TRAF6, which also activates the IKK complex. IKK $\beta$ , in particular, is activated upon TCR signaling. IKK $\beta$  directly phosphorylates I $\kappa$ B $\alpha$ , which binds to NF- $\kappa$ B members and restricts their localization to the cytoplasm. Phosphorylation of I $\kappa$ B $\alpha$  promotes its ubiquitination and degradation, freeing NF-

$\kappa$ B subunits to translocate to the nucleus and bind promoter targets.

As mentioned previously,  $\beta$ TMDmut CD8 T cells have a specific defect in TCR-dependent NF- $\kappa$ B signaling. MUT T cells do not properly recruit and colocalize the TCR and PKC $\theta$  at the immunological synapse (IS) (Teixeiro et al., 2009). The IS is the contact site between a T cell and an APC, and it functions in both the propagation and cessation of TCR signaling (Cemerski and Shaw, 2006; Lee et al., 2003). Translocation of PKC $\theta$  to the IS is required for its activity (Kong et al., 2011; Wang et al., 2003). As a result of these defects, translocation of p65-NF- $\kappa$ B to the nucleus and its DNA binding is impaired in  $\beta$ TMDmut CD8 T cells (Teixeiro et al., 2009). Through the use of the  $\beta$ TMDmut T cell model, this study connected TCR-dependent NF- $\kappa$ B signaling with memory CD8 T cell development.

NF- $\kappa$ B signaling is known to regulate multiple processes in T cells. NF- $\kappa$ B signaling is involved in thymic selection, survival and activation of naïve T cells, and memory responses. Importantly, not all NF- $\kappa$ B signaling components have the same function or affect CD4 and CD8 T cells similarly. This is significant as many of the studies investigating the function of NF- $\kappa$ B signaling in T cells are performed in CD4 T cells in the context of deletion or overexpression of a single NF- $\kappa$ B signaling molecule.

In the thymus, NF- $\kappa$ B signaling has a critical role for survival of thymocytes. In addition, it regulates both positive and negative selection of T cells, especially of the CD8 T cell lineage (Boothby et al., 1997; Hettmann et al., 1999; Voll et al., 2000). Experimental evidence suggests that the amount of NF-

$\kappa$ B activation differentially regulates thymic selection in CD8 T cells, with high NF- $\kappa$ B signaling inducing thymocyte apoptosis while weak NF- $\kappa$ B signaling supports survival (Jimi et al., 2008; Park et al., 2010). After thymic selection, NF- $\kappa$ B signaling is required for naïve T cell survival, partially through the regulation of IL-7R expression and anti-apoptotic molecules (Miller et al., 2014; Schmidt-Supprian et al., 2003; Silva et al., 2014). The homeostatic cytokine IL-7 regulates the expression of Bcl-2, an anti-apoptotic molecule required for naïve T cell survival (Schluns et al., 2000). Interestingly, Bcl-2 is also a known NF- $\kappa$ B target. This suggests that NF- $\kappa$ B can regulate naïve T cell survival both directly and indirectly (Catz and Johnson, 2001; Miller et al., 2014; Silva et al., 2014; Zheng et al., 2003).

NF- $\kappa$ B signaling regulates the genetic programs required for peripheral T cell activation, proliferation, differentiation, and function. NF- $\kappa$ B supports expression of the high-affinity IL-2 receptor (CD25) (Ballard et al., 1988), and IL-2 responsiveness is required for T cell proliferation (Lai et al., 2009). In addition, there is some experimental evidence that suggests NF- $\kappa$ B as a regulator of effector T cell function. In conjunction with Notch, NF- $\kappa$ B is recruited to the promoter of effector molecules perforin and Granzyme B (Cho et al., 2009). Loss of Notch prevented NF- $\kappa$ B binding to these promoters and led to decreased Granzyme B and perforin protein expression in CD8 T cells (Cho et al., 2009). Inhibition of NF- $\kappa$ B signaling also impairs IFN $\gamma$  production in CD4 T cells (Cho et al., 2009; Palaga et al., 2003). As in T cell selection, the amount of NF- $\kappa$ B signaling is important in determining peripheral T cell responses. Loss of NF- $\kappa$ B

signaling prevents proper activation and differentiation of effector T cells (Greve et al., 2007; Krishna et al., 2012). On the other hand, chronic activation of NF- $\kappa$ B signaling induces T cell death and impairs effector function (Krishna et al., 2012).

A recent study has clearly defined a role for non-canonical NF- $\kappa$ B signaling component NIK in CD8 T cell memory development (Rowe et al., 2013). Infectious challenge led to small but significant reduction in the expansion of NIK-KO versus WT T cells. However, there was a drastic reduction in the number of NIK-KO CD44<sup>hi</sup> memory T cells after infection. Surprisingly, the low number of NIK-KO memory T cells responded upon secondary challenge (Rowe et al., 2013). These results suggest that NIK, a non-canonical NF- $\kappa$ B signaling component, regulates memory CD8 T cell differentiation but not responses. Importantly, TCR signaling primarily induces activation of canonical NF- $\kappa$ B signaling (Vallabhapurapu and Karin, 2009). How canonical NF- $\kappa$ B signaling members regulate CD8 T cell memory differentiation has not been elucidated. Expression of a dominant negative I $\kappa$ B $\alpha$ , which cannot be phosphorylated and degraded leading to impaired NF- $\kappa$ B signaling, led to a decreased frequency of memory phenotype CD8 T cells in the peripheral T cell compartment (Hettmann et al., 2003). Genetic loss of IKK $\beta$  also prevented the development of CD44<sup>hi</sup> memory phenotype CD8 T cells (Schmidt-Supprian et al., 2003). These studies, however, did not investigate whether memory differentiation was affected in the context of infection. This is possibly due to the defects in mature, naïve CD8 T cell development in these models (Hettmann et al., 2003; Schmidt-Supprian et al., 2003). We have previously shown that MUT T cells, which have a defect in

TCR-dependent NF- $\kappa$ B signaling, also have impaired memory generation (Teixeiro et al., 2009). The direct link between NF- $\kappa$ B signaling and memory differentiation was not demonstrated, however. The role that NF- $\kappa$ B signaling plays in the regulation of CD8 T cell memory development requires further investigation.

### **TCR Affinity**

The nature of the TCR interaction with pMHC largely defines the outcome of an immune response. This interaction and the resulting strength of TCR stimulation is affected by multiple factors, including the quantity or density of pMHC, the duration of pMHC-TCR encounter, and affinity of pMHC-TCR binding (Corse et al., 2011). In general, stronger TCR signals governed by the above factors (large quantity of pMHC, long duration of pMHC-TCR encounter, and high-affinity pMHC-TCR interaction) promote greater activation of CD8 T cells than a weaker TCR signals. This greater activation is characterized by increased proliferation and cytotoxic function (Khanna et al., 2008; Prlic et al., 2006; Shaulov and Murali-Krishna, 2008). Surprisingly, both strong and weak TCR signals induce T cell differentiation *in vivo* (Khanna et al., 2008; Leignadier and Labrecque, 2010; Prlic et al., 2006; Shaulov and Murali-Krishna, 2008; Zehn et al., 2009). The studies presented here mainly focus on the affinity of the pMHC-TCR interaction in regulation of T cell differentiation.

TCR affinity refers to the strength of the interaction/binding between the TCR and pMHC. The affinity of this interaction is important for a T cell during

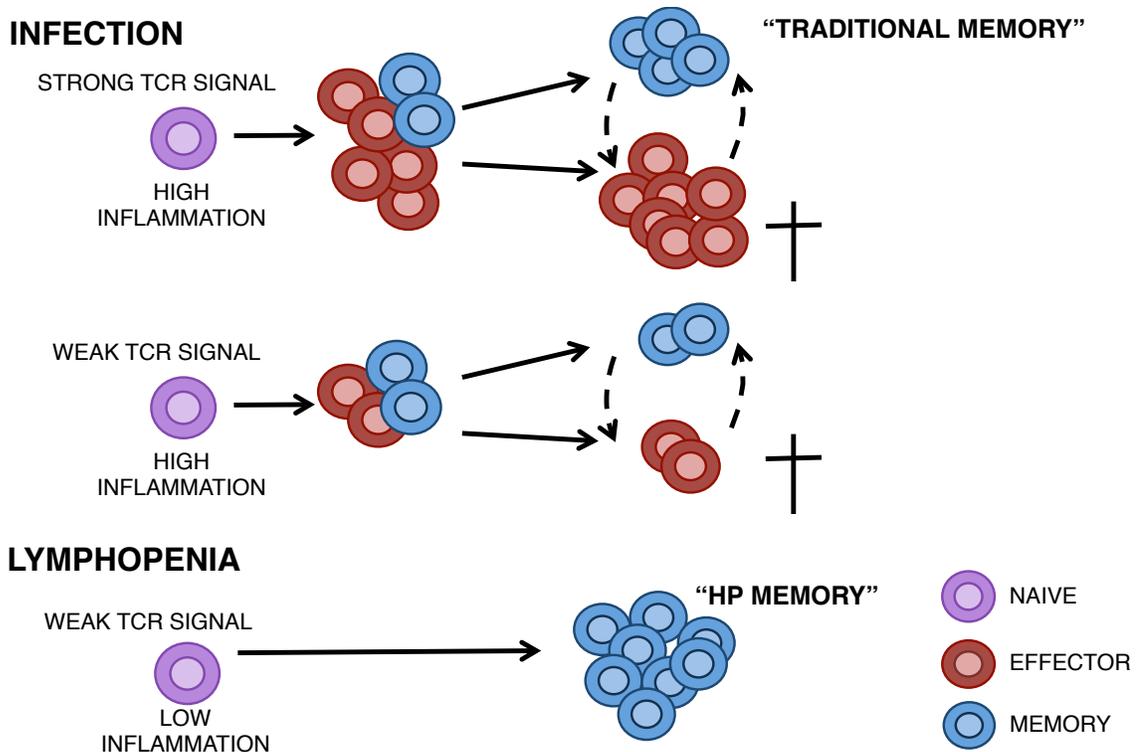
distinct processes such as thymic selection and naïve T cell activation. Thymic selection is the method by which developing T cells are selected to not respond to self-peptides to prevent autoimmunity, termed immunological tolerance (Starr et al., 2003). Thymic selection includes multiple steps involving recognition of self-pMHC by the TCR. Very weak or negligible TCR-pMHC interactions do not support thymocyte survival, called death by neglect; overtly strong TCR-pMHC encounter induces apoptosis, receptor editing, or alternative lineage development, termed negative selection; and an pMHC-TCR binding of “moderate” strength promotes mature naïve T cell differentiation and survival, or positive selection (Starr et al., 2003). Studies using the monoclonal OT-I T cell receptor reported that the weakest negative selector was only two-fold more potent than the strongest positive selector (Daniels et al., 2006). This indicates that the pMHC-TCR affinity threshold to induce T cell death or survival in the thymus is very narrow. Interestingly, TCR encounter with positive and negative selecting peptides in the thymus induce qualitatively distinct activation of proximal TCR signaling molecules (Daniels et al., 2006). In addition, simply titrating the concentration of these peptides to alter the amount of pMHC-TCR interaction does not change the survival decision of the T cell (Daniels et al., 2006). Thus, the TCR is not merely an on/off switch but a sensitive machine that can distinctly signal depending on the strength or quality of pMHC-TCR encounter. Furthermore, minor differences in pMHC-TCR affinity can affect a T cells’ ability to survive.

Once a T cell progresses through thymic selection, it emigrates from the thymus to peripheral secondary lymphoid organs. In addition to other changes, the switch from thymocyte to mature naïve CD8 T cell alters sensitivity and response to different pMHC-TCR affinity interactions. Instead of inducing apoptosis, as in the thymus, naïve TCR encounter with a high-affinity pMHC promotes activation, proliferation, and differentiation. TCR interaction with very low-affinity, self-pMHC supports naïve T cell survival (Takeda et al., 1996; Tanchot et al., 1997). As in thymocytes, the TCR distinctly signals based on the strength of interaction with pMHC in the periphery. Specifically, stimulation of T cells with APLs, peptides with varying affinity for a given TCR, leads to partial phosphorylation of the CD3 $\zeta$  ITAMs (Madrenas et al., 1995; Sloan-Lancaster et al., 1994). This prevents proper recruitment and activation of ZAP-70 and alters distal TCR signaling, such as activation of ERK (Madrenas et al., 1995; Sloan-Lancaster et al., 1994; Stefanová et al., 2003). Despite inducing different (sometimes considered weaker) TCR signaling, weak/low-affinity pMHC encounter (above a certain affinity) by peripheral CD8 T cells can induce activation and effector and memory differentiation (Sabatino et al., 2011; Zehn et al., 2009). Furthermore, low-affinity-stimulated T cells do contribute to an immune response by producing effector cytokines (Sabatino et al., 2011). Further work is required to determine whether weak pMHC-TCR encounter translates to differences in T cell programming and differentiation during infection.

### **Memory CD8 T Cell Ontogeny and TCR Affinity**

The majority of studies investigating CD8 T cell memory development have been performed in the context of infection using cognate, or high-affinity, antigen and high levels of inflammation. However, it is known that other conditions promote memory CD8 T cell generation (Figure 1-6).

Lymphopenic conditions occur in neonates, in humans undergoing chemotherapy, during viral infections, or in immunodeficient or irradiated mice (Freitas and Rocha, 2000; Mackall et al., 1997; Selin et al., 1996; Tumpey et al., 2000). Lymphopenia is characterized by an increased amount of space in the immune compartment, high levels of homeostatic cytokines IL-7 and IL-15, and less competition for self-pMHC complexes. In these conditions, naïve CD8 T cells undergo spontaneous proliferation and differentiation to gain a memory phenotype (Goldrath et al., 2000; Goldrath et al., 2002; Kieper and Jameson, 1999). These naïve T cells do not progress through an effector phase, making this process unique from what is typically described during infection (Goldrath et al., 2004). Despite this, the requirements for functional memory development in lymphopenia do have parallels to memory generation during infection. Naïve T cells undergoing lymphopenia-induced proliferation in mice genetically deficient in *Rag*, which do not have B or T cells and have little inflammation, do not develop into functional memory (Hamilton and Jameson, 2008b; Hamilton et al., 2006b). This is due to the absence of inflammation and CD4 T cell help (Hamilton and Jameson, 2008b; Hamilton et al., 2006b). Both are required for protective memory CD8 T cell generation and maintenance during infection (Agarwal et al., 2009; Hamilton and Jameson, 2008; Hamilton et al., 2006;



**Figure 1-6. Conditions for memory generation of CD8 T cells.** CD8 T cell generation occurs during infection and lymphopenia. Memory generation during infection is induced in response to both high and low-affinity pMHC-TCR encounter and high levels of inflammation. The composition of the T cell pool at the peak of the effector response as well as the ability of these cells to differentiate into memory is different depending on the affinity of the antigen encountered during priming. In contrast, memory development during lymphopenia occurs in response to weak TCR signals and low inflammation. Naïve T cells do not pass through an effector phase during memory generation in lymphopenic conditions (Goldrath et al., 2004).

Schmidt and Mescher, 2002; Shedlock and Shen, 2003; Sun and Bevan, 2003; Xiao et al., 2009).

It was initially thought that strong TCR signals, provided by cognate or high-affinity antigen, were required for effector and memory generation during infection. Recent studies, however, have shown that low-affinity CD4 T cell clones are recruited during polyclonal responses to both infectious challenge and autoimmune conditions (Sabatino et al., 2011). In fact, the number of low-affinity clones present in the immune response was similar to high-affinity clones. Additionally, the low-affinity CD4 T cells did contribute to the immune response by producing significant amounts of effector cytokines (Sabatino et al., 2011). There has also been investigation into the memory development of CD8 T cells upon low-affinity antigen challenge. Weak antigens, in range of self-peptide, induce CD8 T cell differentiation during infection (Sabatino et al., 2011). The CD8 T cell response is curtailed in number of generated effector and memory T cells; however, the low-affinity antigen-generated memory is protective (Zehn et al., 2009). Costimulation and inflammation are present during the response against low-affinity ligands, but the role they play in low-affinity T cell memory generation was not investigated. Collectively, there are three distinct conditions for memory CD8 T cell ontogeny: infection with cognate antigen (high-affinity), infection with low-affinity antigen, and lymphopenia-derived proliferation (Figure 1-6).

## **Summary**

The development of CD8 T cell memory is required for long-term immunity. While the importance of memory generation has been recognized for over 30 years, the mechanism by which memory CD8 T cells arise during immune responses is still not fully understood. We know that TCR signaling is necessary for activation and differentiation of CD8 T cells. Furthermore, our laboratory has previously shown that different TCR signals distinctly program effector and memory generation (Teixeiro et al., 2009). However, the mechanism by which the TCR signal regulates both memory development and memory maintenance is unknown.

To address the question of how TCR signals program CD8 T cell memory, we employed OT-I TCR transgenic T cells. This is in part due to the availability of a defined panel of altered peptide ligands with differing affinities for the OT-I TCR (Table 1-1) (Alam et al., 1999; Daniels et al., 2006; Hogquist et al., 1994). This panel includes the cognate antigen SIINFEKL, also called OVA or N4. Of note, all of the APLs used in these studies have the same affinity for MHC (Zehn et al., 2009). In order to induce a robust and well-described CD8 T cell response, recombinant *Listeria monocytogenes* expressing the aforementioned altered peptide ligands was utilized for infection (Pope et al., 2001; Zehn et al., 2009). In addition, we utilized the  $\beta$ TMDmut OT-I CD8 T cells as a unique tool for investigating the specific impact of the TCR signal on memory generation (Teixeiro et al., 2009). As  $\beta$ TMDmut T cells have a specific defect in NF- $\kappa$ B signaling, this also led us to investigate the role that TCR-dependent NF- $\kappa$ B signaling plays in memory CD8 T cell differentiation.

	Peptide Sequence	OT-I Sensitivity EC50 APL/N4 (CD25 Induction) <sup>1</sup>	OT-I Sensitivity EC50 APL/N4 (IFN $\gamma$ Production) <sup>2</sup>	Designation in Thymus	Designation in Periphery
<b>OVA (N4)</b>	SIINFEKL	1	1	Negative Selector	High Affinity
<b>Q4</b>	SIIQFEKL	*	18.3	Negative Selector	High Affinity
<b>Q4R7</b>	SIIQFERL	*	*	Negative Selector	High Affinity
T4	SIITFEKL	27.9	70.7	Border	Border
<b>Q4H7</b>	SIIQFEHL	63.4	*	Positive Selector	Low Affinity
<b>Q7</b>	SIINFEQL	*	*	Positive Selector	Low Affinity
<b>V4</b>	SIIVFEKL	*	680	Positive Selector	Low Affinity

**Figure 1-7. OT-I TCR ligands.** The OT-I TCR specifically recognizes the SIINFEKL peptide of ovalbumin protein, termed OVA or N4. Numerous altered peptide ligands have been generated with varying affinity for the OT-I TCR. Terminology for the APLs is determined by their mutated amino acids (shown in bold in the peptide sequence). The table shows relative potency in peripheral OT-I T cells as determined by CD25 induction in Koehli et al. 2014 (1) or IFN $\gamma$  production in Zehn et al. 2009 (2). \* indicates APLs that were tested in these studies, but listed due to use in the experiments described here. Table also shows the designation of the APLs in the thymus and periphery, as referenced in Daniels et al. 2006 and the following studies. Responses to border peptide T4 are determined by concentration of peptide and thus T4 can be designated as a positive or negative selector in the thymus or high or low-affinity in the periphery. Table is adapted from (Daniels et al., 2006; King et al., 2012; Koehli et al., 2014).

Determining how the TCR signal regulates memory generation and survival is necessary for improving current vaccine protocols and immunotherapies. All vaccines utilize pathogen-specific antigens to induce immunological memory. By understanding how antigenic signals program memory differentiation, it will be possible to specifically manipulate this process. Then, we can produce more effective and longer lasting memory cells.

**II: Low-affinity T cells are programmed to maintain normal primary responses but are impaired in their recall to low-affinity ligands.**

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N. P. Goplen generated the data used in Figure 2-7 A, B, E, F and Figure 2-8 A.

## Rationale

By utilizing the  $\beta$ TMDmut model, we demonstrated that the TCR signal can distinctly regulate effector and memory CD8 T cell fate (Teixeiro et al., 2009). These studies were performed using high-affinity pMHC-TCR interaction. It was originally thought that strong TCR signals were required for CD8 T cell activation and differentiation. This conclusion was supported by the observation that the antigen-specific T cell response is dominated by high-affinity TCR clones (Malherbe et al., 2004; Slifka and Whitton, 2001; Zehn et al., 2009). However, this view was challenged by the discovery that lymphopenic conditions, in the absence of foreign antigen, promote spontaneous proliferation and differentiation of naïve CD8 T cells into a memory-like phenotype (Goldrath and Bevan, 1999; Goldrath et al., 2000). In addition, it is well-established that CD8 T cells recognize and kill tumors and can mediate autoimmune responses (Hadrup et al., 2012; Walter and Santamaria, 2005). In contrast to infection, T cell activation in these conditions is mediated by self-peptides, which are typically recognized with low-affinity by the endogenous T cell repertoire. Recently, it was described that low-affinity clones are recruited into immune responses and even very weak TCR-pMHC interactions induce memory generation during infection (Sabatino et al., 2011; Zehn et al., 2009). Importantly, memory T cells generated in response to low-affinity ligands are fully functional and respond upon re-challenge with cognate antigen (Zehn et al., 2009). However, whether TCR affinity differentially regulates effector and memory programming and differentiation is not known.

The goal of the following study was to determine whether memory CD8 T cell differentiation and function was affected by the TCR signal strength. In addition, we examined how high and low-affinity-generated memory cells functioned under two different infectious conditions: in response to cognate antigen and in response to low-affinity, pseudo-self peptides. These experiments allowed us to make predictions about the relevance of low-affinity memory T cells to support T cell immunity against future infections and mediate an autoimmune response.

## **Abstract**

T cell responses to low-affinity TCR ligands occur in the context of infection, tumors and autoimmunity despite diminished TCR signal strength. The processes that enable such responses remain unclear. We show that distinct mechanisms drive effector/memory development in high and low-affinity T cells. Low-affinity cells preferentially differentiate into memory precursors of a central memory phenotype that are IL-12R<sup>lo</sup>, IL-7R<sup>hi</sup> and Eomes<sup>hi</sup>. Strikingly, in contrast to naïve cells, low-affinity memory cells were impaired in the response to low but not high-affinity ligands, indicating that low-affinity cells are programmed to generate diverse immune responses while avoiding auto-reactivity. Affinity and antigen dose directly correlated with IL-12R signal input and T-bet but not Eomes expression since low-affinity signals were more potent inducers of Eomes at high antigen dose. Our studies explain how weak antigenic signals induce complete primary immune responses and provide a framework for therapeutic intervention.

## **Introduction**

CD8 T cell memory contributes to the health of an individual by providing protection against re-exposure to intracellular pathogens and tumors. High-affinity interactions between the TCR and peptide-MHC ligands lead to acquisition of effector function and generation of long-term memory (Corse et al., 2011; Gourley et al., 2004). Interactions with low-affinity self-peptide MHC molecules are necessary for the survival of naïve peripheral T cells (Jameson, 2005; Sprent et al., 2008). Strikingly, these low-affinity interactions are sufficient

for the acquisition of effector function and memory differentiation (Zehn et al., 2009). This challenges the notion that central tolerance leads to complete non-responsiveness to self and suggests an important role for low-affinity T cell interactions in the peripheral lymphoid compartment.

The biological significance of generating low-affinity memory T cells becomes evident when considering the following: First, low-affinity T cells are significant contributors to the effector response against auto-antigens, tumors and pathogens (Pardoll, 2002; Sabatino et al., 2011). Second, low-affinity T cells can participate in the maintenance of a diverse memory repertoire (Intlekofer et al., 2006). This may be especially relevant with age considering the drop in thymic output upon puberty (Ahmed et al., 2009; den Braber et al., 2012; Hale et al., 2006; Naylor et al., 2005). Additionally, this may be advantageous to control microbes that mutate T cell epitopes in an effort to evade the immune response (van Gisbergen et al., 2011). Therefore, understanding a T cells' ability to respond to low-affinity ligands is a critical aspect of T cell immunity.

How low-affinity TCR-peptide-MHC interactions lead to T cell effector and memory differentiation is not known. Studies of TCR signaling in response to low-affinity ligands have demonstrated that changes in potency are quantitatively and qualitatively unique and cannot be explained by simple antigen dose effects. Seminal studies with altered peptide ligands have shown that the TCR has the ability to trigger specific signaling events that support some functions but not others depending on the nature of the TCR-peptide-MHC interaction (Daniels et al., 2006; Davis et al., 1998; Jameson and Bevan, 1995; Jameson et al., 1993;

Koniaras et al., 1999; Madrenas et al., 1995; Sloan-Lancaster et al., 1994; Sloan-Lancaster et al., 1994). Therefore, low-affinity TCR interactions do not necessarily imply an overall dampening of TCR signal but rather may lead to qualitative changes in signal output that are sufficient to support unique T cell responses. Additionally, in the context of an immune response, TCR and pro-inflammatory signals cooperate to regulate T cell differentiation (Mescher et al., 2006a). However, whether this is dependent on TCR affinity is poorly defined.

The T-box transcription factors T-bet and Eomes are crucial for effector and memory differentiation in T cell immune responses (Intlekofer et al., 2005). Both are induced by high-affinity TCR ligands and modulated by the pro-inflammatory cytokine IL-12 (Takemoto et al., 2006). Studies with T-bet and Eomes deficient mice suggest that both can act redundantly to induce effector functions. However, while T-bet suppresses Eomes expression and promotes terminal short-lived effector differentiation, Eomes supports self-renewal of antigen-specific memory cells, without affecting T-bet expression (Banerjee et al., 2010a; Intlekofer et al., 2007). Thus, T-bet and Eomes expression profiles may be useful to predict the efficiency of generating a memory pool. Curiously, there is little insight into how TCR affinity impacts T-bet and Eomes expression profiles for effector/memory differentiation.

Central and peripheral tolerance mechanisms regulate the delicate balance between protective immunity and autoimmunity during an immune response. Central tolerance is regulated by a sharp border that defines the difference between positive and negative selection (Daniels et al., 2006; Naeher

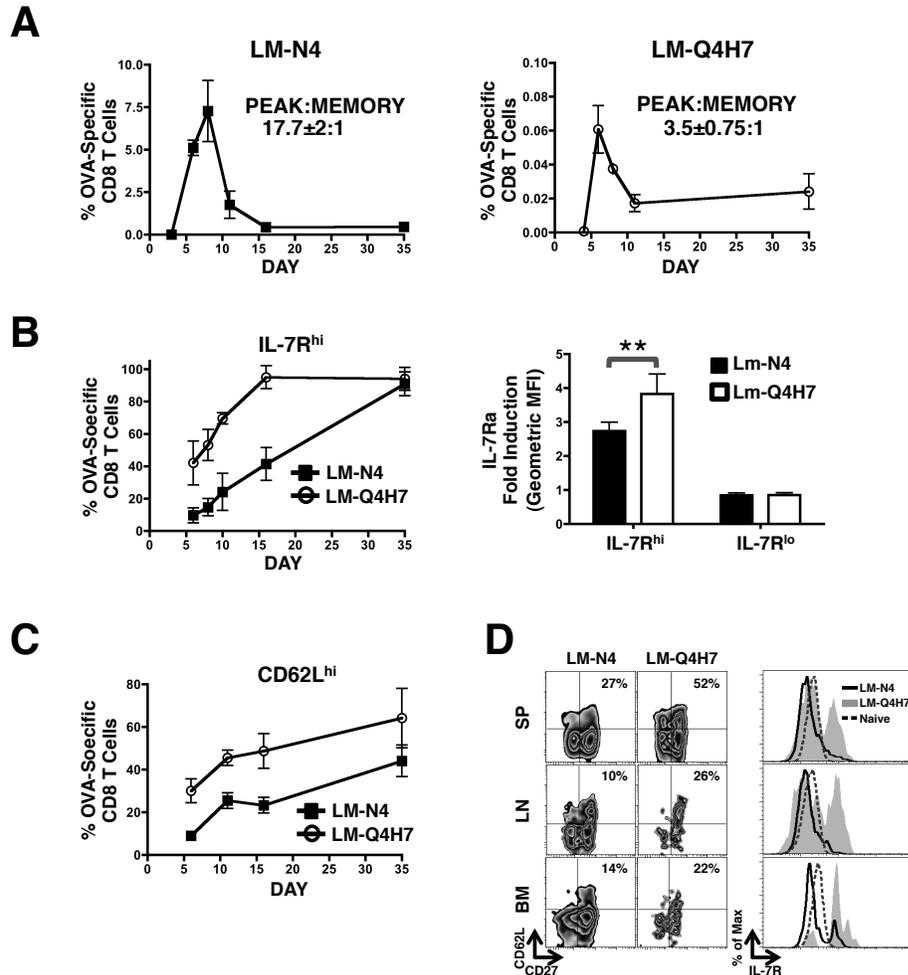
et al., 2007). This is an imperfect process, and there is considerable evidence for self-reactive clones that escape negative selection and can cause autoimmunity (Enouz et al., 2012; Hogquist et al., 2005; Liston et al., 2005). These cells, then, are kept in check by peripheral tolerance mechanisms to avoid autoimmunity. Among these mechanisms, TGF $\beta$  signaling is critically important to control low-affinity T cell responses (Gorelik and Flavell, 2002; O'Sullivan et al., 2011; Zhang and Bevan, 2012). Therefore, for low-affinity T cells to develop effector function and memory without causing autoimmunity, one could envision a scenario where the quantity and/or quality of TCR signals regulate susceptibility to inflammation and tolerance, shaping memory programming in a way that maximizes diversity of the memory pool and limits induction of autoimmunity.

We tested this hypothesis with the OT-I TCR transgenic system. We compared immune responses to the strongest negative selecting peptide, N4 (agonist), and the strongest positive selecting ligand, Q4H7 (pseudo-self), and studied how differences in TCR affinity influence T cell memory programming and the susceptibility of T cells to inflammatory and regulatory signals. We show that high and low-affinity T cells are different in their effector/memory programming. We provide mechanistic data about how TCR affinity, IL-12 and TGF $\beta$  signals interplay to modify this distinct program. We propose that quantity (antigen dose) and quality of the signal triggered by the stimulating TCR ligand not only determine the nature of the primary immune response but also have a profound impact on programming the sensitivity to low-affinity ligands during the secondary immune response.

## Results

*T cells responding to high and low-affinity ligands are phenotypically different.*

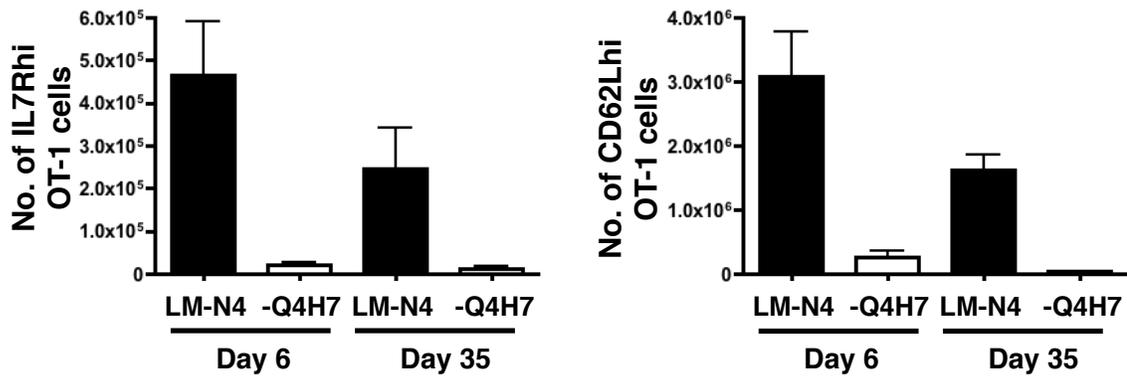
Numerous studies have led to the general conclusion that activation of a T cell is proportional to TCR ligand affinity (Gottschalk et al., 2010). Contrary to this, low-affinity T cells significantly contribute to the effector response against autoantigens and pathogens by developing into effector and memory cells (Zehn et al., 2009), which may pose a risk for autoimmunity. Thus, we sought to understand how very low-affinity TCR ligands (in the range of positive selectors) are able to support these T cell fates. For this, we compared the responses against the cognate antigen for the OT-1 TCR, N4 (SIINFEKL), and the strongest positive selecting ligand Q4H7 (SIIQFEHL). Q4H7 selection potential was determined by analysis of a large panel of N4 variants in fetal thymic organ culture. It was found to have the highest affinity and potency and still induce positive selection of OT-1 T cells (Daniels et al., 2006). We deliberately chose Q4H7 since it would potentially present the highest risk for a positive selecting ligand to induce dangerous auto-reactivity. As expected, *Listeria monocytogenes* (LM) expressing Q4H7 induced a much weaker response than LM-N4. Both LM-N4 and Q4H7 T cell responses gave rise to stable T cell memory pools. However, the ratio of effector to memory in response to LM-N4 and LM-Q4H7 were different (Figure 2-1A). As expected, 5-10% of the cells present at the peak of the LM-N4 response survived contraction and established the memory pool (Williams and Bevan, 2007). Interestingly, in LM-Q4H7 responses, an



**Figure 2-1. Low-affinity cells preferentially differentiate into central memory precursors.**

**(A–D)** OT-I T cells ( $1 \times 10^3$ ) were transferred into congenic B6 hosts and challenged with  $1 \times 10^3$  colony-forming units (CFU) LM-N4 or LM-Q4H7. **(A)** Frequency of cells during the immune response to LM-N4 (left) or LM-Q4H7 (right) in blood. Ratio shown as mean±SD. **(B)** Frequency of cells expressing IL-7R<sup>hi</sup> in blood (left). For all data points except day 35,  $p < 0.001$ . Bar graph represents expression of IL-7R (mean fluorescence intensity, MFI) in IL-7R<sup>hi</sup> and IL-7R<sup>lo</sup> populations in lymph nodes at day 6 (right). Geometric MFIs were normalized to a naive control. **(C)** Frequency of cells expressing CD62L<sup>hi</sup> in blood. For all data points except day 35,  $p < 0.0007$ . Day 35,  $p < 0.05$ . **(D)** CD27, CD62L, and IL-7R expression in lymph nodes, spleen, and bone marrow at day 6. All graphs show mean±SD and represent  $n \geq 3$  independent experiments, with  $n \geq 3$  mice per experiment (\* $p < 0.05$ , \*\* $p < 0.005$ , \*\*\* $p < 0.001$ ).

approximately 6-fold higher ratio of T cells present at the peak ended up in the memory pool. This suggests that while low-affinity effector cell numbers were diminished, the percentage of low-affinity memory cells generated was comparatively increased. Looking further, we measured IL-7Ra expression as a surrogate marker for memory precursors during the immune response. T cells responding to LM-Q4H7 (low-affinity) exhibited a substantially higher percentage of memory precursors than their LM-N4 (high-affinity) counterparts (Figure 2-1B, left)(King et al., 2012). Furthermore, low-affinity responders expressed higher levels of IL-7Ra than naïve or high-affinity responders at the peak of the response (Figure 2-1B, right), supporting the idea that antigen controls IL-7Ra levels (Hammerbeck and Mescher, 2008). IL-7R<sup>hi</sup> cells responding to LM-Q4H7 exhibited an activated phenotype (CD44<sup>hi</sup>, Granzyme B<sup>hi</sup>, etc), discarding the possibility they were un-stimulated naïve T cells (data not shown). The percentage of CD62L<sup>hi</sup> cells was significantly higher in response to LM-Q4H7 than to LM-N4, suggesting a correlation between TCR affinity and central memory phenotype (Figure 2-1C). Yet, the number of cells with IL-7R<sup>hi</sup> and CD62L<sup>hi</sup> phenotypes was significantly lower in low-affinity T cells (Figure 2-2). Low-affinity IL-7R<sup>hi</sup> CD62L<sup>hi</sup> T cells were present at similar frequencies in spleen, lymph nodes and bone marrow, indicating they do not preferentially accumulate in one of these organs. Interestingly, a higher frequency of low-affinity cells remained CD27<sup>hi</sup> (Figure 2-1D), correlating with the idea that its expression is required for low-affinity T cells to develop into memory (van Gisbergen et al., 2011). Collectively, these data show that T cells responding to high and low

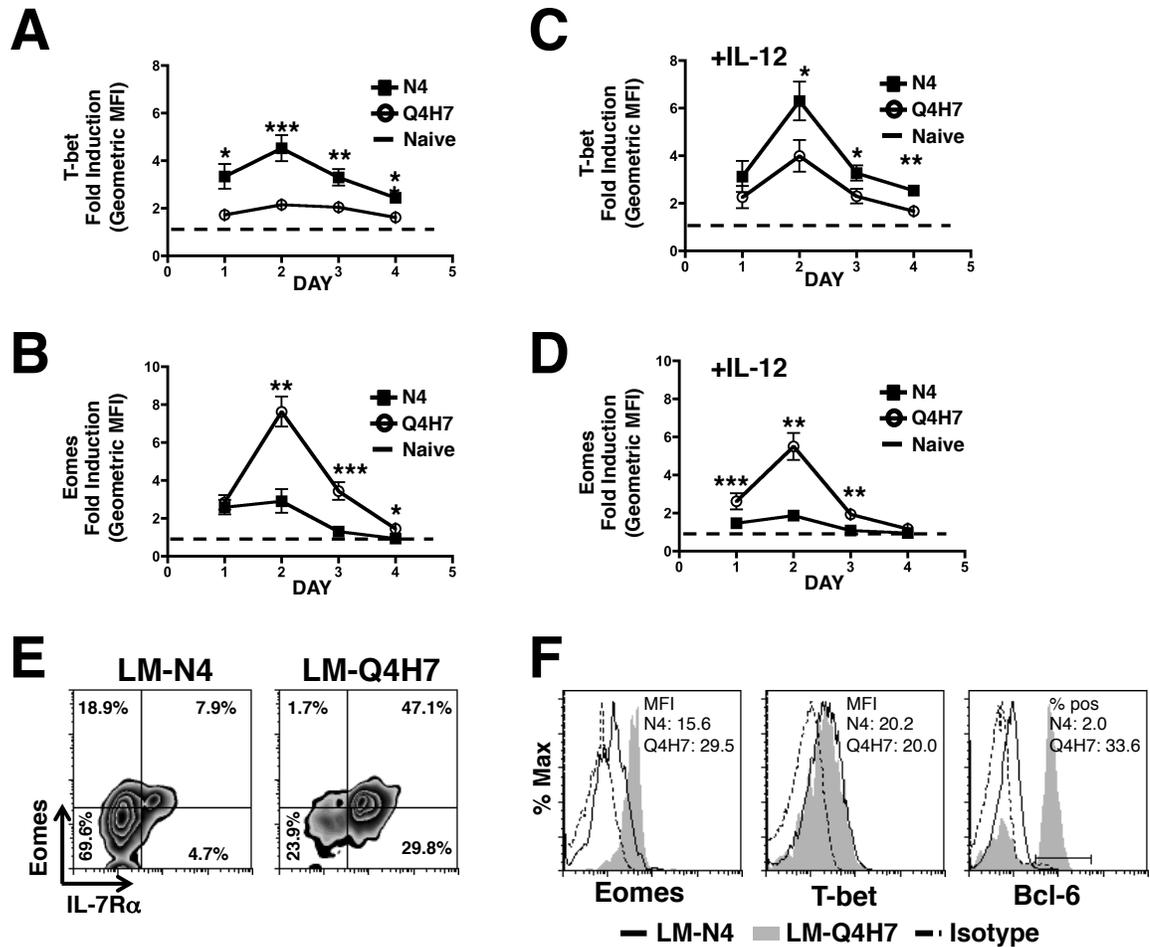


**Figure 2-2. Number of IL-7R<sup>hi</sup> and CD62L<sup>hi</sup> high-(LM-N4) and low-affinity (LM-Q4H7) cells at days 6 and 35 in the immune response.** Experiments were performed as in Figure 2-1. Numbers were calculated in spleen in n≥3 experiments with n≥3 mice per experiment. Graph shows mean±SEM (\*p < 0.05, \*\*p < 0.005, \*\*\*p < 0.001).

affinity TCR ligands are phenotypically different during the course of listeria infection and low-affinity T cells primarily develop into memory precursors with a central memory phenotype.

*Low-affinity TCR ligands trigger a distinct effector/ memory program.*

These results suggest that low-affinity T cells preferentially differentiate into memory at the expense of effectors or, alternatively, they are less efficiently recruited into the effector pool while memory development remains intact (or both). To distinguish between these possibilities, we investigated how low-affinity TCR ligands support effector and memory programming by examining the expression of T-bet and Eomes (Kallies, 2008; Rutishauser and Kaech, 2010). Naïve OT-1 T cells were stimulated with 20nM of N4 or Q4H7 peptide pulsed-APCs and T-bet and Eomes expression was measured from days 1-4. We found that T cells responding to Q4H7 expressed very low levels of T-bet (Figures 2-3A). In contrast, low-affinity T cells expressed Eomes at much higher levels than high-affinity cells (Figure 2-3B). This was not exclusive to Q4H7, since we observed similar results with a lower affinity pseudo-self TCR ligand (Q7) that we have previously described in (Figure 2-4) (Daniels et al., 2006). Similar frequencies of CD25<sup>+</sup> and CD44<sup>+</sup> cells were observed for Q4H7 (or Q7) and N4 stimulation, ruling out that the differences in T-bet and Eomes expression resulted from unstimulated naïve OT-1 T cells (Figure 2-4). Thus, as TCR affinity decreased, T-bet expression diminished, while the induction of the memory-associated factor Eomes increased.

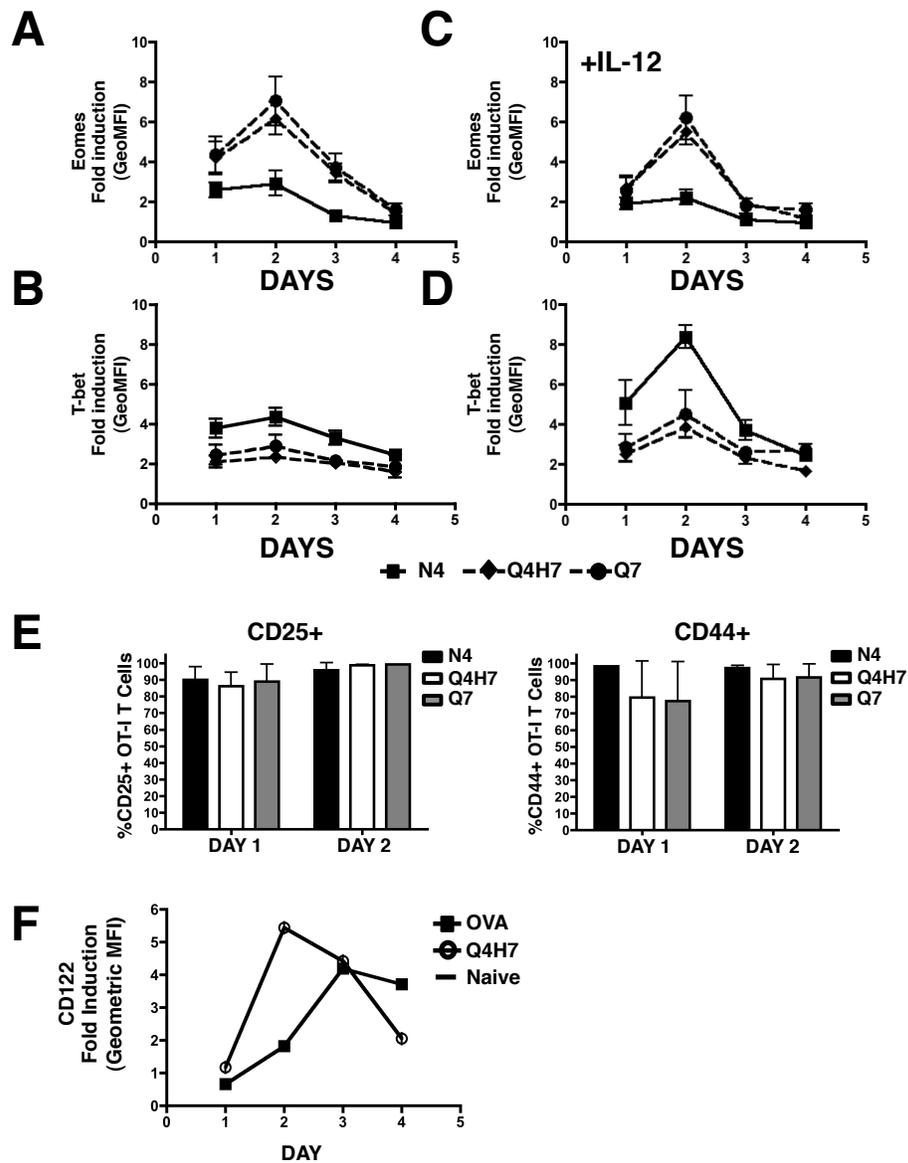


**Figure 2-3. Induction of Eomes, T-bet, and Bcl-6 expression in low-affinity cells. (A–D)**

Naive OT-I splenocytes were stimulated with 20nM N4 or Q4H7 peptide-pulsed APCs in the absence (A, B) or presence (C, D) of 2 ng/ml IL-12. Expression of T-bet (A, C) and Eomes (B, D) were determined by flow cytometry on OT-1 T cells. Naive control (dashed line). Graphs show mean±SD and represent  $n \geq 5$  independent experiments (\* $p < 0.05$ , \*\* $p < 0.005$ , \*\*\* $p < 0.001$ ). (E–F) OT-I naive T cells were transferred into congenic B6 hosts and challenged as in Figure 2-1. (E) Frequency of cells expressing IL-7R $\alpha^{\text{hi}}$ Eomes $^{\text{hi}}$  at the peak of the LM-N4 and LM-Q4H7 immune responses (days 6–8) in blood. Dot plots are representative of 5 independent experiments,  $n \geq 3$  mice per condition. (F) Eomes, T-bet, and Bcl-6 expression determined as in (A) on OT-1 donors at the peak of the LM-N4 and LM-Q4H7 immune responses (days 6–8). Isotype or naive control, dashed line. Histograms and values are representative of 3 independent experiments,  $n \geq 3$  mice per condition. Values show MFI (median) for Eomes and T-bet and percentage of Bcl-6 $^+$  cells.

Since inflammation, and more specifically IL-12, is required for CD8 effector and memory development (Xiao et al., 2009), we also tested whether high levels of exogenous IL-12 differentially affected the induction of T-bet and Eomes depending on TCR affinity. IL-12 increased the expression of T-bet for T cells responding to low and high-affinity TCR ligands. However, IL-12 did not enable Q4H7-stimulated cells to reach the T-bet levels induced by N4 (Figure 2-3C, 2-4). More importantly, IL-12 did not alter Eomes expression for either ligand (Figure 2-3D, 2-4).

Next, we compared Eomes and T-bet expression levels in T cells responding to high and low-affinity TCR ligands in the context of infection. At the peak of both immune responses, low-affinity cells exhibited a 6 fold greater frequency of cells expressing high levels of IL-7R and Eomes than their high-affinity counterparts (Figure 2-3E). Furthermore, on a per cell basis, low-affinity T cells expressed higher Eomes levels than high-affinity cells, confirming the *in vitro* results. We also observed a higher frequency of low-affinity T cells expressing high levels of Bcl-6, a transcription factor linked to memory development (Crotty et al., 2010; Ichii et al., 2002). Interestingly, T-bet expression *in vivo* was comparable between high and low-affinity cells (Figure 2-3F). Overall, these data show that, in the context of infection, low-affinity TCR ligands are able to induce T-bet levels comparable to high-affinity ligands. More importantly, they are uniquely capable of inducing very high levels of memory transcription factors Eomes and Bcl-6. This suggests that low-affinity TCR ligands induce a transcriptional program that favors memory development.

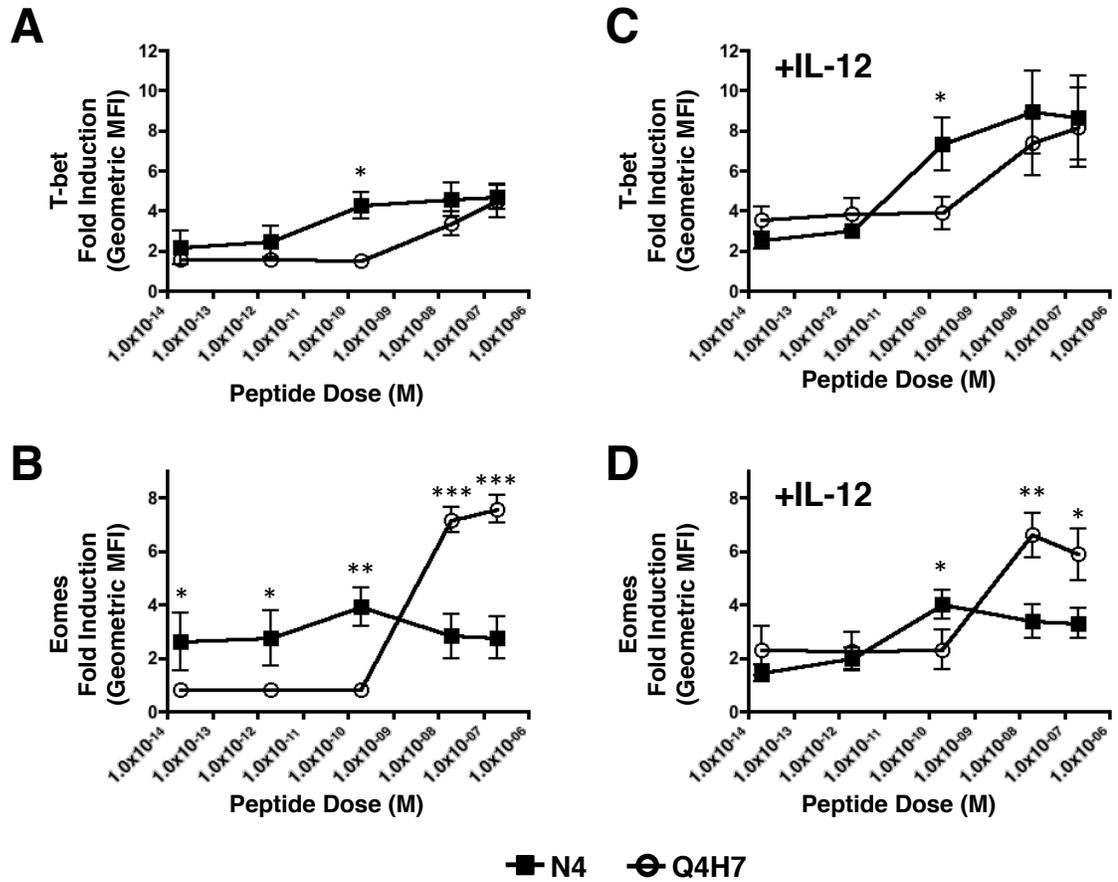


**Figure 2-4. Phenotype of T cells stimulated with different low-affinity TCR ligands. (A–F)** Eomes and T-bet expression kinetics (**A–D**) for a panel of negative selecting ligands (N4) and positive selecting ligands (Q4H7, Q7) for the OT-1 TCR. Naive OT-I splenocytes were stimulated with APCs pulsed with 20nM of the peptides indicated in the absence (**A, B**) or presence (**C–F**) of 2ng/ml IL-12. Expression of markers was determined by flow cytometry on OT-1 T cells. Geometric MFIs were normalized to a naive control. Graphs show mean±SEM or mean±SD and represent n≥5 independent experiments. (\*p < 0.05, \*\*p < 0.005, \*\*\*p < 0.001).

*TCR signals differentially regulate Eomes and T-bet expression.*

It seemed counterintuitive that weak TCR signals could induce T-bet and Eomes equal to or better than strong cognate antigen. Since antigen dose and/or inflammation shape a T cell response, we examined the ability of OT-1 T cells to induce T-bet and Eomes depending on antigen dose, in the absence or presence of excess exogenous IL-12. T-bet expression was directly proportional to TCR ligand affinity and antigen dose. While N4 was generally better at the induction of T-bet, there was no difference in T-bet expression between high and low-affinity ligands at the highest antigen doses (Figure 2-5A). On the other hand, there was not a direct correlation between antigen dose and TCR affinity for Eomes expression. At low antigen doses ( $\leq 10^{-10}$ M), Q4H7 induced lower levels of Eomes than the high-affinity ligand N4. For N4, increase in antigen dose led to more Eomes expression; however, at the highest doses ( $\geq 10^{-9}$  M), Eomes expression was repressed. Strikingly, at these highest doses ( $\geq 10^{-9}$  M), Q4H7 induced much higher Eomes expression than N4. Importantly, high-affinity ligands were unable to reach these levels of Eomes expression at extremely low antigen doses (Figure 2-5B). This cannot be explained by simple differences in TCR occupancy as it only takes 5 fold higher concentration of low-affinity ligands to reach the same level of occupancy of their high-affinity counterparts (Daniels et al., 2006). Taken together, these data suggest that the quality of the TCR signal has a greater affect on Eomes expression than the quantity.

Next, we evaluated the effect of IL-12 on the pattern of T-bet and Eomes expression. T-bet expression was increased in a linear fashion across all doses

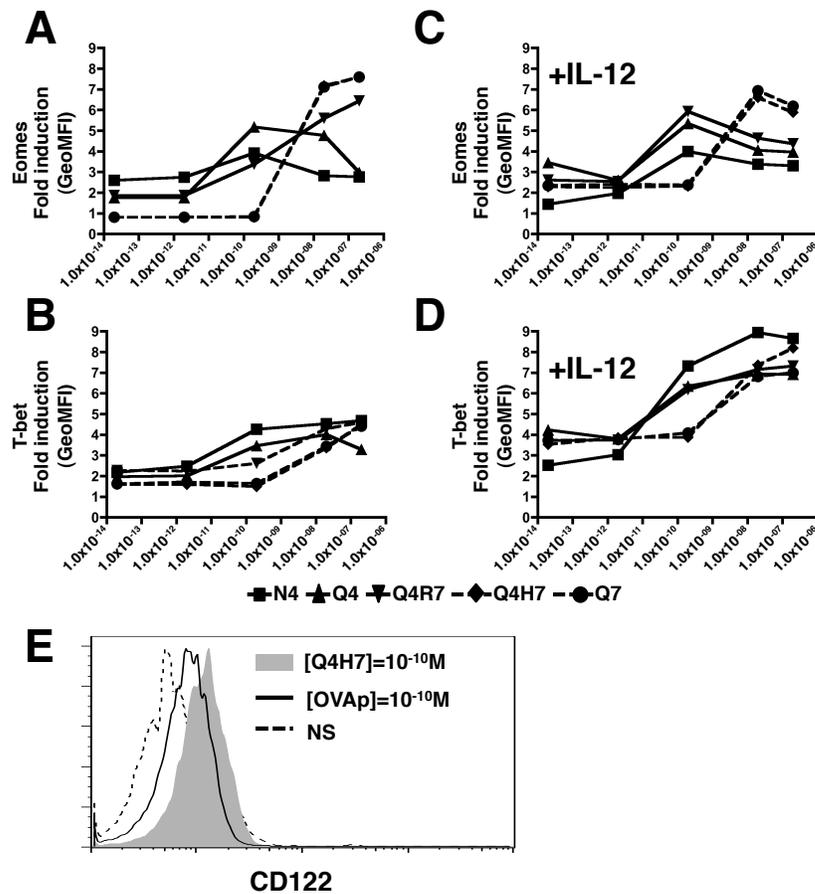


**Figure 2-5. TCR signal strength regulates T-bet and Eomes in distinct ways.** (A–D) Naive OT-I splenocytes were stimulated with APCs pulsed with varying concentrations of N4 or Q4H7 peptides in the absence (A, B) or presence (C,D) of 2ng/ml IL-12. Expression of T-bet (A, C) and Eomes (B, D) was determined by flow cytometry as in Figure 2-3. Graphs show mean±SEM and are representative of n≥4 independent experiments. \*p < 0.05, \*\*p < 0.005, \*\*\*p < 0.001.

for both TCR ligands with no change in sensitivity. Furthermore, similar to results in the absence of exogenous IL-12, low-affinity ligands only reached T-bet levels comparable to the high-affinity ligands at the highest antigen doses (Figure 2-5C). In contrast, we observed two unique patterns of Eomes expression in the presence of exogenous IL-12. At the lowest antigen doses, IL-12 decreased Eomes expression for N4, while it increased Eomes levels for the low-affinity ligand Q4H7. At high antigen doses, IL-12 did not have any significant effect on Eomes expression for either ligand. Yet, Eomes levels were much higher for low than high-affinity ligands (Figure 2-5D). Importantly, we found similar results with other high (negative selectors) and low-affinity (positive selectors) ligands, indicating that these results are not restricted to N4 and Q4H7 peptides (Figure 2-6). Of note, even at very low antigen doses ( $2 \times 10^{-10}$  M), low and high-affinity cells were similarly activated (Figure 2-6). Yet, Eomes and T-bet expression were significantly different between high and low-affinity cells, reinforcing the idea that both TCR affinity and antigen dose regulate Eomes and T-bet expression in distinct ways. Additionally, these results predict that the T-bet and Eomes expression profile observed in low-affinity cells in vivo (Figure 2-3) may correspond to T cells that have encountered high levels of low-affinity antigens.

#### *TCR signals regulate the susceptibility to IL-12.*

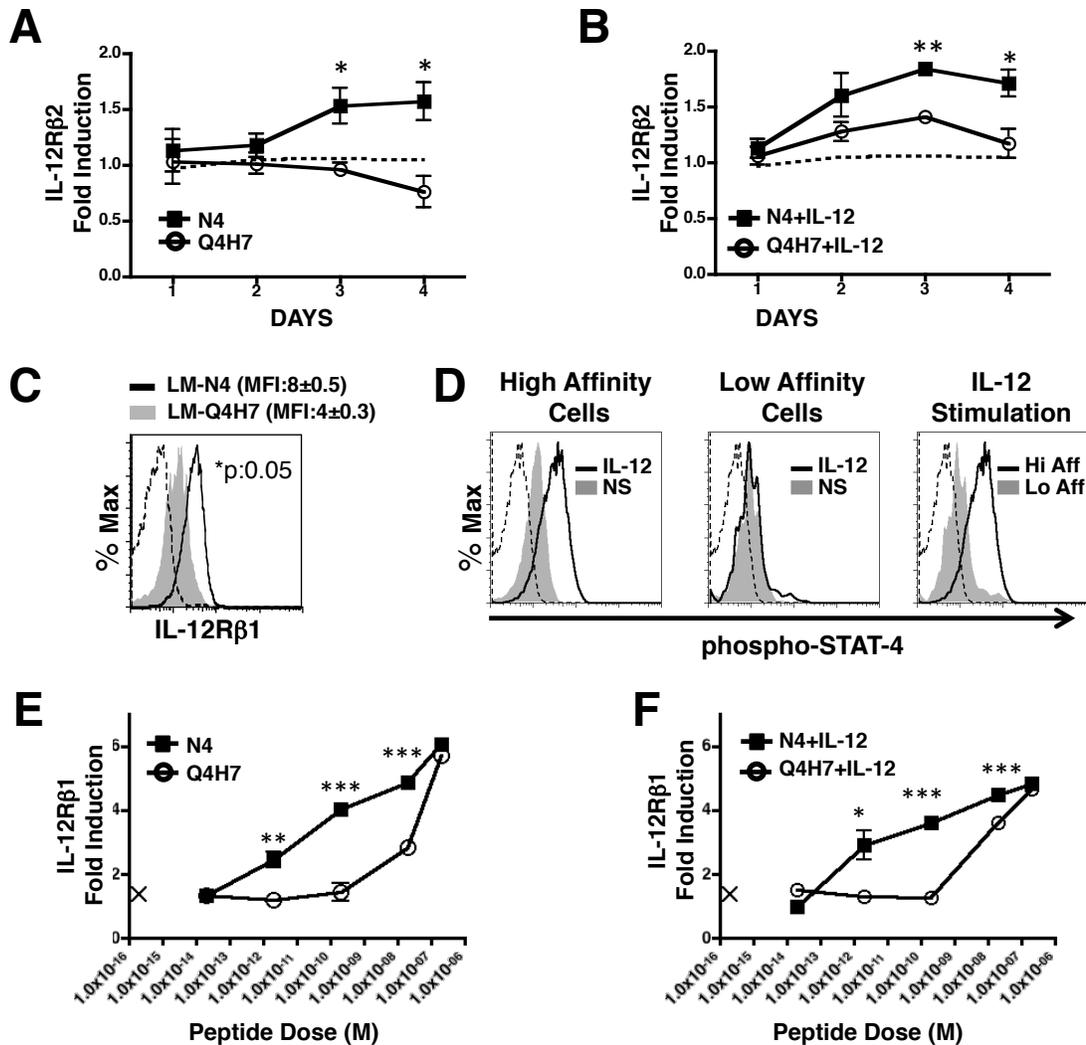
Given the effect of IL-12 on T-bet and Eomes was dependent on TCR affinity and antigen dose, we hypothesized that TCR signals regulate the ability of T cells to receive IL-12 signals. To test this, we evaluated whether TCR affinity



**Figure 2-6. Eomes and T-bet expression dose-response curves for high- and low-affinity T cells. (A–D)** *Eomes* and *T-bet* expression dose response curves for a panel of negative selecting ligands (N4, Q4, Q4R7) and positive selecting ligands (Q4H7, Q7) for the OT-1 TCR. Naive OT-1 splenocytes were stimulated with APCs pulsed with increasing concentrations of peptides shown in the absence (A, B) or presence (C, D) of 2ng/ml IL-12. *Eomes* (A, C) and *T-bet* (B, D) expression was determined as in Figure 2-3. Graphs show mean values for n≥4 independent experiments. Error bars are not shown for clarity. Data were analyzed using the multiple t tests platform (Holmes method correction for multiple comparisons, alpha = 5.000%), utilizing Prism software (GraphPad). The curves were analyzed by 2-way Anova and linear regression to determine the significance of the dose response curves. (E) *CD122* expression of high and low-affinity cells. Histogram shows *CD122* expression of OT-1 cells stimulated with 2x10<sup>-10</sup> M-peptide pulsed APCs at day 1.

and antigen dose could regulate the expression of IL-12R. Naïve OT-1 T cells were stimulated with 20nM N4 or Q4H7 peptide-pulsed APCs in the presence or absence of exogenous IL-12. In the absence of exogenous IL-12, N4 stimulation up-regulated IL-12R expression by day 3. In contrast, IL-12R expression was not induced upon stimulation with the low-affinity ligand Q4H7 (Figure 2-7A). The addition of exogenous IL-12 increased the expression of IL-12R for both TCR ligands over naive levels by day 2 (Figure 2-7B). Yet, IL-12R expression induced by the low-affinity ligand Q4H7 did not reach the levels induced by the high-affinity TCR ligand N4. This was also observed upon infection (Figure 2-7C). Importantly, low IL-12R levels led to low STAT-4 phosphorylation upon IL-12 stimulation of low-affinity T cells (Figure 2-7D). Since antigen dose had a profound effect on T-bet and Eomes expression for low-affinity ligands (Figure 2-5), we next tested whether this also applied to IL-12R expression. The low-affinity TCR ligand Q4H7 was only able to induce levels of IL-12R comparable to the high-affinity ligand N4 at the highest antigen doses in both the presence and absence of exogenous IL-12 (Figure 2-7E,F). Collectively, these data clearly show that TCR signal strength (affinity and antigen dose) regulates the ability of T cells to respond to IL-12. Since IL-12 regulates T-bet and Eomes expression, this suggests that TCR signals regulate the input of IL-12 signals to instruct effector/memory programming, and thereby, T cell fate.

*TCR signals regulate the susceptibility to TGF $\beta$  signals.*

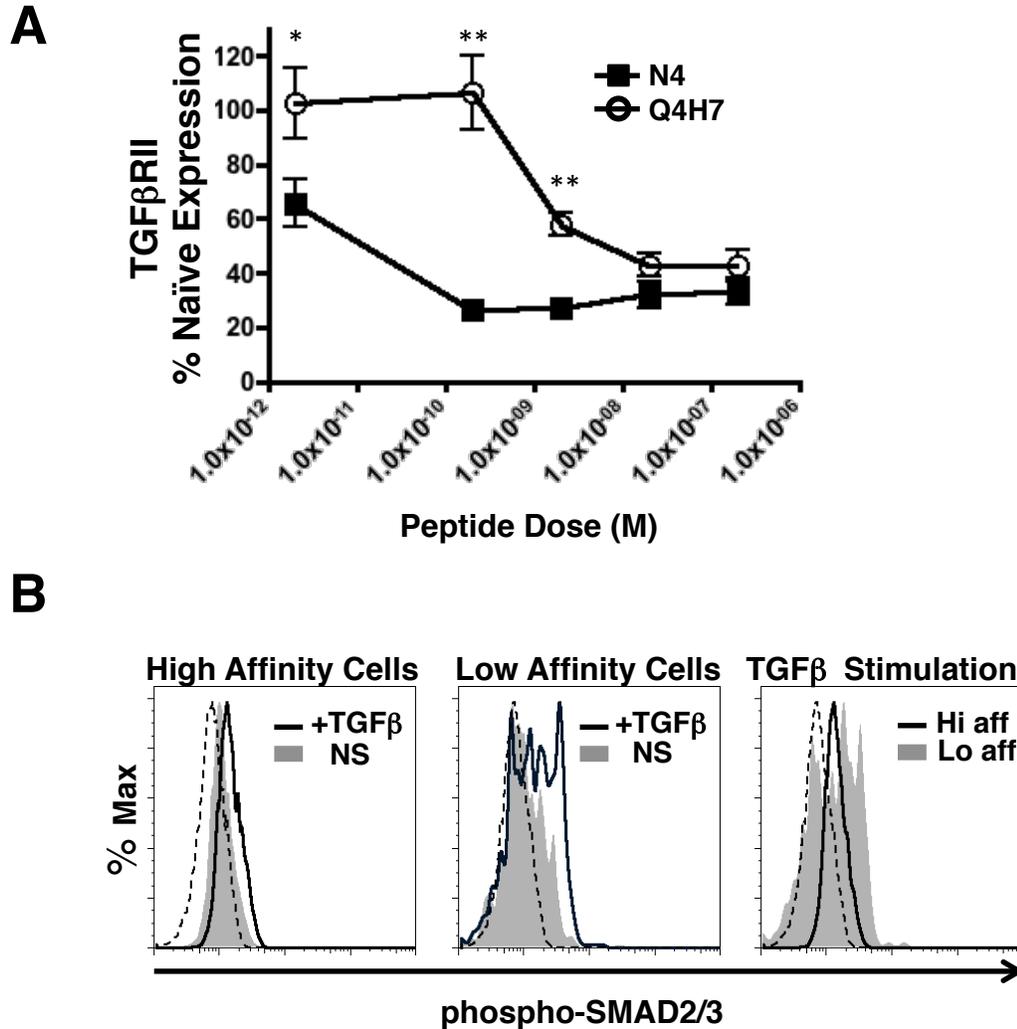


**Figure 2-7. TCR affinity regulates IL-12R expression and signaling.** (A and B) Naive OT-1 splenocytes were stimulated with 20nM N4 or Q4H7 peptide-pulsed APCs in the absence (A) or presence (B) of 2ng/ml IL-12. Expression of IL-12Rβ2 (A, B) was determined by flow cytometry. Naive control (dashed line). (C) IL-12Rβ1 expression on donor OT-1 cells challenged as in Figure 2-1 at day 5 of the immune response, in blood. Representative of n=4. (D) Phosphorylation of STAT-4 was determined on high- and low-affinity cells upon 30 min of 2ng/ml IL-12 stimulation. Isotype control (dashed line). Histograms representative of 2 independent experiments. (E and F) Naive OT-1 splenocytes were stimulated as in Figure 2-5 and IL-12Rβ1 expression was determined as in (A). X denotes IL-12Rβ1 expression in naive T cells. All graphs show mean±SEM and are representative of n≥4 independent experiments (\*p < 0.05, \*\*p < 0.005, \*\*\*p < 0.005). Data generated in collaboration with Nicholas P. Goplen.

TGF $\beta$  receptor signals negatively regulate the response of T cells against low-affinity TCR ligands (Johnson and Jameson, 2012; Zhang and Bevan, 2012a). Thus, we tested whether the susceptibility to TGF $\beta$  signals was dependent on TCR signal strength by measuring TGF $\beta$ RII expression in experiments analogous to Figure 4. Interestingly, the low-affinity TCR ligand Q4H7 only induced the down regulation of TGF $\beta$ RII to the levels of the high-affinity TCR ligand N4 at the two highest antigen doses (Figure 2-8A). Furthermore, the difference in TGF $\beta$ RII expression between high and low-affinity cells resulted in differences in TGF $\beta$ R signaling, as measured by phosphorylation of SMAD2/3 (Figure 2-8B). These data strongly suggest that TCR signal strength also dictates the susceptibility of T cells to TGF $\beta$  regulation.

*Low-affinity memory T cells are impaired in their effector function in response to low-affinity TCR ligands.*

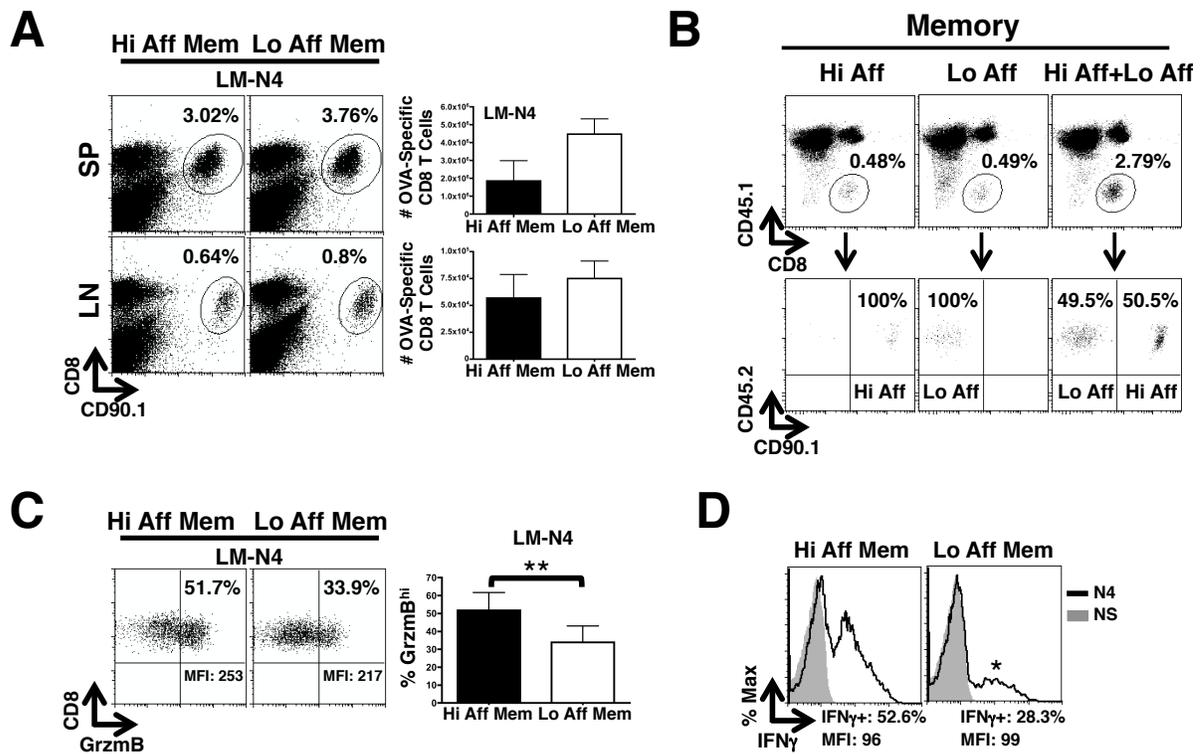
The previous findings indicate that OT-1 T cells responding to low-affinity TCR ligands in the presence of inflammation fully support effector and memory programs. This could explain why, in vivo, naïve T cells responding to low-affinity ligands are able to differentiate into effector and memory T cells (Zehn et al., 2009). However, memory T cells are expected to be higher in frequency and more sensitive to antigen than naïve T cells (Williams and Bevan, 2007). Thus, we wondered whether low-affinity memory T cells would be more reactive to very low-affinity antigens (pseudo-self) and, hence, a higher risk for autoimmunity. For



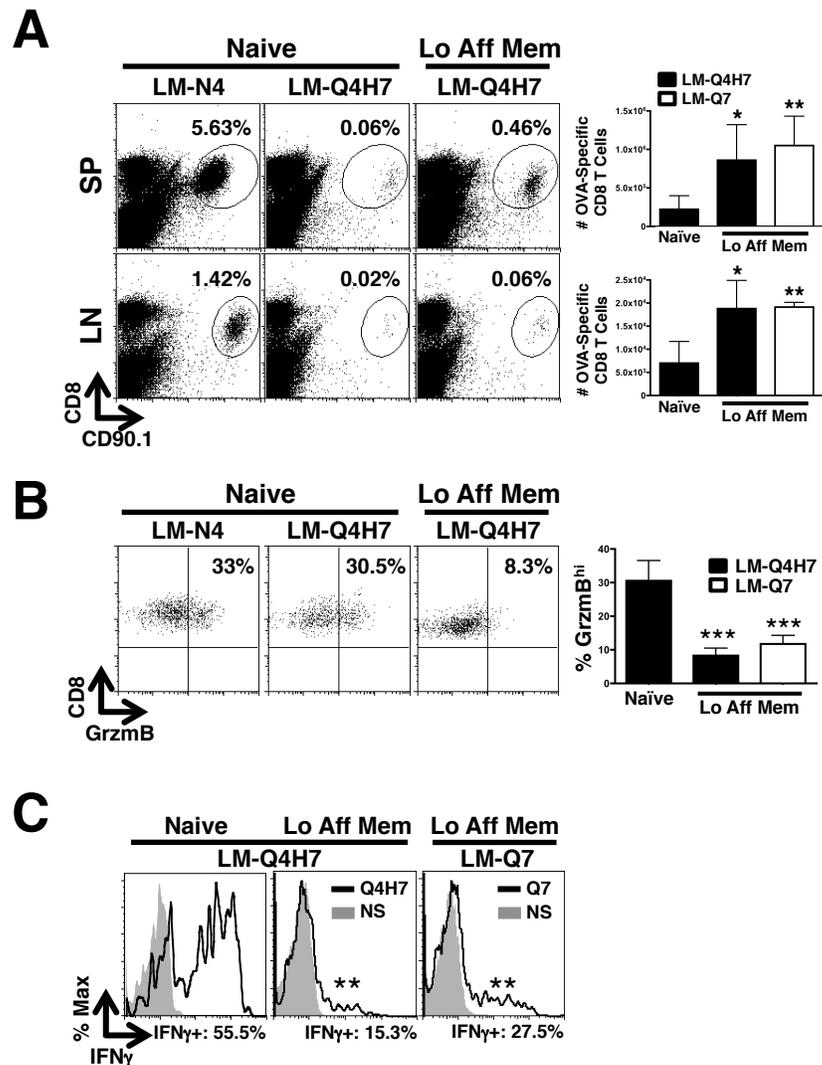
**Figure 2-8. TCR affinity regulates TGF $\beta$ RII expression and signaling. (A)** Naive OT-I splenocytes were stimulated as in Figure 2-5, in the presence of 2ng/ml IL-12 for 3 days. Expression of TGF $\beta$ RII was determined by flow cytometry. Graph shows percentage of downregulation relative to naive T cells (mean $\pm$ SEM, n=5 independent experiments) (\*p < 0.05, \*\*p < 0.005, \*\*\*p < 0.001). **(B)** Naive OT-I splenocytes were stimulated with 0.2nM N4 or Q4H7 peptide- pulsed APCs for 3 days and stimulated with 5ng/ml TGF $\beta$  for 30 min. Phosphorylation of SMAD2/3 was determined by flow cytometry. Isotype control (dashed line). Histograms representative of n=2 independent experiments. *Data generated in collaboration with Nicholas P. Goplen.*

this, we compared the response of naïve T cells and memory OT-1 T cells generated against LM-Q4H7 (low-affinity memory). Low-affinity memory cells (CD44<sup>hi</sup>, IL-7R<sup>hi</sup>) were isolated from recipient mice. Then, equal numbers of naïve or low-affinity memory cells were transferred into naïve congenic hosts and their responses were compared upon infection with either LM-Q4H7 (low-affinity ligand) or LM-N4 (high-affinity ligand). As a control, high-affinity OT-1 memory cells (generated in response to LM-N4) were also re-challenged with high or low-affinity ligands. In response to LM-N4, low-affinity memory cells expanded similarly to high-affinity memory cells (Figure 2-9A). Similar results were observed in competition experiments where equal numbers of high and low-affinity memory cells were transferred into the same congenic recipient followed by challenge with LM-N4 (Figure 2-9B). For memory effector function, we observed that low-affinity memory cells expressed Granzyme B and IFN $\gamma$  at levels comparable to their high-affinity counterparts. However, the percentage of low-affinity memory cells able to perform these effector functions was slightly but significantly lower (Figure 2-9C,D). This suggests that low-affinity memory T cells provide protection against high-affinity antigens although not to the same extent as high-affinity memory T cells.

Next, we evaluated the response of low-affinity memory T cells to low-affinity ligands (LM-Q4H7 and LM-Q7). Low-affinity memory cell re-expansion in response to LM-Q4H7 or -Q7 was substantially better than naïve T cells and similar to the re-expansion of high-affinity memory cells (Figure 2-10A and data not shown). Strikingly, we found that low-affinity memory T cells were impaired in



**Figure 2-9. Low-affinity memory T cells respond to high-affinity ligands.** OT-I CD8<sup>+</sup> T cells ( $2 \times 10^5$ ) were transferred into congenic B6 hosts and challenged with  $1 \times 10^4$  CFU LM-N4 or LM-Q4H7, and N4-primed (high-affinity memory [Hi Aff. Mem]) or Q4H7-primed (low-affinity memory [Lo Aff. Mem]) memory cells were isolated after  $\geq 30$  days. **(A, C, and D)** Hi Aff. Mem or Lo Aff. Mem cells ( $1 \times 10^4$ ) were transferred into naive hosts and rechallenged with  $1 \times 10^5$  CFU LM-N4. Frequency and total number of responding cells **(A)**, Granzyme B **(C)**, and IFN $\gamma$  expression **(D)** were determined in Hi and Lo Aff. Mem cells at day 6 postinfection (p.i.). Average MFI and percentage of positive cells for Granzyme B and IFN $\gamma$  are indicated. Graphs show mean $\pm$ SD. **(B)**  $1 \times 10^4$  Hi Aff Mem (CD45.2+CD90.1+), Lo Aff. Mem (CD45.2+CD90.1-) or a mixture of  $1 \times 10^4$  Hi Aff. Mem and  $1 \times 10^4$  Lo Aff. Mem cells were transferred into naive hosts and rechallenged with  $1 \times 10^5$  CFU LM-N4. Frequency of responding cells was determined in the blood 5 days p.i. Dot plots, histograms, and graphs are representative of  $n \geq 2$  experiments, with  $n = 3-6$  mice each. \* $p < 0.05$ , \*\* $p < 0.005$ , \*\*\* $p < 0.001$ .

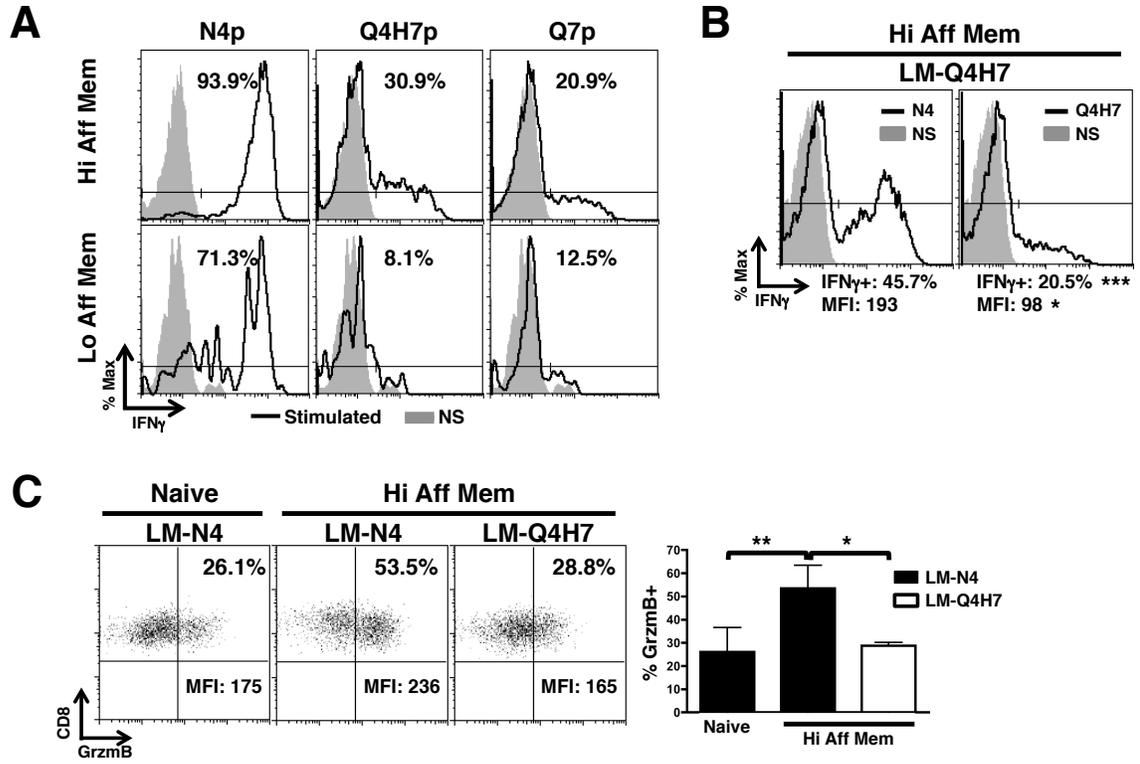


**Figure 2-10. Low-affinity memory T cells are impaired in their effector function against low-affinity ligands (pseudo-self).** (A–C) Lo Aff. memory cells were obtained as in Figure 2-9. Lo Aff. Mem or naive OT-I cells ( $1 \times 10^4$ ) were transferred into naive hosts and challenged with  $1 \times 10^5$  (memory) or  $1 \times 10^4$  (naive) CFU LM-N4, LM-Q4H7, or LM-Q7. Frequency and total number of responding cells (A), expression of Granzyme B (B), and IFN $\gamma$  (C) were determined by flow cytometry in naive or Lo Aff Mem cells at day 6 p.i. Values shown in dot plots are averages. Histograms and graphs show mean $\pm$ SD. Data are representative of  $n \geq 2$  experiments, with  $n = 3-6$  mice each. Significance shown (\* $p < 0.05$ , \*\* $p < 0.005$ , \*\*\* $p < 0.001$ ) denotes comparison versus naive OT-I challenged with LM-Q4H7.

the expression of Granzyme B, IFN $\gamma$ , T-bet and Eomes (Figure 2-10B,C and data not shown). These results are in marked contrast to the response of naïve T cells against LM-Q4H7, which is characterized by the expression of Granzyme B and IFN $\gamma$  at levels comparable to their high-affinity counterparts (Figure 2-10B,C and 2-11) (Zehn et al., 2009). These data suggest, unexpectedly, that low-affinity memory T cells are less efficient effectors than naïve T cells in response to low-affinity ligands. For high-affinity memory cells responding to low-affinity ligands, expression of IFN $\gamma$  was also lower while Granzyme B was similar to naïve cells (Figure 2-11). Collectively, these results suggest that while both high and low-affinity memory T cells undergo normal secondary expansion, they are impaired in their effector function against ligands in the range of positive selectors. However, they remain reactive (although not equally) to high-affinity ligands.

## **Discussion**

In the context of infection, low-affinity cells are able to acquire effector function and develop into memory. However, the mechanisms that enable such responses were unclear. We show that low and high-affinity T cells are unexpectedly different in their effector/memory differentiation. Low-affinity cells expressed higher levels of Eomes and lower to equal levels of T-bet compared to high-affinity cells. This results in low-affinity cells primarily developing into memory precursors that rapidly acquire a central memory phenotype. TCR signal strength (affinity and antigen dose) distinctively regulated the expression of these



**Figure 2-11. Effector function of high-affinity memory T cells in response to low-affinity TCR ligands. (A)** *IFN $\gamma$  expression of high and low-affinity memory cells challenged with low-affinity ligands ex vivo.*  $2 \times 10^5$  OT-I CD8 T cells were transferred into congenic B6 hosts and challenged with  $1 \times 10^4$  CFU LM-N4 or LM-Q4H7. After  $\geq 28$  days, N4-primed (Hi Aff Mem) or Q4H7-primed (Lo Aff Mem) memory cells were isolated and stimulated with 2nM N4, Q4H7, or Q7 peptide pulsed APCs. IFN $\gamma$  production was determined by flow cytometry. Histograms and corresponding values are representative of 2 independent experiments. **(B and C)** *High-affinity memory effector function against low-affinity ligands.*  $2 \times 10^5$  OT-I CD8 T cells were transferred into congenic B6 hosts, challenged with  $1 \times 10^4$  CFU LM-N4 and memory cells (Hi Aff Mem) were isolated after  $\geq 30$  days.  $1 \times 10^4$  Hi Aff Mem or naive OT-1 cells were transferred into naive hosts and challenged with  $1 \times 10^5$  (memory) or  $1 \times 10^4$  (naive) CFU LM-N4 or LM-Q4H7. **(B)** IFN $\gamma$  was determined by flow cytometry in Hi Aff Mem cells at day 6 post-infection (p.i.) upon 4 hr of stimulation with N4 or Q4H7 peptides. **(C)** Expression of Granzyme B was determined by flow cytometry. MFI for Granzyme B and IFN $\gamma$  are indicated in the figure. All graphs show mean $\pm$ SD. Dot plots, histograms and graphs are representative of n=2 experiments, with n=3-6 mice each. (\* p < 0.05, \*\*p < 0.005, \*\*\*p < 0.001).

transcription factors, in part through the control of IL-12R signals. Strikingly, and in contrast to naïve T cells, both high and low-affinity memory T cells are partially desensitized to low-affinity TCR ligands (in the range of positive selectors) in secondary responses.

Most of our knowledge about T cell memory is based on data generated in response to high-affinity ligands. From these studies, a Goldilocks model of antigenic signal strength was proposed, where ‘too little’ stimulation would lead to poor memory development and ‘too much’ would lead to CD8 memory exhaustion (Gourley et al., 2004). This model, however, cannot explain how, in lymphopenic conditions, naïve T cells develop into functional memory in response to self peptide (low-affinity) and low inflammatory signals. Furthermore, it cannot explain how, during infection, low-affinity TCR ligands can generate memory T cells (Zehn et al., 2009). The data presented here shows that, transcriptionally, very low-affinity TCR ligands have the full potential to support effector function and develop into memory (Cordaro et al., 2002; Turner et al., 2008; Zehn et al., 2009). More importantly, low-affinity TCR ligands favor memory by uniquely inducing high levels of Eomes and Bcl-6. How very weak TCR signals are inherently better at inducing Eomes is unclear. It may be related to a poor induction of T-bet. In fact, in T-bet deficient animals, IL-7R expression is higher, the number of memory precursors is increased, Eomes expression levels are higher and central memory differentiation is enhanced upon infection (Intlekofer et al., 2007). Consistent with this, low-affinity T cells express higher levels of IL-7R, exhibit a higher frequency of memory precursors that persist

throughout an immune response and generate a higher percentage of cells with a central memory phenotype (CD44<sup>hi</sup>, CD62L<sup>hi</sup>) than high-affinity cells. Low-affinity T cells are also poor inducers of T-bet in vitro and require high doses of the low-affinity ligand to reach maximum T-bet levels. Although we did not observe low expression of T-bet in low-affinity cells in vivo, it may be that this is a sequential process and a T-bet deficiency manifested earlier leads to a later overexpression of Eomes. Alternatively, IL-12 independent signals, such as IL-7 or CD27, may play a role in T-bet or Eomes induction respectively (Dong et al., 2012; Li et al., 2011a). Curiously, we have made similar observations in polyclonal responses where B6 mice were challenged with LM-OVA. T cells that exhibited low binding to Kb-OVA tetramer had an activated phenotype, were higher for Eomes but equal for T-bet expression. However, we cannot discard the fact that these T cells might have also cross-reacted with a *Listeria* epitope.

Pro-inflammatory signals have a profound impact on T cell differentiation (Agarwal et al., 2009; Li et al., 2006; Zhang and Bevan, 2012). Thus, high levels of inflammation are required to drive effector function, but they also favor terminal effector differentiation (Harty and Badovinac, 2008; Joshi et al., 2007; Mescher et al., 2006; Pipkin et al., 2010). This is thought to be in part because IL-12 enhances T-bet expression, which, in turn, can repress Eomes levels (Intlekofer et al., 2007; Takemoto et al., 2006). While this may be true for high-affinity ligands, our data provide an important insight into how Eomes expression is differentially regulated depending on affinity, antigen dose and the level of IL-12R. We find that, contrary to what has been described for high-affinity ligands

(Rao et al., 2010a), high IL-12 does not repress Eomes for low-affinity ligands and, actually, has the opposite effect at low antigen doses. Strikingly, at high antigen doses, IL-12 does not repress Eomes for either TCR ligand. This indicates that IL-12-dependent regulation of Eomes depends on TCR signal strength. This may be in part linked to the regulation of IL-12R signals by TCR signal strength, since low-affinity TCR ligands are poor inducers of IL-12R. Alternatively, it may be that Eomes expression is negatively regulated by very strong TCR signals (independently of IL-12) and low-affinity ligands, even at the highest antigen doses, are not able to trigger the mechanisms that induce this repression. Another possibility could be that low-affinity TCR ligands are inherently better inducers of a specific signaling pathway that drives Eomes expression. Notably, TCR affinity does not regulate T-bet expression in the same way as Eomes. T-bet levels increase proportionally to TCR affinity. The positive influence of IL-12 in T-bet expression equally applies to both high and low-affinity ligands. Collectively, these findings show novel evidence that TCR affinity can differentially regulate transcription factors (T-bet and Eomes) that are associated with different T cell fates. This also supports previous data indicating that the TCR signaling requirements for effector function and memory development are different (Smith-Garvin et al., 2010; Teixeira et al., 2009) and raises the intriguing idea that TCR signals could be manipulated to direct a T cell towards a specific fate.

It has been reported that inflammation regulates TCR sensitivity to antigen (Richer et al., 2013). Remarkably, our data demonstrate that TCR signal strength

also regulates the response of a T cell to inflammatory signals. Hence, since there is a mutual regulation and cooperation of both signals, neither inflammation nor antigenic signals can be considered alone to fully understand CD8 T cell differentiation.

For low-affinity T cells, negative regulation by TGF $\beta$  signals has an important impact in their immune response (Johnson and Jameson, 2012; Zhang and Bevan, 2012). We have observed that low-affinity T cells are unable to down-regulate TGF $\beta$ RII (except at the highest antigen doses), even in the presence of IL-12. TGF $\beta$ R signals have been shown to regulate CD8 short-lived effector cell survival but do not affect memory cell development (Sanjabi et al., 2009). This adds credence to the idea that low-affinity TCR signals favor memory differentiation, maybe at the expense of effector differentiation. The fact that TCR signal strength regulates both IL-12R and TGF $\beta$ R signals suggest that low-affinity T cells need to be tightly regulated (low for IL-12R signals and high for TGF $\beta$ R signals) to limit overt activation in the context of infection. This may explain in part why low-affinity T cells are able to differentiate to a certain extent but do not cause stable autoimmunity (King et al., 2012).

In the thymus, a sharp border of TCR affinity defines the threshold between positive selection of tolerant T cells and the deletion of auto-reactive T cells (Daniels et al., 2006a). In the periphery, these positively selected T cells are expected to react against their cognate antigen and remain tolerant to self. Because of this, it is striking that weak TCR signals from positive selecting ligands (low-affinity) can induce complete immune responses (Zehn et al., 2009).

Based on our data, we propose that the purpose of these responses is to maintain the diversity of the memory repertoire. This could be crucial to protect against viral escape variants and heterologous infections in secondary immune responses (van Gisbergen et al., 2011) or tumors. Intriguingly, low-affinity memory cells re-expanded in response to low-affinity challenge but in contrast to naïve T cells responding to low-affinity ligands in the range of positive selectors, they were impaired in their effector function (IFN $\gamma$  and Granzyme B expression). This may be explained in part by the fact that low-affinity memory cells exhibit higher levels of TGF $\beta$ RII than their high-affinity counterparts (O'Sullivan et al., 2011). Alternatively, this could be a consequence of their memory programming during the primary immune response. Interestingly, low-affinity memory cells were able to respond against high-affinity TCR ligands but not to the same extent as high-affinity memory cells. This suggests that low and high-affinity memory cell responses are not equal, an idea that is also inferred in studies comparing true memory T cells (generated against strong antigens) and HP-memory T cells (generated against self-antigens in lymphopenic conditions) (Cheung et al., 2009; Jellison et al., 2012).

Others have shown that monoclonal or polyclonal T cells with low functional avidity can escape negative selection and after, being primed and/or re-challenged with TCR ligands that are at or below the threshold of negative selection, can cause autoimmunity to tissue expressing self-antigen at or above this threshold (Enouz et al., 2012; von Herrath et al., 1994; Zehn and Bevan, 2006). Our data suggest that reactivity against ligands below the threshold of

positive selection could exist if certain biochemical criterion is met. Thus, low-affinity T cells were able to integrate TCR and IL-12R signals to support an effector program (high levels of T-bet and Eomes) similar to high-affinity ligands only at the highest doses of antigen in the presence of inflammation. Interestingly, low-affinity primed-OT-I memory T cells, which are in principle more reactive than naïve T cells, were impaired in their ability to express effector molecules IFN $\gamma$  and Granzyme B but not in their ability to proliferate upon re-challenge with positive selecting ligands Q4H7 and Q7. This suggests that T cells mount restricted secondary responses to ligands in the range of positive selectors. Is it possible that restriction could be overcome and lead to autoimmunity? Polyclonal T cells from RIP-OVA mice challenged or re-challenged with a pathogen that expresses OVA caused autoimmunity in 30% of the RIP-OVA mice (Enouz et al., 2012). Curiously, also in the RIP-OVA model, King et al. showed that only at high doses of the very low-affinity ligand (Q4H7), autoimmunity was transiently induced (King et al., 2012), again suggesting there may be a specific tolerance mechanism to keep these responses in check below the threshold of negative selection. The differences between these two systems highlight the importance of understanding the mechanisms that underlie breaks in self-tolerance and indicate that the equilibrium between reactivity to foreign and self is more complex than originally thought.

In summary, we show that high and low-affinity memory T cells are differentially programmed. Despite the fact that low-affinity T cells are recruited into the memory pool, they undergo normal secondary expansion, yet they are

impaired in their effector response to positive selecting ligands. Hence, we propose that the sharp TCR affinity threshold that distinguishes positive and negative selection is relevant for memory responses. This information has important implications for vaccine development, tumor immunotherapies and autoimmunity.

## Conclusion

In these studies, we examined the mechanism by which high and low-affinity pMHC-TCR signals program effector and memory generation. We utilized OT-I CD8 T cells for these experiments. The OT-I TCR transgenic system has a well-defined panel of altered peptide ligands. OT-I APLs contain substitution mutations in the amino acid sequence of SIINFEKL, the cognate antigen for the OT-I TCR. These amino acid substitutions impact the binding affinity of the corresponding pMHC to the TCR of the transgenic T cell. As previously described (Zehn et al., 2009), high-affinity pMHC-OT-I TCR interactions induce a massive expansion phase after TCR stimulation. The majority of these high-affinity-primed OT-I T cells die during contraction, leading to the establishment of a small but stable memory pool. In contrast, infection with low-affinity OT-I APLs surprisingly skewed a larger proportion of the T cell pool to develop a central memory phenotype and survive contraction.

The disparity seen between effector and memory generation in response to strong versus very weak pMHC-TCR signals correlated with the distinct induction of transcription factors important for T cell programming. Challenge with OT-I cognate antigen promoted the expression of effector-associated factor T-bet while low-affinity antigen induced the expression of memory-associated transcription factors Eomes and Bcl-6. Interestingly, T-bet and Eomes expression were differentially regulated by quantity and affinity of pMHC-TCR signals, respectively. In addition to affecting the programming of T cells, TCR affinity also

regulated the expression of receptors for the IL-12 and TGF $\beta$ . This result importantly indicates that the TCR signal controls the integration of other signals during immune responses.

How low-affinity-primed those memory cells responded to re-challenge depended on the affinity of the antigen in the secondary response. Low-affinity-primed memory T cells, similar to high-affinity memory T cells, expanded and produced effector molecules in response to cognate antigen re-challenge. In contrast, low-affinity memory T cells were unable to gain effector function in response to low-affinity antigen re-challenge. This is surprising since memory T cells have been shown to be more sensitive to antigen than naïve T cells (Croft et al., 1994; Mehlhop-Williams and Bevan, 2014; Pihlgren et al., 1996; Rogers et al., 2000; Slifka and Whitton, 2001; Zimmermann et al., 1999).

These studies demonstrate that the strength of the TCR signal alone regulates memory programming, differentiation, and response during infection. Furthermore, they suggest that memory generation and function are differentially regulated. While a broad range of pMHC-TCR affinity induce memory generation, how these memory T cells respond to re-challenge is programmed by the strength of the priming pMHC-TCR interaction. This regulation may allow for maintenance of a diverse repertoire of memory T cells while preventing aberrant responses against self.

## **Materials and Methods**

### *Mice and Reagents*

C57BL/6, B6.SJL, and OT-I and OT-1.PL (Thy1.1<sup>+</sup>) TCR transgenic mice were bred and maintained according to the University of Missouri OAR and ACUC. Peptides were purchased from New England Peptides. rmlL-12 was from Peprotech. IL-2 (X63-IL-2 hybridoma) were used at 50U/ml. TGF $\beta$  was from R&D Systems.

### *Bacteria*

Listeria strains were generously provided by M. Bevan (University of Washington). Listeria strains were grown to an OD<sub>600</sub> of 0.5 and were grown as described in (Zehn et al., 2009).

### *Adoptive Transfer and Infections*

Donor naïve ( $1 \times 10^3$ - $1 \times 10^4$ ) or memory OT-1 T cells were purified from the lymph nodes of OT-1 or host mice and transferred intravenously (i.v.) into congenic mice. All infections were performed i.v. at least one day after adoptive transfer of transgenic T cells.

### *Flow Cytometry and Antibodies*

Anti-CD8 $\alpha$  (53-6.7), CD90.1 (OX-7), IL-7R $\alpha$  (A7R34), CD62L (MEL-14), V $\beta$ 5 (MR9-4), IL-12R $\beta$ 1 (114), IL-12R $\beta$ 2 (HAM10B9), IFN $\gamma$  (XMG1.2) and phospho-

STAT-4 were from BD Pharmingen. Anti-CD45.1 (A20), CD45.2 (I04), CD27 (LG.7F9), Eomes (Dan11Mag) were from eBioscience. Anti-T-bet (4B10) and Bcl-6 (Clone 7D1) were from Santa Cruz Biotechnology and secondary antibodies and Granzyme B (GB12) were from Invitrogen. Anti-phospho-SMAD2/3 was from Cell Signaling. Flow cytometry was performed on a FACSCalibur flow cytometer (Becton Dickinson), and the data was analyzed with FlowJo FACS Analysis Software (Tree Star, Inc.). For determining induction of Eomes and T-bet, geometric mean fluorescent intensity was normalized to a naïve control. For determining induction of IL-12R $\beta$ 2 and IL-12R $\beta$ 1, geometric mean fluorescent intensity was normalized to an isotype control.

#### *Intracellular Staining*

Splenocytes were washed in FACS buffer (1% FCS, 0.02% sodium azide in PBS) and stained for CD8 and congenic markers (CD45.1 or CD45.2 or CD90.2 or CD90.1) to identify donor cells. Cells were washed in FACS buffer, then fixed and permeabilized using Cytofix/ Cytoperm or Phosflow (Perm. Buffer III) kit according to the manufacturer's instructions (BD Biosciences). Next, cells were stained with specific antibodies. For re-stimulation and determining cytokine production, splenocytes were incubated in 96-well round-bottom plates with 1  $\mu$ l/ml Golgistop (BD Biosciences) in the presence of 250nM OVA or Q4H7 or Q7 peptides for 4h at 37°C. Data was analyzed by flow cytometry.

### *Statistical Analysis*

For statistical analysis, unless otherwise stated in figures, two-tail unpaired Student's t test was applied using Prism software (GraphPad). Significance was set at  $p < 0.05$ . For T-bet and Eomes dose response curves (Figure 2-5, 2-6, and 2-7), data were analyzed using the multiple t-tests platform (Holmes method correction for multiple comparisons,  $\alpha=5.000\%$ ) utilizing Prism software (GraphPad).

### **Acknowledgements**

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**III: The signals for the generation of T cell memory are qualitatively different depending on TCR ligand strength.**

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S. E. Hamilton generated the data used in Figure 3-3.

## Rationale

The T cell receptor has the ability to signal differently depending on the strength of pMHC-TCR interaction (Rabinowitz et al., 1996; Sloan-Lancaster and Allen, 1996; Sloan-Lancaster et al., 1994). In the previous chapter, we showed that altering pMHC-TCR affinity, by utilizing high and low-affinity APLs for the OT-I TCR, affected effector and memory generation of CD8 T cells. In particular, low-affinity pMHC-TCR interactions skewed CD8 T cells toward memory during infection. These results demonstrated that low-affinity TCR signals distinctly promote memory CD8 T cell differentiation.

$\beta$ TMDmut T cells have been used to examine the role of TCR signals in memory CD8 T cell development (Teixeiro et al., 2009; Teixeira et al., 2004; Teixeira et al., 1999). Strikingly, mutating a single residue in the  $\beta$ TMD of the TCR prevents memory generation during infection (Teixeiro et al., 2009). This previous study demonstrated that different TCR signals promote effector and memory differentiation. All of this work, however, was performed during stimulation or infection with high-affinity antigen. Whether these same TCR signals regulate memory generation during weak pMHC-TCR interaction was not determined.

The following study was performed to further elucidate whether the rules to generate memory change depending on TCR signal strength. Specifically, we investigated whether altering TCR affinity affected  $\beta$ TMDmut T cell memory differentiation.  $\beta$ TMDmut T cells were activated with low-affinity antigens during infection and lymphopenia, conditions that favor memory development (Goldrath

et al., 2000; Goldrath et al., 2004; King et al., 2012; Knudson et al., 2013b).

Furthermore, we explored the impact of weak pMHC-TCR interactions on the induction of TCR-dependent NF- $\kappa$ B signaling, the specific signaling pathway impaired in MUT T cells (Teixeiro et al., 2009).

## **Abstract**

CD8 T cell memory critically contributes to long-term immunity. Both low and high-affinity TCR signals are able to support the differentiation of memory CD8 T cells. However, it is unclear whether the requirements for memory development change when TCR signal strength is altered. To gain further insight into this question, we used a TCR $\beta$  transmembrane domain mutant ( $\beta$ TMDmut) model that is defective in the generation of memory in response to high-affinity ligands. Surprisingly, lowering TCR signal strength, by stimulation with low-affinity ligands, resulted in normal memory development. Restoration of memory correlated with recovery of TCR-dependent NF- $\kappa$ B signaling. Thus, these data provide novel evidence that the requirements for memory are qualitatively different depending on TCR signal strength.

## **Introduction**

Memory CD8 T cells provide protective immunity against intracellular pathogens and tumors. It has been assumed that high-affinity TCR ligands are required for naïve CD8 T cells to acquire effector function and differentiate into long-lived memory T cells. However, there is compelling evidence that low-affinity TCR ligands support memory development (de Visser et al., 2000; Kieper and Jameson, 1999; Smith-Garvin et al., 2010; Zehn et al., 2010). In the context of infection, T cells responding to low-affinity TCR ligands are able to differentiate into functional memory T cells (Zehn et al., 2009). Furthermore, in lymphopenic conditions, T cells homeostatically proliferate (HP) and develop into memory cells

in response to self-peptide-MHC complexes (low-affinity), high levels of homeostatic cytokines and low levels of inflammation (Goldrath and Bevan, 1999; Hamilton et al., 2006; Surh and Sprent, 2008). These HP memory T cells are similar to antigen-experienced (true) memory cells at clearing pathogens upon infection and generating secondary memory (Cheung et al., 2009). Curiously, T cells differentiating into memory under lymphopenic conditions exhibit an expression profile that is comparable to antigen-experienced cells. However, it remains unclear whether the biochemical mechanisms required to induce the memory program are the same for T cells stimulated by high versus low-affinity TCR ligands.

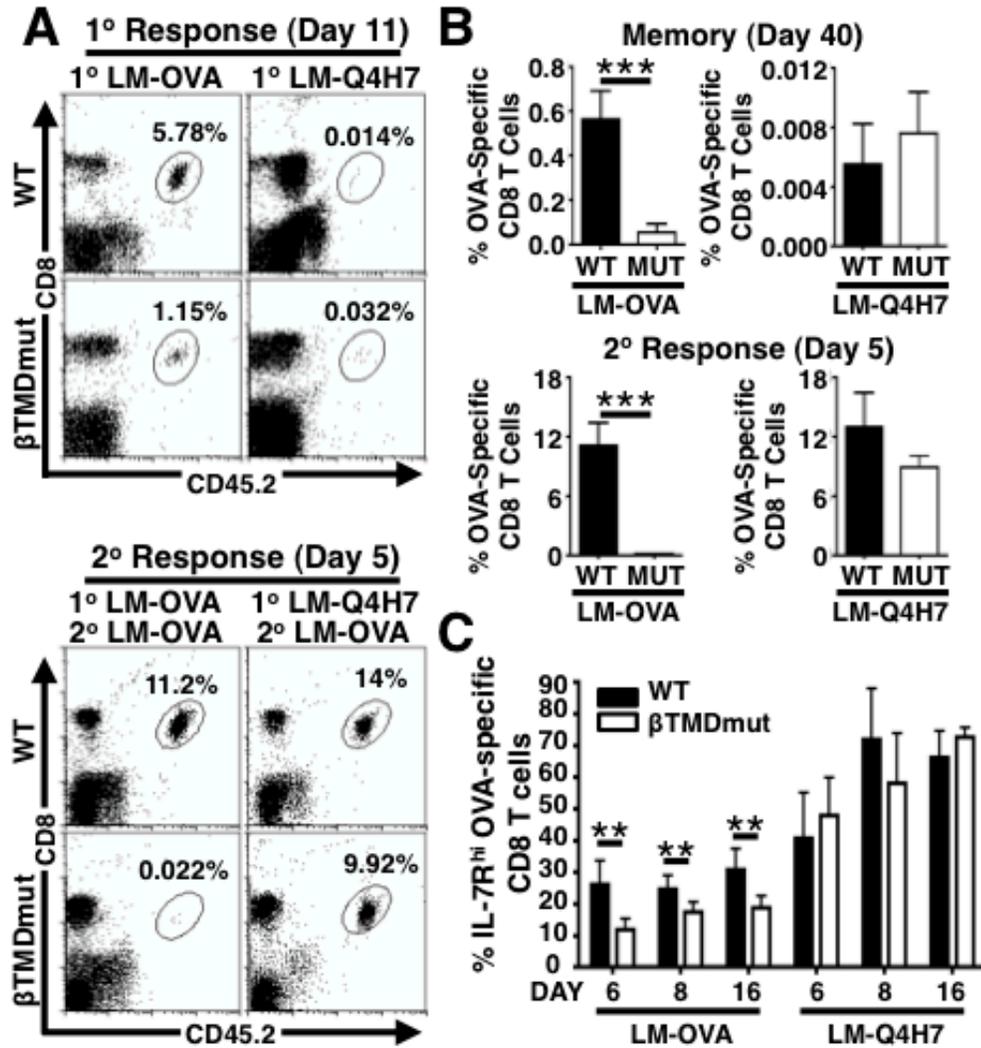
Indeed, the TCR is able to differentially signal depending on the ligand it recognizes (Corse et al., 2011; Daniels et al., 2006; Edwards and Evavold, 2011). In line with this, recent data suggest that the TCR can selectively regulate the transduction of signals that drive T cell memory (Leignadier and Labrecque, 2010; Smith-Garvin et al., 2010; Teixeira et al., 2009). We have previously shown that CD8 T cells bearing a point mutation in the TCR $\beta$  transmembrane domain ( $\beta$ TMDmut or MUT) acquire effector function but are uniquely defective in the development of memory in response to high-affinity TCR ligands (Teixeiro et al., 2009). Here we show unexpectedly that lowering TCR affinity leads to a recovery in memory programming in MUT T cells. This process was dependent on NF- $\kappa$ B signaling, which was efficiently triggered by low but not high-affinity TCR ligands in MUT CD8<sup>+</sup> T cells. These findings support the idea that depending on TCR

signal strength, there is differential assembly of the signaling machinery needed to generate memory.

## **Results**

### *Memory-defective T cells recover memory development in response to low-affinity TCR ligands.*

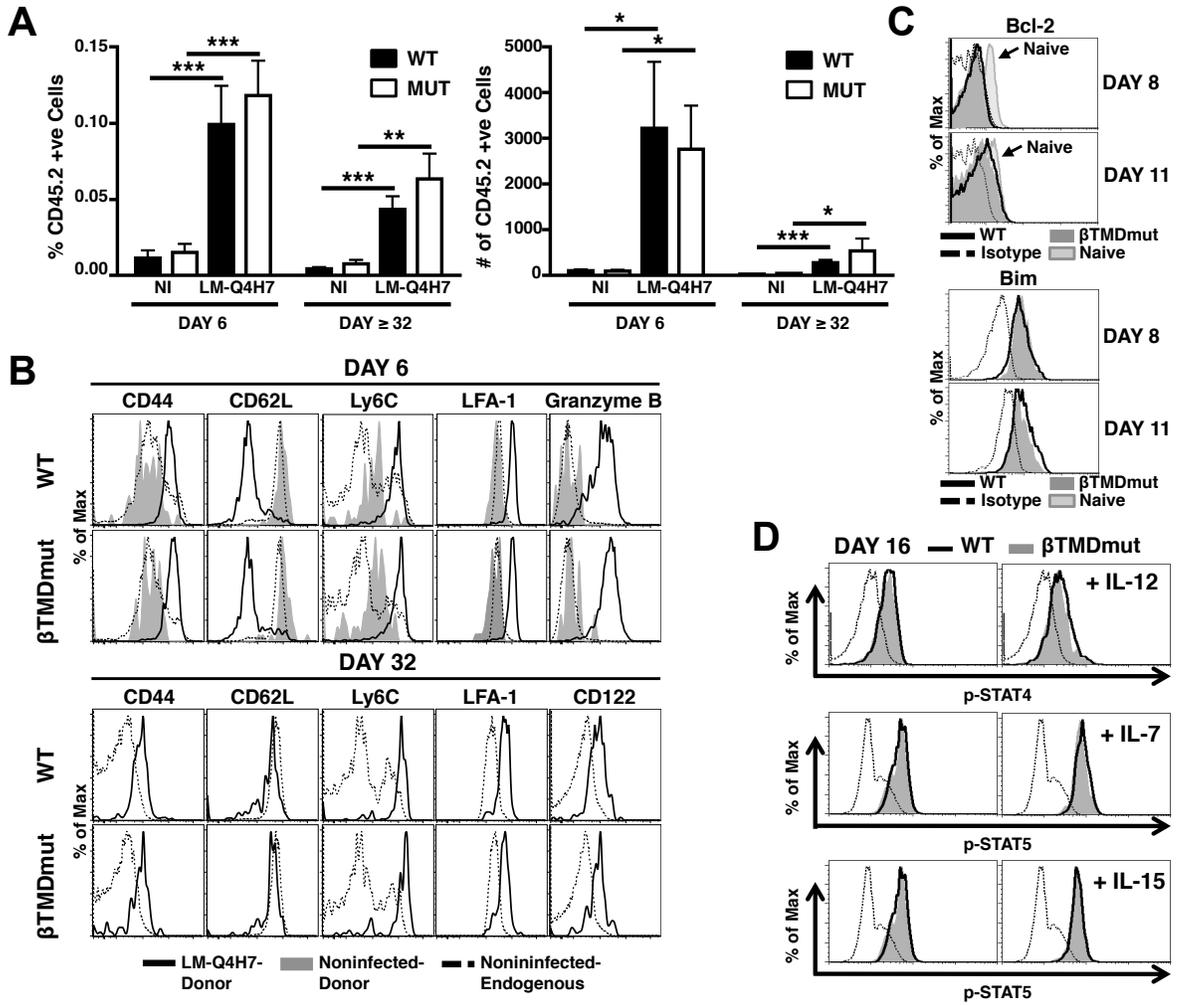
In previous studies, we reported that OT-1  $\beta$ TMDmut cells (MUT) are defective in memory development upon infection with *Listeria monocytogenes* expressing the high-affinity ligand OVA (LM-OVA) (Teixeiro et al., 2009). Interestingly, T cells responding to low-affinity ligands are able to support memory development in the context of infection (Zehn et al., 2009). To test whether the requirements for memory change depending on TCR signal strength, we determined whether MUT T cells differentiate into memory in response to low-affinity TCR ligands. For this, we chose a LM strain that expresses Q4H7, an OVA peptide variant that exhibits very low-affinity for the OT-1 TCR (Daniels et al., 2006) and induces memory differentiation of OT-I T cells (King et al., 2012; Zehn et al., 2009). Wild type (WT) and MUT CD8 T cells were adoptively transferred into congenic hosts and challenged with LM-OVA (strong) or LM-Q4H7 (weak). As expected, challenge with LM-OVA led to a marked decrease in the frequency of MUT CD8 T cells developing into memory and in the ability of MUT memory cells to respond to re-challenge (lower panels, Figure 3-1A,B)(Teixeiro et al., 2009). Surprisingly, in response to LM-Q4H7 infection, MUT T cells generated memory T cells at frequencies comparable to WT (Figure



**Figure 3-1. MUT memory defect is restored in response to low-affinity TCR ligands.** Naïve T cells were transferred into B6.CD45.1<sup>+</sup> hosts and challenged with  $1 \times 10^3$  CFU LM-OVA or LM-Q4H7. **(A)** Representative plots show frequencies of OVA-specific CD8 T cells in blood at day 11 of 1° response (upper panel) and day 5 of 2° response (lower panel). **(B)** Graphs show frequencies of OVA-specific cells in blood at day 40 of 1° response (upper panel) and day 5 of 2° response (lower panel) (mean±SD) in experiments analogous to (A). **(C)** Graph shows frequencies of IL-7R<sup>hi</sup> cells in blood determined by flow cytometry (mean±SD). Data is representative  $n \geq 3$  independent experiments;  $n \geq 3$  mice per group. \* $p \leq 0.05$ , \*\* $p \leq 0.005$ , \*\*\* $p \leq 0.001$ .

3-1B, upper panel). We confirmed this using magnetic bead enrichment procedures (Figure 3-2A) (Akue et al., 2012). WT and MUT cells reached similar frequencies and numbers at the peak (day 6) and memory points of the LM-Q4H7 immune response. The frequencies and number of T cells in the LM-Q4H7 response were substantially higher than in uninfected control mice (transferred in parallel with equal number of naïve WT and MUT donor T cells). Furthermore, in response to LM-Q4H7, both WT and MUT displayed an effector (day 6) and memory (day 32) phenotype clearly distinct from matched non-infected controls where donor cells remained naïve (Figure 3-2B). Importantly, MUT memory T cells primed with the low-affinity TCR ligand underwent robust secondary responses (to LM-OVA), similar to WT cells. This is in stark contrast to MUT cells primed with the high-affinity ligand OVA (lower panels Figure 3-1A,B), where MUT memory generation is defective. Hence, these data indicate that MUT cells are unexpectedly able to generate functional memory T cells in response to low-affinity TCR ligands.

Next, we measured the frequency of T cells differentiating into IL-7R<sup>hi</sup> memory precursors. MUT cells exhibited a significantly lower frequency of IL-7R<sup>hi</sup> memory precursors during the immune response to LM-OVA. In contrast, weak (low-affinity) TCR signals overcame this MUT defect, since frequencies of MUT IL-7R<sup>hi</sup> memory precursors were restored to WT levels in response to LM-Q4H7 (Figure 3-1C). These data suggest that mutant T cells are defective in memory programming in response to strong TCR ligands but recover the ability to program memory in response to weak TCR signals.

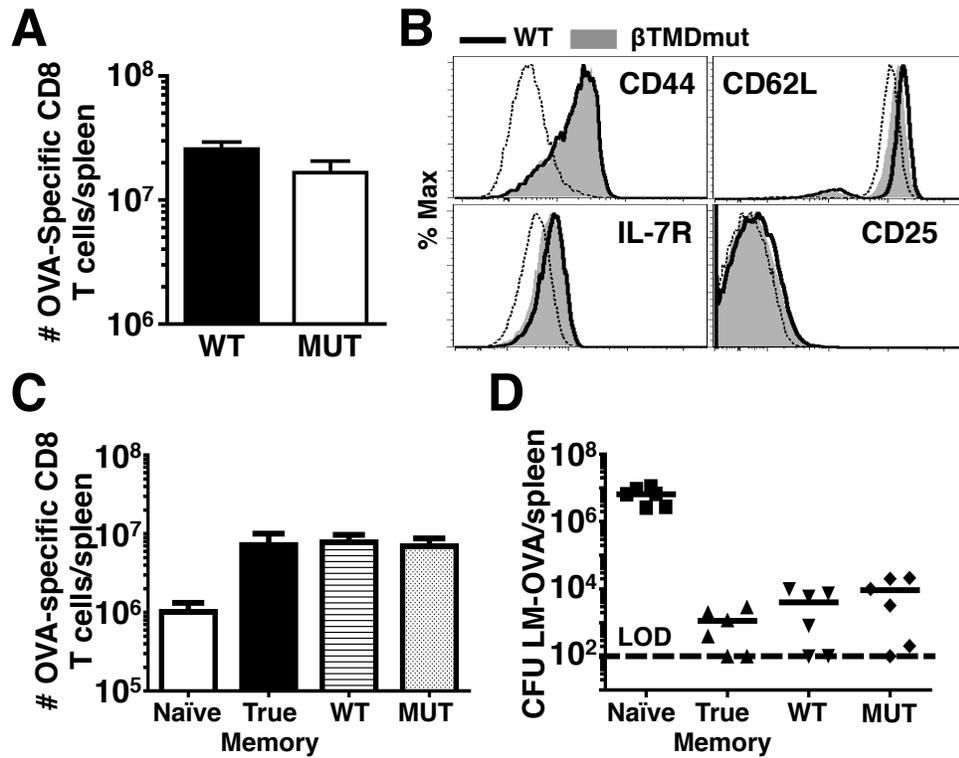


**Figure 3-2. Comparing WT and MUT cells upon infection with LM-OVA and LM-Q4H7. A and B. WT and MUT cells become activated and generate memory T cells in response to LM-Q4H7.**  $1 \times 10^3$  WT or MUT donor CD45.2+CD8 T cells were transferred into CD45.1+ hosts and challenged with  $1 \times 10^3$  CFU LM-Q4H7 or PBS (noninfected-NI). At day 6 or day  $\geq 32$ , CD45.2+ donor cells were subjected to magnetic bead enrichment and frequency, numbers (A) and phenotype (B) from the bound fraction were determined by flow cytometry. Due to lack of detectable non-infected donor cells, endogenous CD8+ T cells from non-infected mice were used as controls at day  $\geq 32$ . Values in (A) are shown as mean  $\pm$  SEM (\* $p < 0.05$ , \*\*\* $p < 0.001$ ). Graphs and histograms represent 3 independent experiments,  $n = 3$  mice each. *Data obtained in collaboration with Dr. Sara E. Hamilton.* **C. WT and MUT cells exhibit similar expression of anti- and pro-apoptotic factors in the contraction phase of the LM-OVA immune response.** Mice were challenged as in Fig. 2-1. Bcl-2 and Bim expression of WT and MUT cells in the blood at day 8 and 11. **D. MUT cells are not defective in IL-12R, IL-7R or IL-15R signaling.** Mice were challenged as in Fig. 2-1. Phosphorylation of STAT-4 and -5 determined by intracellular staining at day 16 of the LM-OVA response and upon stimulation with the cytokines shown. Dashed line represents isotype control. Histograms representative of  $n \geq 3$  experiments,  $n = 3-5$  mice each.

The inability of MUT cells to differentiate into memory against strong TCR ligands could not be attributed to altered apoptosis since WT and MUT exhibited similar levels of the regulators of contraction Bim and Bcl-2 (Figure 3-2C) (Kurtulus et al., 2010). In addition, we did not find defects in IL-12R, IL-7R and IL-15R signaling in MUT cells as measured by levels of phosphorylation of STAT-4 (as a readout for IL-12R signaling) and STAT-5 (as a readout of IL-7R and IL-15R signaling) (Figure 3-2C). Thus, these data demonstrate that increased apoptosis or impaired cytokine signal input cannot account for the defective mutant memory phenotype. Rather, MUT cells are defective in the TCR signals that regulate memory programming in response to high-affinity TCR ligands. Overall, lowering TCR signal strength leads to a recovery in mutant memory differentiation. These findings unexpectedly reveal that the signals necessary for programming memory are qualitatively different depending on TCR signal strength.

*Memory-defective T cells recover memory development in lymphopenic conditions.*

In lymphopenic conditions, T cell memory development also occurs in response to low-affinity TCR ligands but in the context of IL-7, IL-15 and low levels of inflammation (Goldrath and Bevan, 1999; Hamilton and Jameson, 2008). To determine whether MUT cells were able to differentiate into HP-memory, we adoptively transferred wild type (WT) or MUT OT-1 CD8 T cells into sublethally-irradiated B6 hosts. MUT T cells underwent normal homeostatic

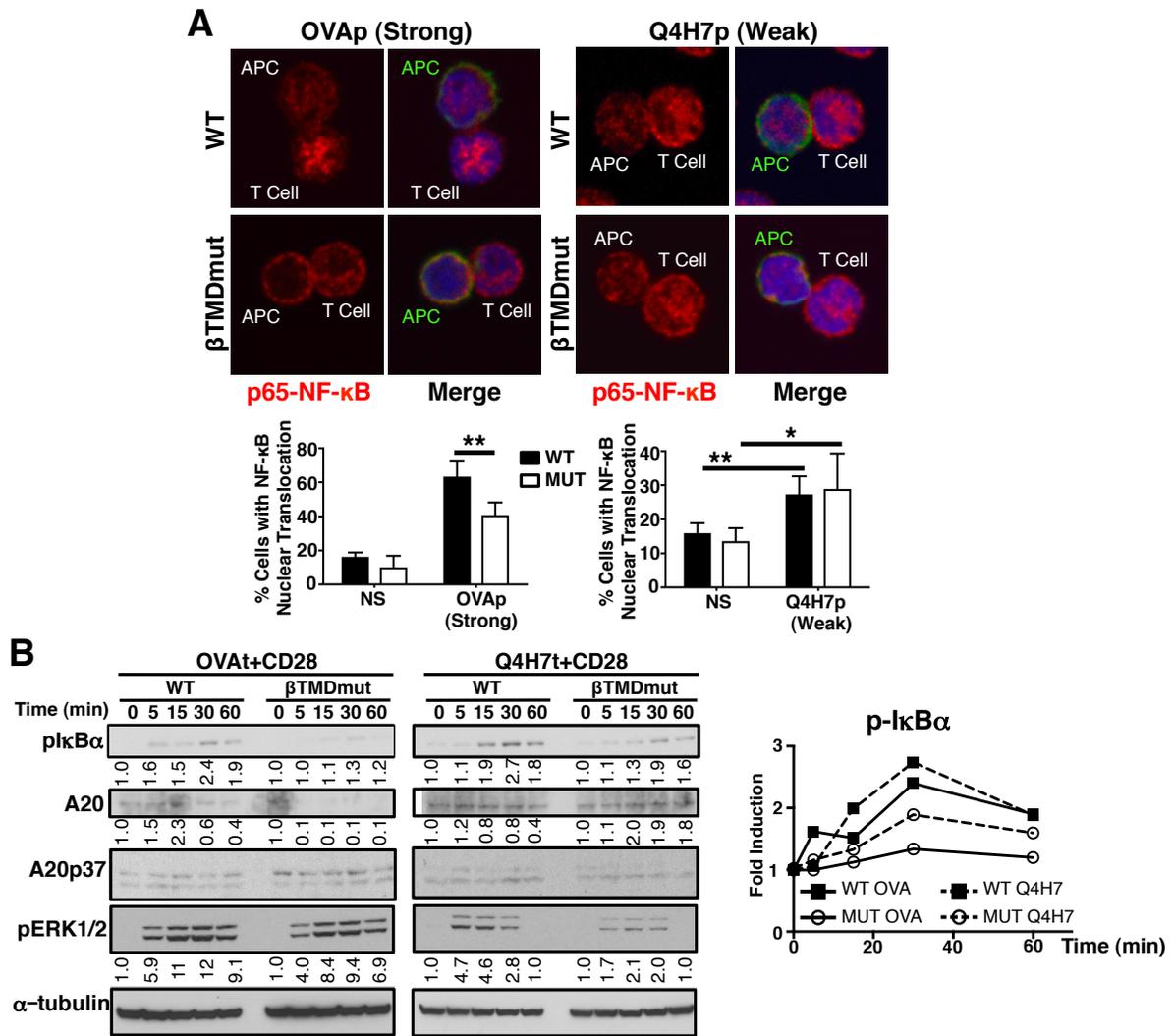


**Figure 3-3. Memory-deficient MUT T cells recover memory development in response to lymphopenia.**  $1-3 \times 10^6$  naïve CD45.2<sup>+</sup> T cells were transferred into sublethally-irradiated hosts and allowed to differentiate into HP-memory. **(A)** Representative graphs show total cell number at day 28 (mean  $\pm$  SD). **(B)** Memory phenotype determined at day 28. Dashed line = naïve control. **(C)**  $3 \times 10^5$  HP-memory cells from (A) were transferred to B6.CD45.1<sup>+</sup> and challenged with  $1 \times 10^5$  CFU LM-OVA. Graphs show total cell numbers (means  $\pm$  SD) **(C)** and LM-OVA titers **(D)** at day 5 p.i. Data is representative of  $n \geq 3$  independent experiments;  $n \geq 3$  mice per group. *Data generated by Dr. Sara E. Hamilton.*

proliferation and acquired a memory-like phenotype, similar to the results obtained in the LM-Q4H7 response (Figure 3-3A,B). To test HP-memory cell function, equal numbers of WT and MUT HP-memory cells were adoptively transferred into naïve congenic hosts and challenged with LM-OVA. Importantly, MUT HP-memory T cells were able to expand and clear LM-OVA in the spleen comparable to WT and the true-memory control (Figure 3-3C,D). Thus, MUT T cells also recovered memory development in response to lymphopenia. These data further support that memory development is differentially regulated depending on TCR signal strength, although it is possible that high doses of homeostatic cytokines also contribute to the TCR-dependent recovery of memory in these conditions.

#### *MUT T cells recover NF- $\kappa$ B signaling in response to low-affinity TCR signals*

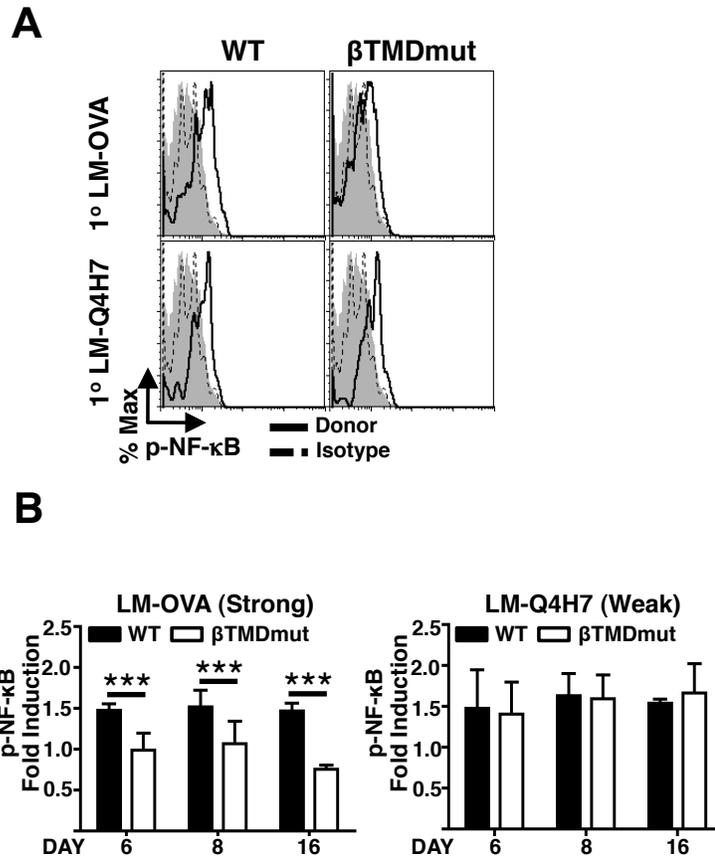
Next, we investigated the mechanism responsible for the recovery of MUT memory with weak TCR ligands (low-affinity). MUT  $\beta$ TMD TCRs are impaired in transducing NF- $\kappa$ B signals in response to strong TCR ligands (Teixeiro et al., 2009; Teixeira et al., 2009; Teixeira et al., 2004; Teixeira et al., 1999). Thus, we tested whether the MUT TCR could activate NF- $\kappa$ B signaling in response to low-affinity ligands. As previously described (Teixeiro et al., 2009), upon OVA stimulation *in vitro*, MUT cells were defective in translocating the p65-NF- $\kappa$ B subunit to the nucleus (Figure 3-4A). This correlated with a defect in the induction of phosphorylated I $\kappa$ B $\alpha$  (p-I $\kappa$ B $\alpha$ ), an upstream regulator of NF- $\kappa$ B signals (Teixeiro et al., 2009; Teixeira et al., 2009; Vallabhapurapu and Karin, 2009)



**Figure 3-4. MUT T cells recover NF-κB signaling in response to low-affinity ligands.**

(A) Nuclear translocation of p65-NF-κB determined by confocal microscopy. CD45.1 (green), NF-κB (red), nucleus (blue). Images are representative of  $n \geq 50$  conjugates from  $n \geq 3$  independent experiments. Graphs show the mean  $\% \pm$  SD of conjugated T cells that exhibit p65 in the nucleus over unstimulated cell levels. (B) Phosphorylation of IκBα, ERK1/2 and expression of A20 in response to high and low-affinity TCR ligands was determined in WT or MUT T cells stimulated with K<sup>b</sup>-OVA or -Q4H7 tetramers and anti-CD28 antibodies by immunoblot. All immunoblots correspond to the same representative experiment. A representative experiment of  $n=4$  is shown. Graph and numbers in the blot show relative induction of p-IκBα, A20, p-ERK1/2 levels over unstimulated (time 0), normalized to α-tubulin (loading control).

(Figure 3B). Remarkably, in response to the low-affinity ligand Q4H7, a similar percentage of WT and MUT cells translocated NF- $\kappa$ B to the nucleus although the percentage observed was lower than upon OVA stimulation (Figure 3-4A). Interestingly, WT and MUT cells stimulated with Q4H7 exhibited induction of p-I $\kappa$ B $\alpha$  levels similar to WT OVA stimulation (Figure 3-4B). Together with the confocal data, this indicates that, on a per cell basis, the NF- $\kappa$ B signal input is higher upon Q4H7 than OVA stimulation. Furthermore, MUT cells were impaired in keeping the balance between degradation and expression of the NF- $\kappa$ B target A20 upon OVA but not Q4H7 stimulation (Krikos et al., 1992) (Figure 3-4B). A20 is also a negative regulator of NF- $\kappa$ B signal and we observed A20 degradation (A20p37 cleaved form)(Duwel et al., 2009). This suggests that the MUT defect occurs upstream of A20. Indeed, we have previously described that MUT cells are defective in the translocation of protein kinase C  $\theta$  (PKC $\theta$ ) to the membrane, an event that is required to assemble the I $\kappa$ B kinase complex (IKK $\epsilon$ ) with the CARMA1/Bcl10/Malt1(CBM) signalosome (Paul and Schaefer, 2013; Teixeira et al., 2009). Of note, there was not an overall enhancement in other TCR signaling pathways in response to Q4H7, as indicated by the levels of ERK phosphorylation (Figure 3-4B). Finally, we determined whether the early NF- $\kappa$ B signaling defect observed in MUT cells *in vitro* would occur and persist *in vivo* in the context of infection. We monitored NF- $\kappa$ B signaling *in vivo* by measuring the IKK complex-mediated phosphorylation of the NF- $\kappa$ B subunit p65 (p-NF- $\kappa$ B) (Hayden and Ghosh, 2008). Notably, MUT T cells were impaired in the induction of p-NF- $\kappa$ B in the immune response to LM-OVA. Conversely, MUT cells



**Figure 3-5. MUT cells are defective in the induction of NF-κB signals in response to high-affinity but not low-affinity TCR ligands upon infection. A.** WT or βTMDmut donor CD8 T cells were adoptively transferred into congenic B6 hosts and challenged with  $1 \times 10^3$  CFU LM-OVA or LM-Q4H7. Histograms show phosphorylation of p65 NF-κB (Ser 536) in antigen-specific T cells at the peak of the responses by flow cytometry. Solid grey histogram represents the naïve control. Histograms shown are representative of  $n=4$  experiments,  $n=3-6$  mice each. **B.** Graphs show induction of p- NF-κB over naïve levels (mean  $\pm$  SD) at different days of the LM-OVA and LM-Q4H7 responses. Data is representative of  $n \geq 3$  independent experiments;  $n \geq 3$  mice per group. \*\*\* $p \leq 0.001$ .

recovered p-NF- $\kappa$ B levels *in vivo* when challenged with LM-Q4H7 (Figure 3-5A,B). Taken together, these data show that TCR-dependent NF- $\kappa$ B signaling can be differentially regulated depending on TCR signal strength.

## **Discussion**

Our previous data indicated that the  $\beta$ TMD has a role in the assembly of the PKC $\theta$ -CARMA1/Bcl10/Malt1(CBM) complex in the membrane (Teixeiro et al., 2009a; Teixeira et al., 2004a). It has been proposed that digitization of NF- $\kappa$ B signaling occurs upstream or at the level of the TCR such that only strong TCR ligands will generate efficient signal to induce gene expression (Kingeter et al., 2010). Instead of this, the data presented here suggest that even weak TCR signals can produce efficient NF- $\kappa$ B signaling that persists during the immune response. It is possible that the  $\beta$ TMD plays a role in integrating the antigenic signal input to determine which cells acquire productive NF- $\kappa$ B signaling required to generate memory. Alternatively, other TCR-dependent downstream mechanisms that control NF- $\kappa$ B induction or act together with NF- $\kappa$ B signals to drive memory programming may be differentially regulated by high and low-affinity TCR ligands. It is still unclear how the TCR regulates early signaling events upstream of the PKC $\theta$ -CBM complex. Thus, defining whether the mechanisms that differentially regulate memory through the TCR lie upstream or downstream of PKC $\theta$ -CBM is an exciting area that needs further investigation.

Several studies have explored the impact of TCR signal strength on T cell memory development. It has been shown that changing the dose of high-affinity

ligands influences T cell memory (Corse et al., 2010; Leignadier and Labrecque, 2010; Prlic et al., 2006; Zehn et al., 2009). In contrast, changing TCR affinity does not (Corse et al., 2010; Leignadier and Labrecque, 2010; Prlic et al., 2006; Zehn et al., 2009). Thus, it is possible that the quantity of TCR signal alone is not sufficient to define a T cell's ability to differentiate into memory. The results presented here provide evidence that weak TCR signals can support memory in conditions where strong TCR signals fail. Hence, we favor the hypothesis that high and low-affinity ligands signal qualitatively different to promote memory programming. Intriguingly, this implies that the diversity of signals generated from the TCR that control T cell memory might be more complex than previously appreciated. This may also contribute to the diversity of the CD8 memory pool (Gerlach et al., 2013; Jameson and Masopust, 2009).

In summary, the results from this study fit a model where T cell memory programming is qualitatively different depending on TCR signal strength. This offers the possibility of specifically targeting TCR antigenic signals to improve memory generation in vaccine regimes. Thus, the  $\beta$ TMD mutant model rises as an ideal platform to provide deeper insight into the structural and molecular mechanisms that can enable the manipulation of T cell memory.

## Conclusion

This set of studies further investigated if pMHC-TCR affinity distinctly regulates memory CD8 T cell generation.  $\beta$ TMDmut CD8 T cells have a specific defect in memory generation against cognate antigens (Teixeiro et al., 2009). Surprisingly, MUT memory precursor generation and memory development was fully recovered to WT levels in response to low-affinity pMHC-TCR encounter during both infection (with low-affinity OT-I APL challenge) and lymphopenia, conditions which favor memory generation (Goldrath et al., 2000; Knudson et al., 2013b).

$\beta$ TMDmut T cells display normal activation of ERK, JNK, NFAT, and calcium signaling upon TCR stimulation (E. Teixeira unpublished data and (Teixeiro et al., 2009)). However, they have a specific defect in TCR-dependent NF- $\kappa$ B signaling (Teixeiro et al., 2009). This defect in TCR-dependent NF- $\kappa$ B signaling was implicated in the impaired MUT T cell memory generation during infection with high-affinity antigen. Correlating with the recovery in CD8 T cell memory development, low-affinity antigen stimulation of MUT T cells was strikingly able to induce high levels of TCR-dependent NF- $\kappa$ B activation. In fact, on a per cell basis, low-affinity antigen stimulation induced more NF- $\kappa$ B activation than strong antigens.

The results presented here suggest that the signals required for memory generation are regulated differently depending on TCR signal strength. In addition these findings support NF- $\kappa$ B signaling as a regulator of memory programming (the development of IL7R<sup>hi</sup> memory precursors). Thus, low-affinity

pMHC-TCR interactions, potentially through induced NF- $\kappa$ B signaling, promote memory differentiation.

## **Materials and Methods**

### *Mice and Reagents*

C57BL/6, C57BL/6 Rag<sup>-/-</sup>, B6.SJL (CD45.1<sup>+</sup>), OT-1 (WT) and MUT OT-1 TCR transgenic mice (Teixeiro et al., 2009a) were bred and maintained in accordance with University of Missouri OAR ACUC or University of Minnesota IACUC. OVA and Q4H7 peptides were from New England Peptides. rmlL-7, rmlL-15, and rmlL-12 were from Peprotech. Antibodies anti-CD90.1, -IL-7R $\alpha$ , -CD44, -CD25, -CD122, -phospho-STAT-4 and STAT-5 and -Bcl-2 were from BD Pharmingen. Anti-CD62L and anti-CD8 $\alpha$  were from BD Pharmingen or Biolegend. Anti-CD45.1, -CD45.2, -CD11a (LFA-1), -Ly6C were from eBioscience. Anti-plkB $\alpha$ , -p-p65-NF- $\kappa$ B (Ser536) and -p-ERK1/2 were from Cell Signaling. Anti-Granzyme B was from Invitrogen. Anti- $\alpha$ -tubulin was from Sigma. Anti- A20 (59A426) was from Millipore. Anti-Bim was from Enzo. Anti-p65 for confocal studies was from Santa Cruz. Intracellular staining was performed as in (Teixeiro et al., 2009a).

### *Flow cytometry*

Cells were prepared for staining and assays as previously described (Teixeiro et al., 2009a). Frequencies and phenotype are based on the live gate (FSC/SCS) for lymphocytes and then, on donor T cells (congenic marker<sup>+</sup> CD8<sup>+</sup>). For determining phospho-NF- $\kappa$ B, geometric mean fluorescent intensity was normalized to a naïve control. Cells were analyzed on a FACSCalibur, LSR II (BD Biosciences) and FLOWJO (TreeStar) software.

### *Adoptive Transfer and Infections*

Naïve ( $1 \times 10^3$ ) CD8 T cells were purified from the lymph nodes of OT-1 or MUT Rag1<sup>-/-</sup> mice and transferred intravenously as performed in (Hamilton et al., 2006). LM-N4 and LM-Q4H7 strains were grown as described in (Zehn et al., 2010). All infections were performed i.v. at least one day after adoptive transfer of naïve transgenic T cells. LM titers in the spleen were obtained as previously described (Hamilton et al., 2006).

### *Magnetic Bead Enrichment*

Single cell suspensions were generated from the spleens and lymph nodes of non-infected and LM-Q4H7-infected CD45.1<sup>+</sup> mice that were previously adoptively transferred with WT and MUT CD45.2<sup>+</sup> donor CD8 T cells. Total cells were stained with CD45.2-FITC antibodies, then incubated with MACS anti-FITC microbeads (Miltenyi Biotech), and passed over magnetic MACS LS Columns (Miltenyi Biotech) to enrich for CD45.2<sup>+</sup> WT or MUT CD8 donor T cells following a protocol similar to (Akue et al., 2012). Bound and unbound fractions from individual uninfected and LM-Q4H7-infected mice were stained and analyzed by flow cytometry for detection and phenotype of CD45.2<sup>+</sup> WT or MUT CD8 T cells. No CD45.2<sup>+</sup> cells were detected in the unbound fraction.

### *Immunoblot analysis*

2-5x10<sup>6</sup> T cells were stimulated with OVA- or Q4H7-tetramer and anti-CD28 were subjected to immunoblot as described in (Daniels et al., 2006) for p-I $\kappa$ B $\alpha$  signaling and p-ERK1/2 and A20 blots. Densitometry of the immunoblots was obtained using ImageJ.

### *Confocal Microscopy*

Microscopy was performed and analyzed as described in (Teixeiro et al., 2009).

### *Statistical Analysis*

For statistical analysis, two-tail unpaired Student's t test was applied using GraphPad Prism software. Significance was set at  $p < 0.05$ .

### **Acknowledgments**

This work was supported by University of Missouri Life Sciences Fellowship (K.M.K), Leukemia and Lymphoma Career Development (S.H.E.) and grants from University of Missouri Mission Enhancement Fund and University of Missouri Research Board Grant. We thank M. Bevan and D. Zehn for providing *Listeria m.* strains.

## **IV: NF- $\kappa$ B Signaling Regulates Eomes and CD8 T Cell Memory Development**

Karin M. Knudson, Amnon Altman, Mark A. Daniels, and Emma Teixeiro

## Rationale

The proper expression of transcription factors is required to induce and maintain the memory program. Two important regulators T cell differentiation are T-box transcription factors T-bet and Eomes. While they are induced upon T cell activation (Pipkin et al., 2010), how the TCR signal regulates their expression is unknown.

In prior chapters, as well as in previously published data, we demonstrated, using a TCR mutant model, that the TCR signal distinctly regulates effector and memory T cell differentiation (Teixeiro et al., 2009). MUT CD8 T cells, which only had a defect in TCR-dependent NF- $\kappa$ B signaling, could not generate memory precursors. This phenotype was reverted by lowering the affinity of the pMHC-TCR interaction and restoring the induction of TCR-dependent NF- $\kappa$ B signaling (Chapter III). These studies also implicated TCR-dependent NF- $\kappa$ B signaling in memory CD8 T cell generation. However, the direct role of NF- $\kappa$ B signaling in memory development was not formally tested. In addition, determining the mechanism by which NF- $\kappa$ B signaling supports CD8 T cell memory programming and differentiation may identify novel targets for vaccine and immunotherapy generation.

While its role in memory development is not fully understood, NF- $\kappa$ B signaling has been shown to regulate memory T cell function. Loss of NF- $\kappa$ B signaling at the memory timepoint impairs rapid recall responses in CD4 T cells, characterized by loss of effector molecule production (Lai et al., 2011). These studies were performed *in vitro*, however. The role NF- $\kappa$ B plays in a CD8 T cell

memory response to infection has not been elucidated. Furthermore, whether NF- $\kappa$ B signaling is required for memory survival remains to be investigated.

The goal of this study was to further investigate how TCR signals control CD8 T cell memory programming. We particularly focused on how TCR-dependent NF- $\kappa$ B signaling regulates the expression of transcription factors Eomes and T-bet. In addition, we investigated the mechanism by which memory T cells survive and respond after differentiation, again focusing on the role NF- $\kappa$ B signaling. We also examined potential TCR-independent mechanisms in the regulation of NF- $\kappa$ B signaling during infection.

## **Abstract**

CD8 T cell memory is critical for long-term immunity. We have previously shown that the T cell receptor (TCR) signal differentially regulates effector and memory generation. In addition, our prior results suggested that TCR-dependent NF- $\kappa$ B signaling is important for memory development. However, the mechanism by which TCR-dependent NF- $\kappa$ B regulates memory programming is unknown. We report that TCR-dependent NF- $\kappa$ B signaling is required for the expression of memory-associated transcription factor Eomes and the generation of memory CD8 T cells. NF- $\kappa$ B signaling also regulates Eomes expression in memory T cells, and sustained NF- $\kappa$ B dependent Eomes is required for memory survival and function. We also describe a novel role for Pim-1 kinase in supporting NF- $\kappa$ B signaling and Eomes expression late in the immune response. These data provide additional insight into the complex regulation of Eomes expression in CD8 T cells as well as a molecular target to alter memory CD8 T cell development.

## **Introduction**

CD8 T cells provide protection against intracellular pathogens and tumors, making them a vital component of the adaptive immune system. The development of immunological memory after infection is required for long-term immunity. In order for full activation and differentiation of a naïve CD8 T cell into memory, it must receive antigenic, costimulatory, and inflammatory signals (Williams and Bevan, 2007). Antigenic signals are essential for T cell memory

development (Teixeiro et al., 2009; Williams and Bevan, 2007). The T cell receptor (TCR) recognizes antigenic peptides in the context of major histocompatibility complex I (MHC). This leads to the induction of several signaling pathways that are key for the activation of a T cell. One of these pathways is NF- $\kappa$ B. NF- $\kappa$ B signaling is coupled to the TCR through PKC $\theta$  (Coudronniere et al., 2000). Upon TCR engagement, PKC $\theta$  mediates the activation of the IKK complex. The IKK complex is composed by the kinases IKK $\alpha$  and IKK $\beta$  and the regulatory subunit IKK $\gamma$ /NEMO. Activated IKK $\beta$  phosphorylates I $\kappa$ B $\alpha$ , a negative regulator of NF- $\kappa$ B signaling, promoting its ubiquitination and degradation. This frees NF- $\kappa$ B subunits to translocate to the nucleus and mediate transcription of various receptors and molecules required for T cell differentiation (Vallabhapurapu and Karin, 2009). Proper NF- $\kappa$ B signaling is essential during a CD8 T cells' lifetime, from thymic selection to peripheral survival of mature T cells and activation in response to TCR stimulus (Gerondakis et al., 2013; Grumont et al., 2004; Miller et al., 2014; Schmidt-Supprian et al., 2003). NF- $\kappa$ B signaling is also important in the development of T cell memory (Miller et al., 2014; Schmidt-Supprian et al., 2003; Schmidt-Supprian et al., 2004; Teixeira et al., 2009). Yet, how NF- $\kappa$ B signaling specifically regulates T cell memory remains to be elucidated.

The T-box transcription factors T-bet and Eomesodermin (Eomes) have are major regulators of effector and memory differentiation, respectively (Belz and Kallies, 2010; Daniels and Teixeira, 2010; Kaech and Cui, 2012). T-bet and Eomes are both important, and potentially redundant, in the regulation of T cell

effector function. They promote effector differentiation by regulating IFN $\gamma$  and Granzyme B expression (Intlekofer et al., 2005b; Pearce et al., 2003).

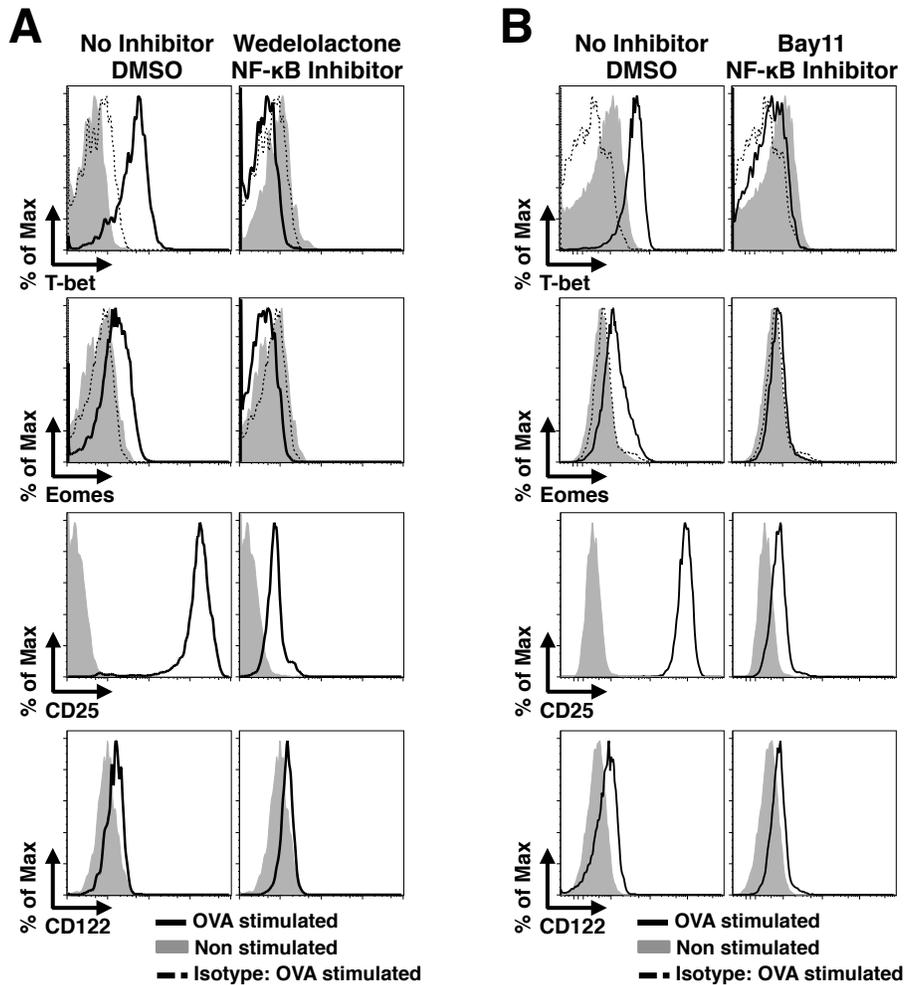
Interestingly, the Eomes/T-bet ratio is not constant during the course of an immune response. The Eomes/T-bet ratio increases as T cells enter in the memory phase (Kaech and Cui, 2012). Indeed, loss of Eomes leads to the development of fewer memory cells, particularly of a central memory phenotype, due to impaired long-term persistence (Banerjee et al., 2010). Along the same line, we have previously shown that responses to low-affinity antigens support high levels of Eomes expression and skews T cells toward memory (Knudson et al., 2013a). Eomes is induced upon TCR stimulation (Pipkin et al., 2010), but how TCR signaling promotes Eomes expression is unknown.

Here, we identify the NF- $\kappa$ B signaling pathway as a key regulator of Eomes expression. We manipulate the activity of different members of the NF- $\kappa$ B pathway to test its role in the regulation of Eomes and development of T cell memory. Our data shows that TCR-dependent NF- $\kappa$ B signaling programs late expression of Eomes in a Pim-1 kinase-dependent manner. Pim-1 and NF- $\kappa$ B activity was critical for the establishment of the memory pool.

## **Results**

### *NF- $\kappa$ B signaling controls Eomes expression in activated CD8 T cells.*

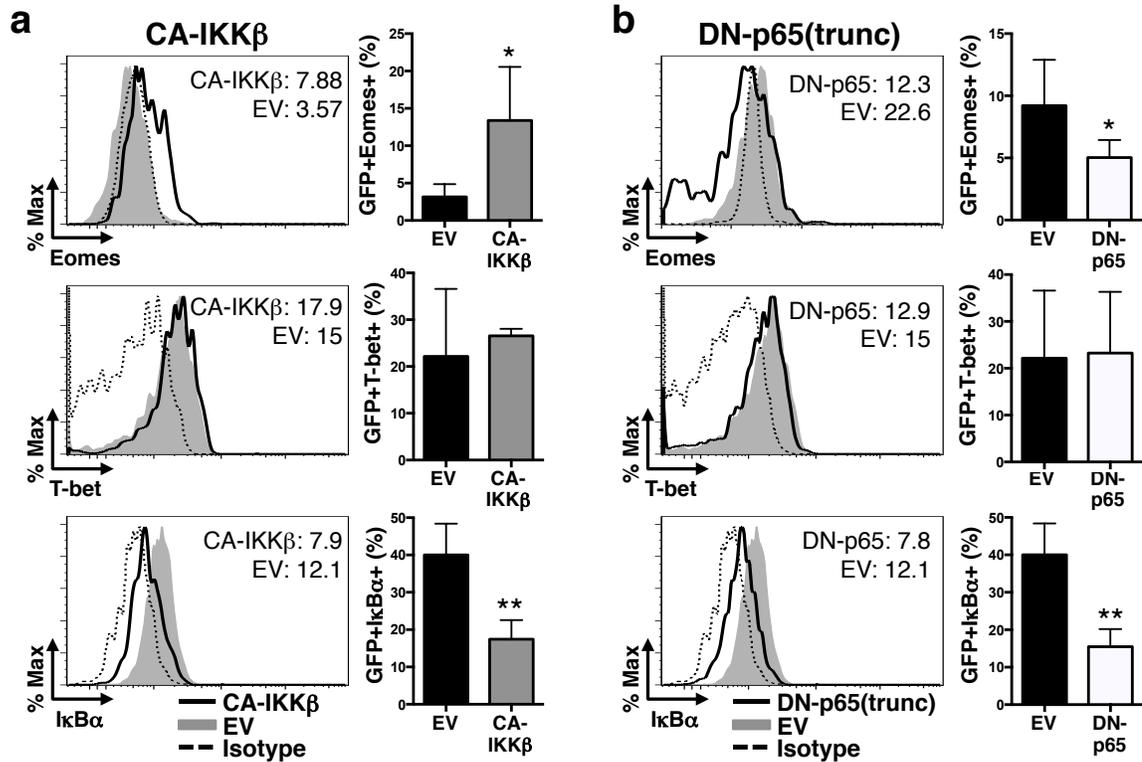
We have previously shown that NF- $\kappa$ B signaling is involved in memory CD8 T cell development (Teixeiro et al., 2009). T cells with impaired NF- $\kappa$ B



**Figure 4-1. Chemical inhibition of NF- $\kappa$ B signaling prevents Eomes expression in activated CD8 T cells.** OT-I T cells were stimulated with 20nM OVA with or without 100uM Wedelolactone (A) or 20uM Bay11 (B). Eomes, T-bet, CD25, and CD122 expression were determined by flow cytometry at day 1 or 2. Histograms representative of  $\geq 2$  independent experiments.

signaling had defects in memory programming, as shown by deficient generation of IL-7R<sup>hi</sup> memory precursors (Knudson et al., 2013b). Thus, we investigated how NF-κB signaling regulates memory programming, through the expression of T-bet and Eomes. T-bet and Eomes are two transcription factors known to regulate effector and memory differentiation of CD8 T cells, respectively (Banerjee et al., 2010; Intlekofer et al., 2005; Joshi et al., 2007). Initial experiments utilizing two chemical inhibitors of NF-κB signaling showed that expression of both Eomes and T-bet, but not CD122 (a negative control), was abrogated in activated T cells with inhibition of NF-κB signaling (Figure 4-1). We confirmed these results by altering NF-κB signaling using two gain/loss of function approaches. First, we retrovirally transduced CD8 T cells with a construct that expresses a constitutive active form of IKKβ to enhance NF-κB signaling (Song et al., 2008). CA-IKKβ GFP<sup>+</sup> retrovirally transduced cells exhibited lower levels of IκBα than their empty vector (EV)-transduced counterparts. This indicated that constitutive activation of IKKβ led to enhanced degradation of IκBα and subsequent increased NF-κB signaling. More importantly, enhanced IKKβ activity increased levels of Eomes. Strikingly, T-bet expression was not altered in cells containing CA-IKKβ (Figure 4-2A).

Next, we assessed the direct role of NF-κB in the regulation of Eomes and T-bet by expressing a dominant negative truncated form of p65-NF-κB (DN-p65(trunc)). The DN-p65(trunc) RV construct encodes for a truncated p65-NF-κB subunit that competes with endogenous p65-NF-κB for NF-κB binding sites in promoters. Introduction of DN-p65(trunc) decreases NF-κB transcriptional activity

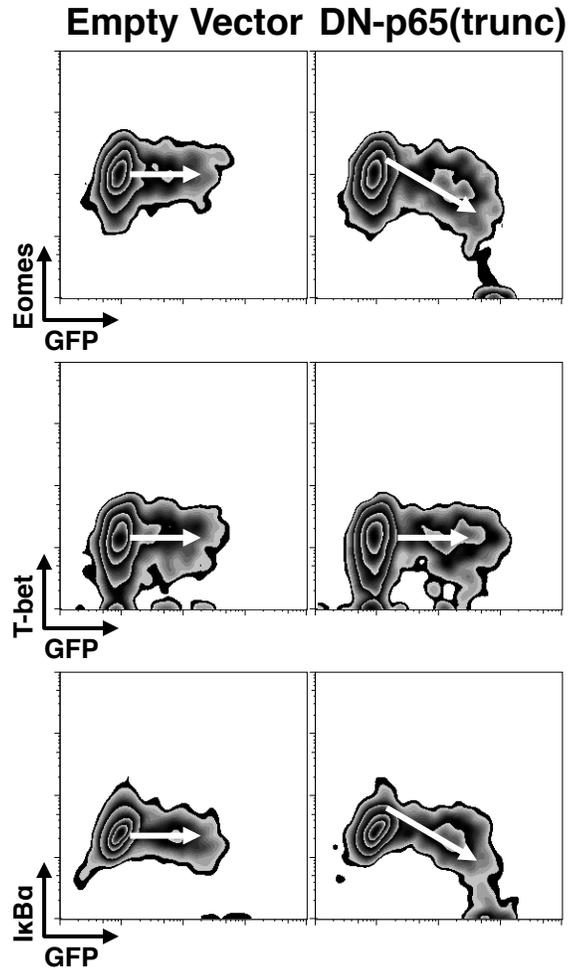


**Figure 4-2. NF- $\kappa$ B signaling regulates Eomes expression in activated CD8 T cells.** OT-I T cells were stimulated with 20nM OVA for 24h and spin transduced with CA-IKK $\beta$  RV (A), DN-p65(truncated) RV (B), or the respective empty vector RV. Eomes, T-bet, and I $\kappa$ B $\alpha$  expression were determined in CD8+GFP+ cells by flow cytometry at day 1 or 2 post-transduction. Numbers in histograms are geometric MFI of indicated population. Graphs show % GFP+ cells with Eomes, T-bet, or I $\kappa$ B $\alpha$  expression over the isotype level. All graphs show mean $\pm$ SD. Histograms and graphs representative of  $\geq 3$  independent experiments. \* $p \leq 0.05$ , \*\* $p \leq 0.005$ .

in the nucleus (Mondor et al., 2005). Since I $\kappa$ B $\alpha$  is an NF- $\kappa$ B dependent gene, we expectedly observed lower levels of I $\kappa$ B $\alpha$  in DN-p65(trunc) transduced T cells (Figure 4-2B) (Ghosh and Baltimore, 1990; Sun et al., 1993). This indicates NF- $\kappa$ B activity was effectively inhibited. Additionally, transduction of OVA-stimulated T cells with DN-p65(trunc) lead to a severe reduction in Eomes levels, confirming the important role of NF- $\kappa$ B in the regulation of Eomes expression (Figure 4-2B). Interestingly, Eomes and I $\kappa$ B $\alpha$  levels correlated with GFP expression, suggesting that the level of p65-NF- $\kappa$ B inhibition directly affects the amount of Eomes and I $\kappa$ B $\alpha$  in activated T cells (Figure 4-3). As seen with CA-IKK $\beta$  expression, no change in T-bet levels occurred with altered NF- $\kappa$ B activity. This suggests that T-bet is not a NF- $\kappa$ B target in activated T cells. Together, these data demonstrate that NF- $\kappa$ B signaling directly regulates Eomes expression in CD8 T cells.

*NF- $\kappa$ B signaling directly regulates Eomes expression and memory development of CD8 T cells.*

Next, we determined whether NF- $\kappa$ B signaling regulates Eomes expression during infection. T cell priming during the first 48 hours after infection is essential to program memory differentiation (Kaech and Ahmed, 2001; van Stipdonk et al., 2003). As we did not want to alter this initial memory programming during retroviral transduction, we generated a novel protocol that does not require re-stimulation of previously-primed T cells. We harvested primed OT-I CD8 T cells 4 days after infection with *Listeria monocytogenes* expressing OVA (LM-OVA). We then transduced these cells without additional

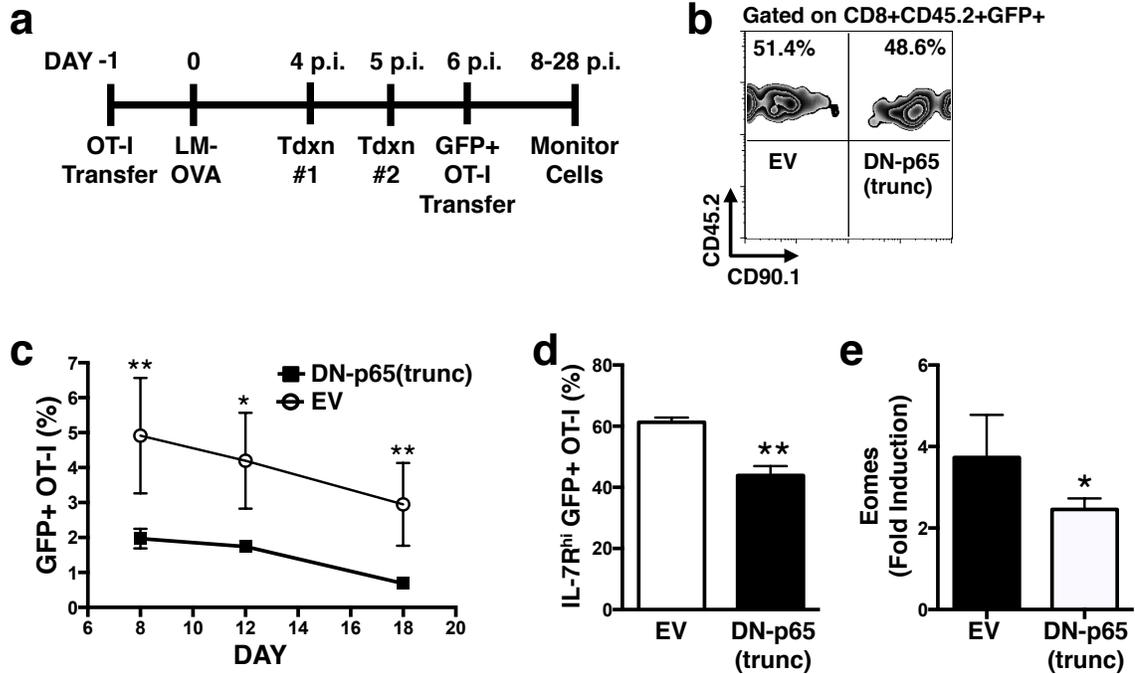


**Figure 4-3. Expression of IκBα and Eomes correlates with NF-κB activity in activated T cells.** OT-I T cells were stimulated with 20nM OVA for 24h and spin transduced with DN-p65(trunc) RV or the respective empty vector control RV. Eomes, T-bet, and IκBα expression were determined in CD8<sup>+</sup> T cells by flow cytometry at day 1 post-transduction. Dot plots show expression of Eomes, T-bet, and IκBα versus GFP level. Arrows show trend of population.

TCR stimulation with either DN-p65(trunc) to inhibit NF- $\kappa$ B-induced transcription or empty vector control. Equal numbers of EV-GFP+ or DN-p65(trunc)-GFP+ cells were co-transferred into WT-LM infection-matched hosts (Figure 4-4A,B). Both empty vector and DN-p65(trunc)-GFP+ transduced OT-1 T cells underwent contraction after adoptive transfer into infection-matched hosts. As expected, EV-transduced T cells persisted and generated a memory pool. In contrast, DN-p65(trunc)-transduced cell frequencies started to decay 2 days after transfer and cells were undetectable at the memory phase (Figure 4-4C). This failure to develop memory cells correlated with a defect in the generation of IL-7R<sup>hi</sup>-expressing memory precursors as well as decreased Eomes expression in those cells transduced with the DN-p65(trunc) retrovirus (Figure 4-4D,E). Together, these data clearly demonstrate that NF- $\kappa$ B signaling directly regulates Eomes expression and memory development of CD8 T cells during infection.

*TCR-dependent NF- $\kappa$ B signaling is required for Eomes expression.*

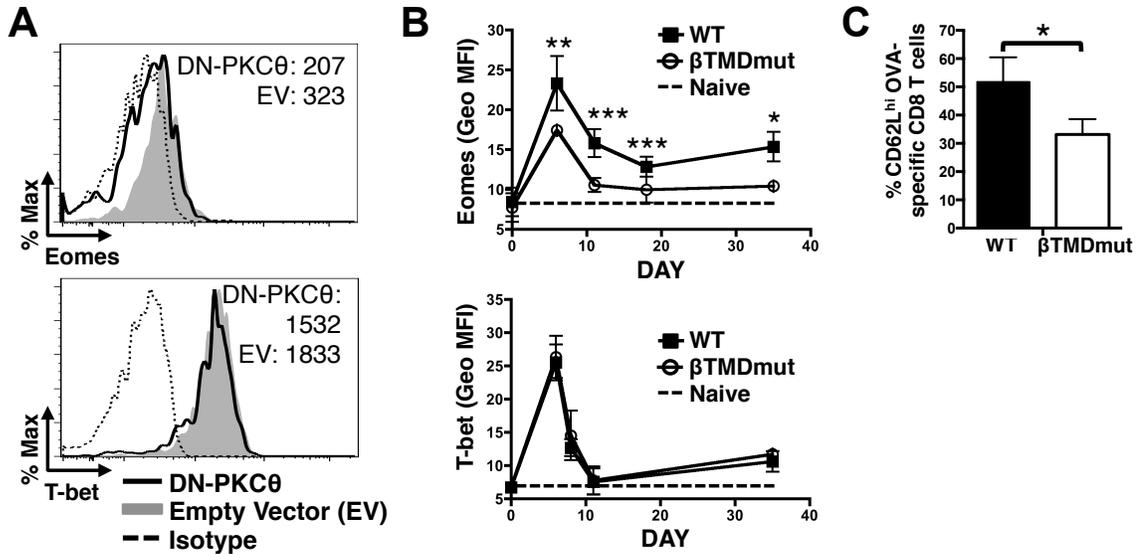
NF- $\kappa$ B signaling can be induced by stimulation through the TCR, cytokine receptors, and costimulatory molecules. Our previous published data in a TCR mutant model showed that T cells specifically impaired in activating TCR-dependent NF- $\kappa$ B signals failed to generate memory precursors. This strongly supported that TCR-dependent NF- $\kappa$ B signaling is required for memory programming (Knudson et al., 2013b; Teixeira et al., 2009). Upon TCR/CD28 stimulation, PKC $\theta$  colocalizes with the TCR in the immunological synapse (IS) enabling the activation of the NF- $\kappa$ B signaling cascade (Kong and Altman, 2013).



**Figure 4-4. NF- $\kappa$ B signaling supports Eomes expression and memory development during infection.** (A-E)  $2 \times 10^5$  OT-I T cells were adoptively transferred into congenic hosts and challenged with  $7 \times 10^6$  CFU Att-LM-OVA (actA-). After 4 days, OT-I T cells were spin transduced (Tdxn) 2 times with empty vector or DN-p65(truncated) RV with 10ng/ml IL-7. 24h after last transduction,  $1 \times 10^5$  EV (CD45.2+CD90.1-) and  $1 \times 10^5$  DN-p65(truncated) (CD45.2+CD90.1+) GFP+ OT-I were mixed (B) and co-transferred into infection-matched hosts (CD45.1+CD90.1-) challenged with WT LM (no OVA). (C) The kinetics of EV and p65(truncated) GFP+ OT-I T cells were determined after adoptive transfer. Frequencies of are % GFP+ cells in the CD8+ pool (mean $\pm$ SEM). Days represent post-infection. (D) Frequency of IL-7R<sup>hi</sup> memory precursors of GFP+ OT-I on day 8. (E) Expression of Eomes on day 8 in GFP+ OT-I was determined by flow cytometry. Graph shows fold induction over an isotype control. All graphs show mean $\pm$ SD. Data representative of 3 independent experiments; n=3-4 mice. \*p  $\leq$  0.05, \*\*p  $\leq$  0.005.

Thus, to investigate whether TCR-dependent NF- $\kappa$ B signaling was required for Eomes expression, we altered the kinase activity of PKC $\theta$  using a previously described kinase dead mutant form of PKC $\theta$  (DN-PKC $\theta$ ) [25, 26]. Transduction of OVA-stimulated OT-I T cells with DN-PKC $\theta$  impaired Eomes expression. However, T-bet expression was not altered (Figure 4-5A). Thus, TCR-dependent NF- $\kappa$ B signaling regulates Eomes at the level of PKC $\theta$  in CD8 T cells.

Upon antigenic stimulation,  $\beta$ TMDmut cells are impaired in the recruitment and colocalization of PKC $\theta$  into the IS, NF- $\kappa$ B signaling and memory development (Teixeiro et al., 2009). Thus, we used this unique model to test the role of TCR-dependent NF- $\kappa$ B signaling in the regulation of Eomes expression during infection. We adoptively transferred WT OT-I or  $\beta$ TMDmut (MUT) naive T cells into congenic hosts and monitored Eomes and T-bet expression after infection with LM-OVA. In WT T cells, the kinetics of Eomes and T-bet expression were similar with the highest levels near the peak of the response and decreasing expression during the contraction phase. Eomes and T-bet levels stabilized during late contraction and were maintained into the memory time point (Figure 4-5B).  $\beta$ TMDmut CD8 T cells had normal levels of the effector-associated transcription factor T-bet after LM-OVA infection, correlating with the fact that MUT cells are normal in their expansion and effector differentiation (Teixeiro et al., 2009c). Yet, Eomes expression was impaired in  $\beta$ TMDmut CD8 T cells throughout the immune response. In fact, expression of Eomes during late contraction was not induced over naïve levels in MUT T cells (Figure 4-5B). Higher Eomes levels have been associated with T cell memory development



**Figure 4-5. TCR-dependent NF-κB signaling is required for expression of Eomes.** (A) OT-I T cells were stimulated with 20nM OVA for 24h and spin transduced with DN-PKCθ RV or control RV. Eomes and T-bet expression were determined in GFP<sup>+</sup> cells by flow cytometry at day 1 post-transduction. Numbers in histograms represent geometric MFI of respective population. Histograms are representative of 2 independent experiments. (B-C)  $1 \times 10^3$  WT or MUT OT-I T cells were adoptively transferred into congenic hosts and challenged with  $1 \times 10^3$  CFU LM-OVA. (B) The kinetics of Eomes (top) and T-bet (bottom) expression were determined in the blood or spleen by flow cytometry. (C) The frequency of CD62L<sup>hi</sup> memory cells were determined  $\geq 28$  days p.i. Graphs show geometric MFI (mean $\pm$ SD). Data is representative of three or more independent experiments;  $n \geq 3$  mice per group. \* $p \leq 0.05$ , \*\* $p \leq 0.005$ , \*\*\* $p \leq 0.001$ .

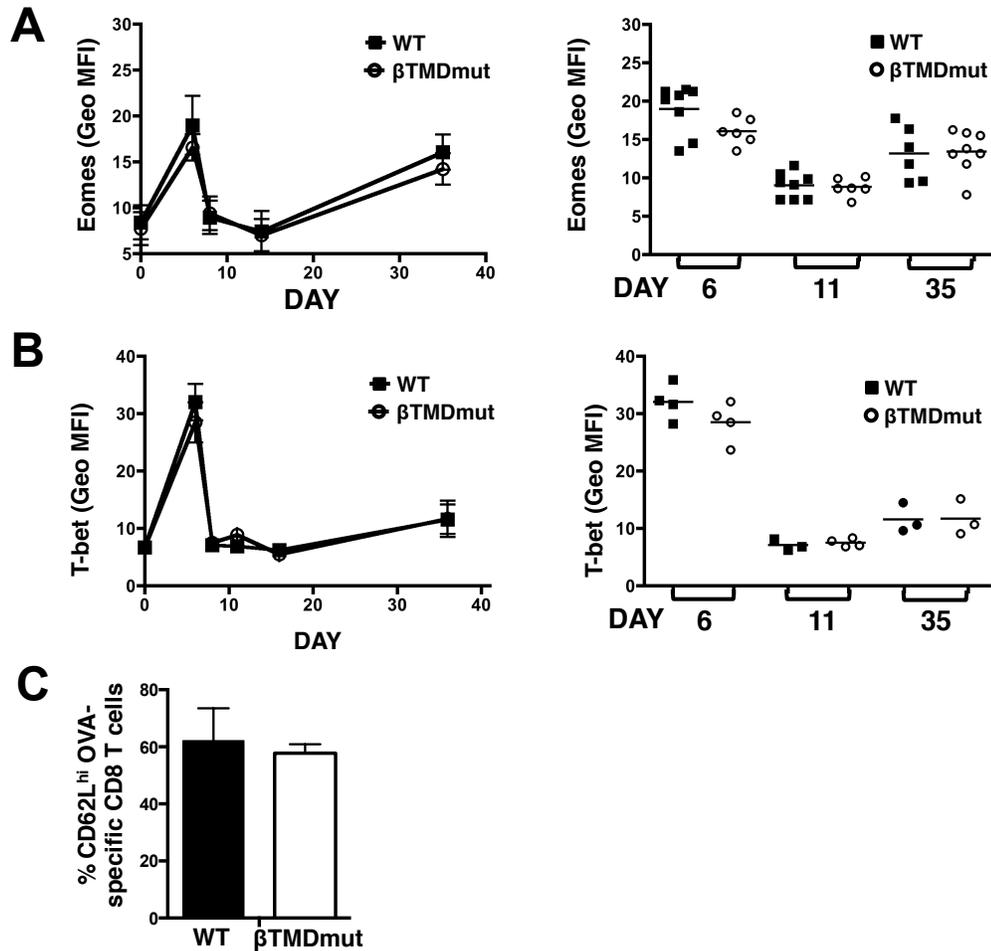
(Banerjee et al., 2010; Belz and Kallies, 2010; Kaech and Cui, 2012).

Conversely, in TCR stimulatory conditions where  $\beta$ TMDmut T cells recover NF- $\kappa$ B signaling and memory differentiation (low TCR signal strength provided by infection with LM-Q4H7 (Knudson et al., 2013b)), Eomes expression was rescued to WT levels (Figure 4-6A). As with LM-OVA challenge, there was no difference in T-bet expression with LM-Q4H7 infection (Figure 4-6B).

Eomes expression is important for the development of central memory CD8 T cells. *Eomes*-deficient memory T cells are skewed toward an effector memory phenotype (Banerjee et al., 2010). Due to the defects seen in Eomes expression in  $\beta$ TMDmut CD8 T cells, we examined the frequency of WT and MUT CD62L<sup>hi</sup> central memory CD8 T cells in the blood after LM-OVA challenge. Correlating with defects seen in Eomes expression, there was a significant reduction in the frequency of CD62L<sup>hi</sup> central memory MUT T cells as compared to WT counterparts (Figure 4-5C). This difference in the frequency of CD62L<sup>hi</sup> cells was recovered after challenge with LM-Q4H7 (Figure 4-6C). Together, these data show that TCR-dependent NF- $\kappa$ B signaling is required for the Eomes levels necessary for proper memory programming and development during infection.

*NF- $\kappa$ B signaling is necessary for Eomes expression in memory CD8 T cells.*

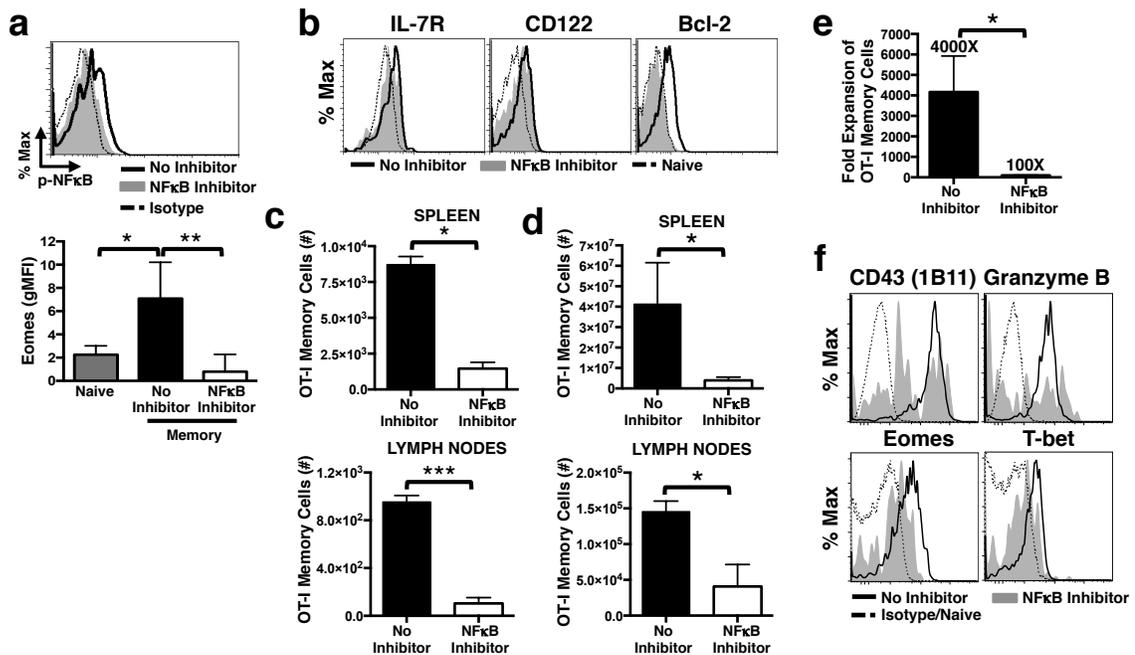
Eomes levels increased after contraction, and it is known that Eomes expression is higher in memory than naïve CD8 T cells (Figure 4-5B) (Banerjee et al., 2010). This led us to hypothesize that NF- $\kappa$ B signaling is involved in



**Figure 4-6. Recovery of NF-κB signaling during low-affinity antigen challenge induces Eomes expression in βTMDmut CD8 T cells.**  $1 \times 10^3$  WT or MUT OT-I T cells were adoptively transferred into congenic hosts and challenged with  $1 \times 10^3$  CFU LM-Q4H7. The kinetics of Eomes (A) and T-bet (B) expression were determined in the blood or spleen by flow cytometry. (C) The frequency of CD62L<sup>hi</sup> memory cells were determined  $\geq 28$  days p.i. Graphs show geometric MFI (mean $\pm$ SD). Data is representative of three or more independent experiments;  $n \geq 3$  mice per group.

supporting Eomes expression in memory cells. To test this, we inhibited NF- $\kappa$ B signaling in memory T cells and determined their capacity to maintain Eomes expression. Since memory T cells have a slow cell division rate, they are not amenable to retroviral transduction. Thus, we used a chemical inhibitor known to specifically inhibit NF- $\kappa$ B signaling in T cells (Kobori et al., 2004; Riou et al., 2007). Use of this inhibitor also impaired Eomes expression in activated T cells (Figure 4-1). First, using phospho-p65- NF- $\kappa$ B (Ser536) (p-NF- $\kappa$ B) as a readout of NF- $\kappa$ B activity (Mattioli et al., 2004), we observed that memory T cells at a resting state exhibited NF- $\kappa$ B signaling activity. Addition of the NF- $\kappa$ B inhibitor decreased the level of p-NF- $\kappa$ B after treatment. Furthermore, Eomes expression was completely lost when NF- $\kappa$ B signaling was impaired (Figure 4-7A). These data suggest that NF- $\kappa$ B signals are necessary to maintain Eomes levels in memory cells.

Since mTOR signaling regulates both Eomes and T-bet expression (Li et al., 2011b; Rao et al., 2010b), we also tested whether this pathway was involved in maintenance of Eomes and T-bet levels in memory T cells. Of note, mTOR activity was higher in memory cells than in naïve T cells (Figure 4-8A). However, treatment of memory CD8 T cells with rapamycin, a chemical inhibitor of mTOR signaling, was unable to alter Eomes or T-bet expression in memory cells. This is in direct contrast to activated T cells, where addition of rapamycin led to decreased phosphorylation of mTOR as well as T-bet and CD122 expression (Figure 4-8B). These data indicate that mTOR and NF- $\kappa$ B pathways play different roles in CD8 T cell memory. While both regulate Eomes expression in the early

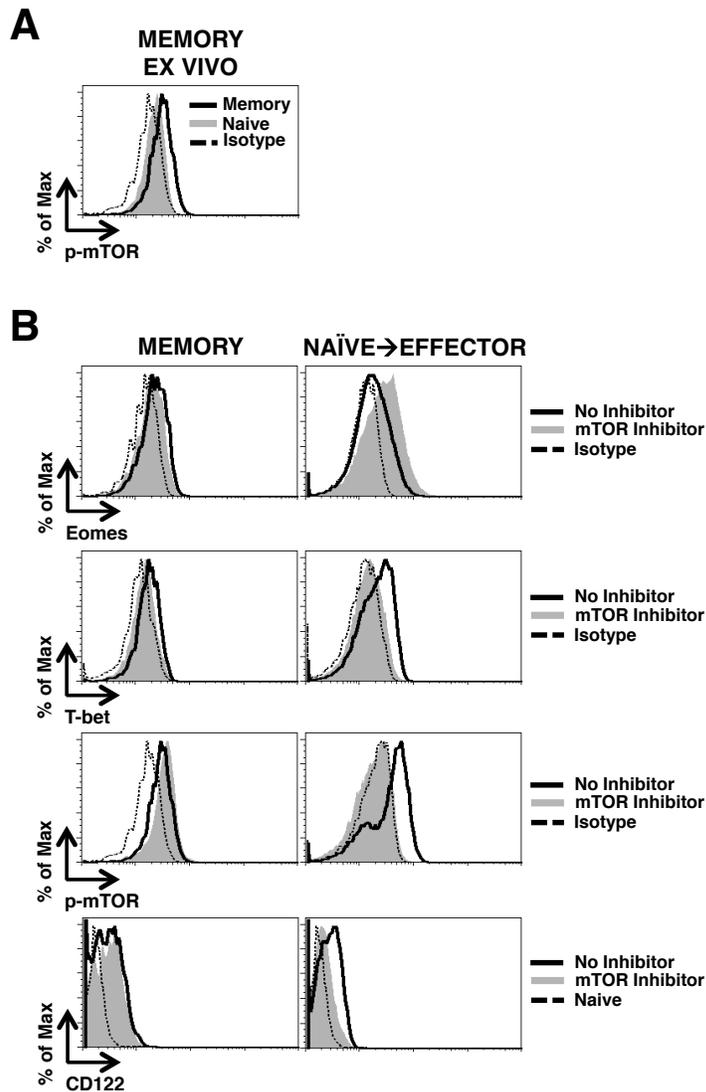


**Figure 4-7. NF-κB signaling is required at memory to maintain memory CD8 T cell survival and function.**  $2 \times 10^5$  naive T cells were transferred into congenic hosts and challenged with  $1 \times 10^4$  CFU LM-OVA. 28 days p.i. memory cells were harvested and treated  $\pm$  100  $\mu$ M NF-κB inhibitor with 0.1 ng/ml IL-7. (A) Phosphorylation of p65- NF-κB and expression of Eomes were determined by flow cytometry after 24h. Graphs show geometric MFI (mean $\pm$ SD). (B) CD122, IL-7R, and Bcl-2 expression were by flow cytometry after 24h. Histograms representative of  $\geq$  3 independent experiments. (C) After 24h treatment, equal numbers of vehicle or NF-κB- inhibited memory cells were transferred into naïve congenic hosts. Graphs show total number of transferred OT-I T cells in lymph nodes and spleens at day 4 (mean $\pm$ SD). (D) Hosts were infected with  $1 \times 10^4$  CFU LM-OVA. Graphs show total number transferred OT-I T cells in lymph nodes and spleens at day 6 p.i. or (E) fold expansion at day 5 (mean  $\pm$  SD). (F) Representative histograms show CD43 (1B11), Granzyme B, Eomes, and T-bet expression in the spleen at day 3 p.i. Data is representative of  $n \geq$  3 independent experiments;  $n \geq$  3 mice per group. \* $p \leq$  0.05, \*\* $p \leq$  0.005, \*\*\* $p \leq$  0.001.

phases of memory programming, only NF- $\kappa$ B is required to maintain Eomes levels in memory T cells.

*Sustained NF- $\kappa$ B signaling is required for memory survival and function.*

Several studies have implicated components of the NF- $\kappa$ B pathway in naïve T cell homeostasis (Schmidt-Supprian et al., 2003; Silva et al., 2014; Zheng et al., 2003). However, the role of NF- $\kappa$ B signaling in T cell memory survival is unclear. Lack of Eomes expression in memory CD8 T cells leads to decreased expression of the pro-survival factor Bcl-2 and impaired maintenance of memory cells (Banerjee et al., 2010). Since NF- $\kappa$ B regulates Eomes in memory T cells, we investigated whether NF- $\kappa$ B signaling was also involved in memory cell survival. Response to homeostatic cytokines IL-7 and IL-15 is required for memory T cell survival (Ku et al., 2000; Lodolce et al., 1998; Schluns et al., 2000), and treatment of memory CD8 T cells with the NF- $\kappa$ B inhibitor did not affect expression of IL-7R nor CD122 (Figure 4-7B). Bcl-2 expression, on the other hand, was significantly reduced in the NF- $\kappa$ B inhibitor-treated cells (Figure 4-7B). This is in agreement with previous reports showing that inhibition of NF- $\kappa$ B activity leads to defects in Bcl-2 levels (Zheng et al., 2003). To formally determine whether loss of NF- $\kappa$ B signaling impacted memory survival, memory T cells were treated for 24h with the NF- $\kappa$ B inhibitor, thoroughly washed, and transferred into naïve hosts. Their maintenance *in vivo* was determined 4 days later. NF- $\kappa$ B inhibitor-treated cell numbers were significantly decreased in the lymph nodes and spleens of recipient hosts as compared to vehicle-treated



**Figure 4-8. mTOR signaling regulates Eomes and T-bet expression in activated, but not memory, CD8 T cells.**  $2 \times 10^5$  naive T cells were transferred into congenic hosts and challenged with  $1 \times 10^4$  CFU LM-OVA. 28 days p.i. memory cells were harvested and treated  $\pm 20 \mu\text{M}$  mTOR inhibitor rapamycin with 0.1 ng/ml IL-7. (A) Phosphorylation of mTOR was determined by flow cytometry in *ex vivo* memory cells. (B) Expression of Eomes, T-bet, and CD122 and phosphorylation of mTOR was determined by flow cytometry in treated memory or activated OT-I T cells 1 or 2 days post-treatment/stimulation, respectively. Histograms representative of 2 independent experiments.

controls (Figure 4-7C). This suggests that NF- $\kappa$ B signaling is required for survival of memory T cells.

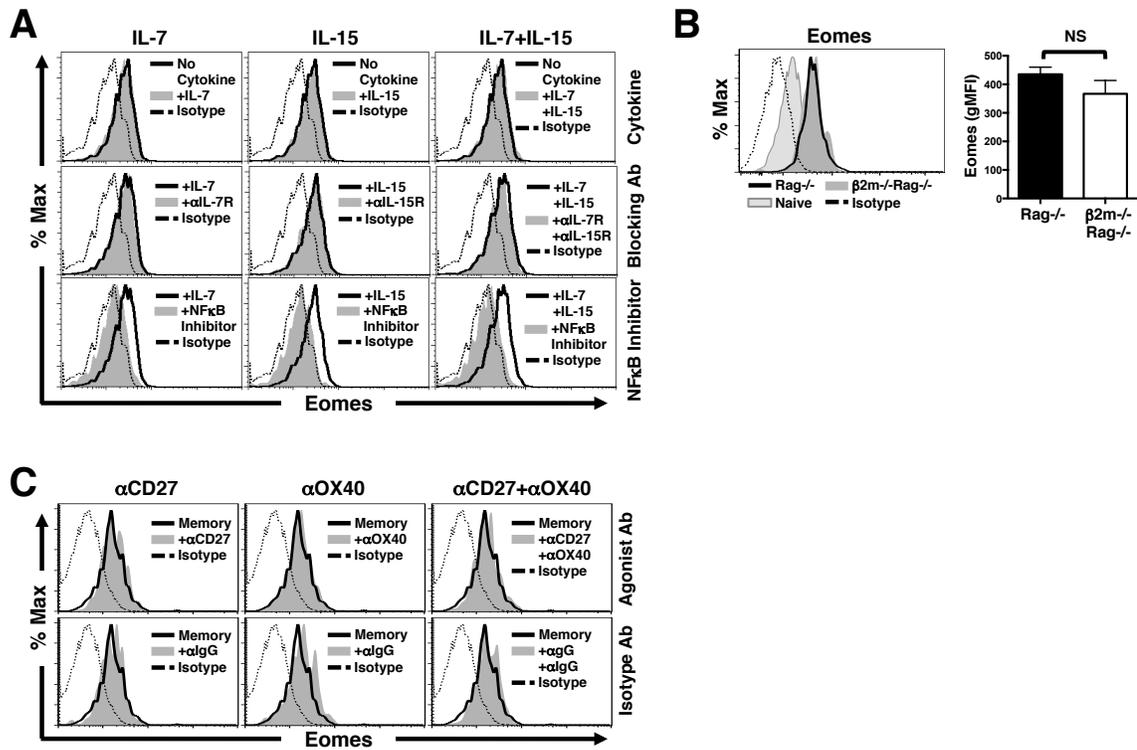
Next, we assessed whether NF- $\kappa$ B signaling was also important in the response of memory T cells. For this, naïve mice containing transferred vehicle- or NF- $\kappa$ B inhibitor-treated memory OT-1 cells were re-challenged with LM-OVA. Vehicle-treated memory cells expanded robustly after infection, but those with inhibited NF- $\kappa$ B signaling were unable to expand to the same extent (Figure 4-7D). Overall, memory cells with inhibited NF- $\kappa$ B signaling proliferated approximately 40 times less in the spleen of infected mice than vehicle-treated memory T cells (Figure 4-7E). Furthermore, NF- $\kappa$ B signaling-inhibited memory cells expressed less of the activation-associated glycoform of CD43 (1B11) and were unable to express Granzyme B, Eomes, and T-bet after LM-OVA re-challenge (Figure 4F). This is consistent with previous findings showing that CD4 T cells deficient in different components of the NF- $\kappa$ B pathway (IKK $\beta$ , Bcl-10) are impaired in recall responses (Schmidt-Suppran et al., 2003). These data indicate that NF- $\kappa$ B signaling regulates Eomes and T-bet levels in memory CD8 T cells. In addition, NF- $\kappa$ B signaling is required for both CD8 T cell memory survival and responses.

*Maintenance of Eomes expression at memory is not regulated by extrinsic factors.*

We and others have shown that as developing CD8 T cells enter the memory phase, their Eomes levels increase and are maintained higher than at

the naïve stage (Figure 4-5) (Paley et al., 2013). The fact that NF- $\kappa$ B signal deficient  $\beta$ TMDmut cells are impaired in upregulating and maintaining Eomes levels supports a role for TCR-dependent NF- $\kappa$ B signaling in the expression of Eomes. What supports the increased Eomes levels after the contraction phase when antigen is most likely cleared? To answer this, we investigated the role of other survival and homeostatic signals that may regulate Eomes levels during the memory phase. We first examined whether the homeostatic cytokines IL-7 and IL-15, which are essential for memory survival (Berard et al., 2003; Schluns et al., 2000), supported Eomes expression in memory cells. Addition of IL-7 and IL-15 to memory cells did not change Eomes levels. Furthermore, Eomes expression was not altered upon inhibition of their signaling using blocking anti-IL-7R and -IL-15R antibodies (Sudo et al., 1993; Tanaka et al., 1991). Of note, IL-7 and IL-15 signaling did not recover Eomes expression in the absence of NF- $\kappa$ B activity (Figure 4-9A). Together, these results suggest that IL-7 and IL-15 do not support NF- $\kappa$ B-dependent Eomes expression in memory T cells.

While it has been shown that self-pMHC-TCR interaction is not required for memory CD8 T cell maintenance (Jabbari and Harty, 2005; Leignadier et al., 2008; Murali-Krishna et al., 1999; Polic et al., 2001), it is not known whether tonic signals via the TCR are required for Eomes expression at memory. To test this, we adoptively transferred memory cells into B6Rag<sup>-/-</sup> or B6Rag<sup>-/-</sup> $\beta$ 2m<sup>-/-</sup> hosts (Daniels et al., 2006b). B6Rag<sup>-/-</sup> $\beta$ 2m<sup>-/-</sup> cells do not express MHC class I molecules, so the transferred memory cells will not encounter self-pMHC. After 28 days, Eomes expression was similar between memory cells regardless of



**Figure 4-9. The role of extrinsic factors in the regulation of Eomes expression in memory T cells.**  $2 \times 10^5$  naive T cells were transferred into congenic hosts and challenged with  $7 \times 10^6$  CFU Att-LM-OVA (actA-). At  $\geq 28$  days p.i. memory cells were used. (A) Memory cells were cultured  $\pm$  20 ng/ml IL-7 and/or 10 ng/ml IL-15 (top panel)  $\pm$  25  $\mu$ g/ml IL-7R $\alpha$  and IL-15R $\beta$  blocking antibodies (middle panel)  $\pm$  NF- $\kappa$ B inhibitor (bottom panel). Eomes levels were determined by flow cytometry after 24 hrs. Histograms representative of 2 independent experiments. (B) Memory cells were cultured with 20 ng/ml IL-7 and 10 ng/ml IL-15  $\pm$  5  $\mu$ g/ml blocking antibodies against CD27, OX40 or both (top panel) or respective isotype controls (bottom panel). Eomes levels were determined by flow cytometry after 24 hrs. Histograms representative of 2 independent experiments. (C)  $5 \times 10^5$  memory OT-I T cells were adoptively transferred into B6Rag<sup>-/-</sup> or B6Rag<sup>-/-</sup>/β2m<sup>-/-</sup> hosts. After 28 days, memory cells were harvested, and Eomes expression was determined by flow cytometry. Graph shows geometric MFI (mean $\pm$ SEM). Data representative of 2 independent experiments; n=3 mice.

whether they were able to receive self-pMHC signals through the TCR (Figure 4-9B).

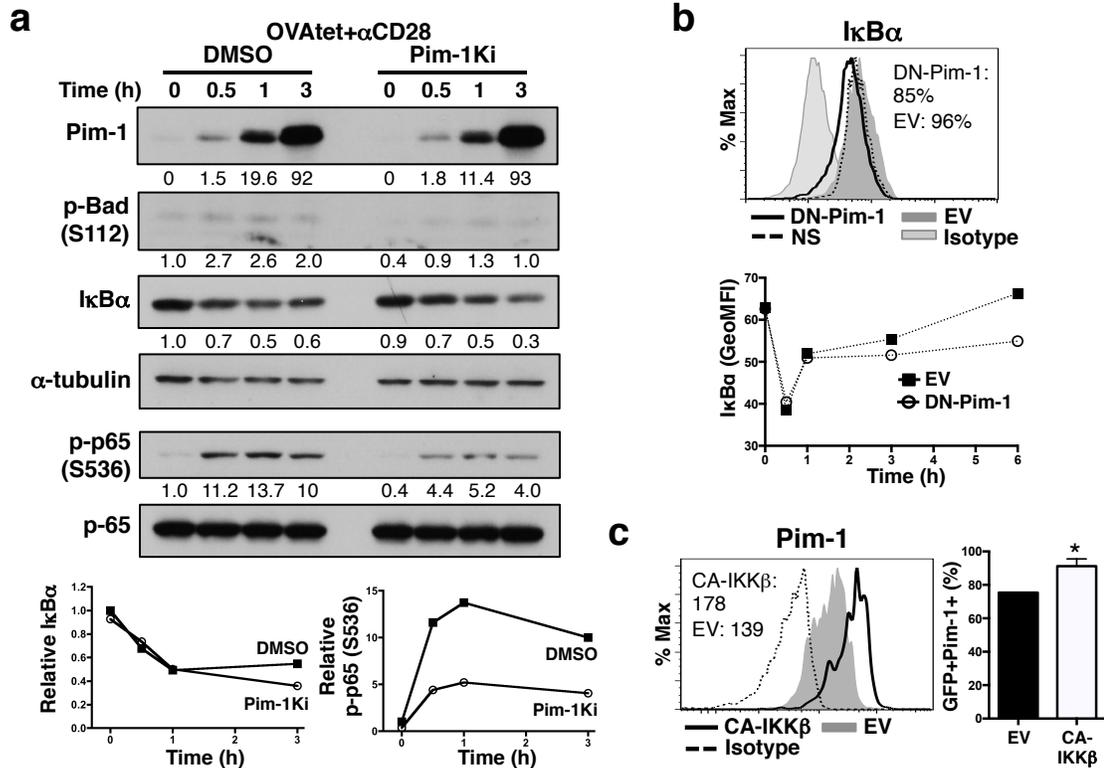
Costimulatory molecules OX40 and CD27 regulate memory generation and secondary responses (Hendriks et al., 2000; Ruby et al., 2007). Furthermore, both are part of the TNFR superfamily and are inducers of NF- $\kappa$ B signaling (Akiba et al., 1998; Ramakrishnan et al., 2004; Song et al., 2008). Activation of CD27 and OX40 signaling with agonist antibodies did not alter Eomes expression in memory T cells (Figure 4-9C). In addition, adoptive transfer of memory cells into mice genetically deficient in the ligand for CD27, CD70, did not affect their expression of Eomes or have an impact on their long-term survival (data not shown). Overall, these results show that the maintenance of NF- $\kappa$ B-dependent Eomes expression is not dependent on extrinsic factors IL-7 and IL-15, self-pMHC-TCR interaction, nor costimulatory molecules CD27 and OX40.

*NF- $\kappa$ B and Pim-1 regulate each other in CD8 T cells.*

Our data indicate that none of the known extrinsic factors involved in memory generation or survival support NF- $\kappa$ B-dependent Eomes expression at memory. This strongly suggests that programming of NF- $\kappa$ B-dependent late Eomes expression occurs during priming. For this to occur, we hypothesized that early TCR-dependent NF- $\kappa$ B signals program the expression of a protein involved in supporting a continuum of NF- $\kappa$ B signaling. Interestingly, Pim-1 kinase is involved in the regulation of NF- $\kappa$ B signaling, expressed upon TCR signaling, and constitutively active-once expressed (Bachmann and Möröy, 2005;

Fox et al., 2003; Fox et al., 2005; Nihira et al., 2009). Thus, we tested the potential role of Pim-1 in the regulation of NF- $\kappa$ B signaling in CD8 T cells. For this, OT-I T cells were stimulated with K<sup>b</sup>-OVA-tetramer (OVA<sub>tet</sub>) and  $\alpha$ -CD28 in the presence of a Pim-1 kinase inhibitor (Pim-1Ki) or vehicle control. The expression of Pim-1 is induced after 30 minutes of TCR stimulation, peaks at three hours and was not affected by the presence of the inhibitor (Figure 4-10A). Phosphorylation of p65- NF- $\kappa$ B (p-p65(S536)) was observed at the same time Pim-1 expression was induced and maintained even at times (3h) when TCR expression was down-regulated (Teixeiro et al., 2009c). However, CD8 T cells with inhibited Pim-1 kinase activity showed a 50% reduction in the phosphorylation of p65- NF- $\kappa$ B. As expected, I $\kappa$ B $\alpha$  levels decreased in CD8 T cells upon 30 min of stimulation and started to return to basal levels at 3 hours as a consequence the known NF- $\kappa$ B feedback loop that regulates I $\kappa$ B $\alpha$  expression (Ghosh and Baltimore, 1990; Sun et al., 1993). On the contrary, in the presence of the Pim-1Ki, I $\kappa$ B $\alpha$  levels continued to decrease as Pim-1 levels increased. The levels of phospho-Bad (p-Bad), a known target of Pim-1 kinase activity, were also decreased in the presence of Pim-1Ki (Figure 4-10A).

To confirm the results seen with the chemical inhibitor, activated OT-I T cells were retrovirally transduced with a dominant negative, kinase dead form of Pim-1 and stimulated with PMA+ionomycin to induce maximal activation of NF- $\kappa$ B signaling (Kim et al., 2010). I $\kappa$ B $\alpha$  degradation and re-expression in empty vector or DN-Pim-1 GFP+ transduced T cells was monitored over 6 hours. As expected, degradation of I $\kappa$ B $\alpha$  was similar between EV- and DN-Pim-1-



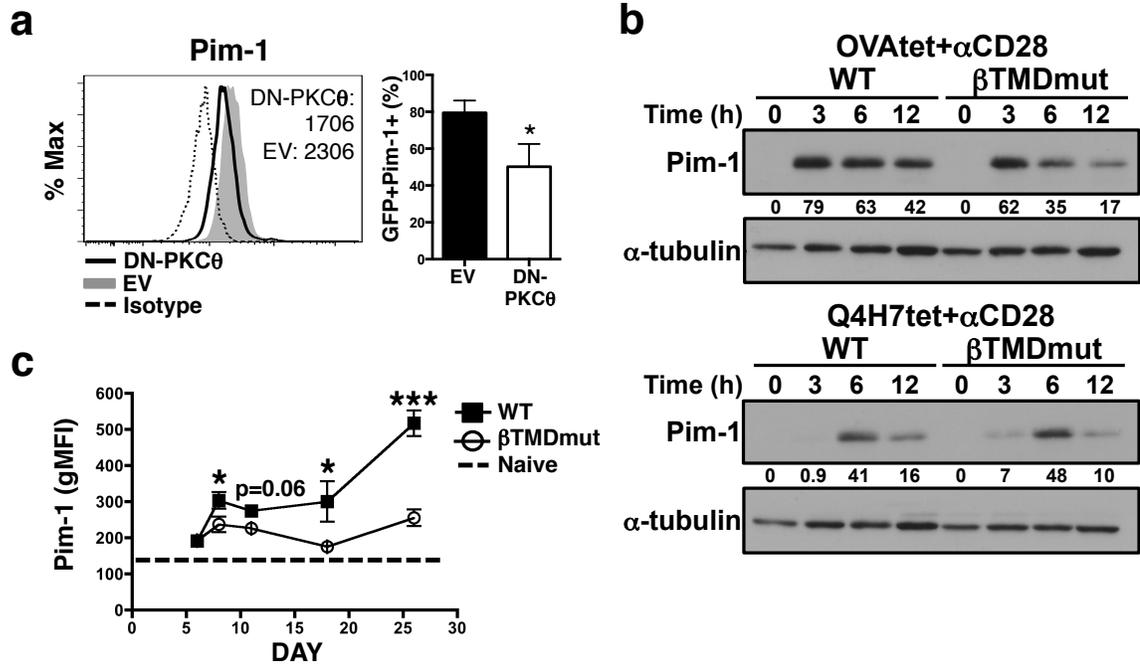
**Figure 4-10. Pim-1 and NF- $\kappa$ B reciprocally regulate each other in CD8 T cells.** (A) Naïve OT-I T cells were pretreated with 10 $\mu$ M Pim-1 kinase inhibitor (Pim-1Ki) or vehicle for 30 minutes and then stimulated with OVA-tetramer+ $\alpha$ CD28 for the indicated times. Phosphorylation of p65-NF- $\kappa$ B and Bad and levels of p65- NF- $\kappa$ B, Pim-1, I $\kappa$ B $\alpha$ , and tubulin were determined by immunoblot. Graphs show kinetics of I $\kappa$ B $\alpha$  degradation and re-expression and phosphorylation of p65- NF- $\kappa$ B. Immunoblots and graphs representative of 2 independent experiments. (B) OT-I T cells were stimulated with 20nM OVA for 24h and spin transduced with DN-Pim-1 or empty vector RV. 24 hours after the 2<sup>nd</sup> spin transduction (d4), OT-I T cells were stimulated with PMA+ionomycin for the indicated times. Expression of I $\kappa$ B $\alpha$  in GFP+ T cells was determined by flow cytometry. Data representative of 1 experiment. (C) OT-I T cells were stimulated with 20nM Q4H7 for 24h and spin transduced with CA-IKK $\beta$  or empty vector RV. Pim-1 expression was determined in GFP+ cells by flow cytometry at day 2 post-transduction. Numbers in histograms are geometric MFI of indicated population. Graph shows % GFP+ cells with Pim-1 over the isotype level (mean $\pm$ SD). Histograms and graphs representative of  $\geq$  2 independent experiments. \*p  $\leq$  0.05.

transduced T cells. Importantly, re-expression of  $\text{I}\kappa\text{B}\alpha$ , shown in both frequency of positive cells and geometric MFI, was impaired when Pim-1 kinase activity was repressed (Figure 4-10B). Together these data show for the first time that Pim-1 regulates NF- $\kappa$ B signaling at late time points upon CD8 T cell stimulation.

We also addressed whether Pim-1 expression was dependent on NF- $\kappa$ B signaling. Pim-1 levels were determined in OVA-stimulated T cells transduced with CA-IKK $\beta$  RV or an empty vector control. Constitutive activation of NF- $\kappa$ B signaling led to up-regulation of Pim-1 expression that was absent in CD8 T cells transduced with the empty vector (Figure 4-10C). This indicates that NF- $\kappa$ B signaling regulates Pim-1 expression in CD8 T cells. Collectively, these results demonstrate that NF- $\kappa$ B and Pim-1 regulate each other in CD8 T cells and suggest this as a mechanism to preserve a continuum of NF- $\kappa$ B signaling when antigenic signals are diminishing.

*TCR-dependent NF- $\kappa$ B signaling is required for Pim-1 expression.*

Pim-1 is expressed upon TCR stimulation (Fox et al., 2005) (Figure 4-10A). We have also shown that NF- $\kappa$ B signaling regulates Pim-1 expression (Figure 4-10C). Next, we determined whether TCR-dependent NF- $\kappa$ B signaling is involved in this process. To examine this, we first inhibited TCR-dependent NF- $\kappa$ B signals by overexpressing DN-PKC $\theta$  (Figure 4-5). Indeed, T cells expressing DN-PKC $\theta$  exhibited lower levels of Pim-1 upon T cell activation than EV-transduced controls (Figure 4-11A). To more precisely connect TCR-dependent NF- $\kappa$ B signaling with Pim-1 expression, we used the TCR mutant model where



**Figure 4-11. TCR-dependent NF- $\kappa$ B signaling is required for Pim-1 expression.** (A) OT-I T cells were stimulated with 20nM OVA for 24h and spin transduced with DN-PKC $\theta$  RV or control RV. Pim-1 expression and phosphorylation of Bad were determined in GFP+ cells by flow cytometry at day 2 post-transduction. Numbers under histograms represent geometric MFI of respective population. Graph shows % GFP+ cells with Pim-1 over the isotype level (mean $\pm$ SD). Histogram and graph are representative of 2 independent experiments. (B) Naïve OT-I and  $\beta$ TMDmut CD8 T cells were stimulated with OVA- or Q4H7-tetramer and  $\alpha$ -CD28 for the indicated times. Pim-1 and  $\alpha$ -tubulin levels were determined by immunoblot. Data representative of 2-3 independent experiments. (C)  $5 \times 10^3$  OT-I and  $\beta$ TMDmut T cells were adoptively transferred into congenic hosts and challenged with  $7 \times 10^6$  CFU Att-LM-OVA (ActA-). The kinetics of Pim-1 expression was determined in the blood by flow cytometry. Data is representative of three independent experiments; n=4 mice per group. \* $p \leq 0.05$ , \*\* $p \leq 0.005$ , \*\*\* $p \leq 0.001$ .

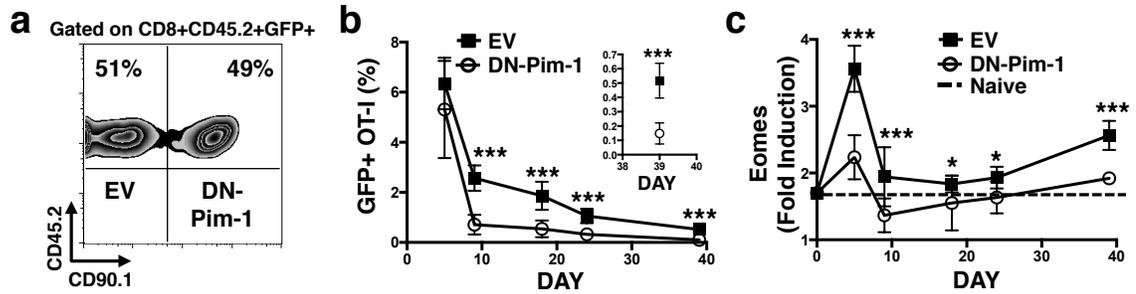
only NF- $\kappa$ B signals are impaired upon strong antigenic stimulation (Knudson et al., 2013b; Teixeira et al., 2009; Teixeira et al., 2004; Teixeira et al., 1999). WT and MUT OT-I T cells were stimulated with K<sup>b</sup>-OVA-tetramer and  $\alpha$ -CD28, and Pim-1 expression was examined. WT and MUT T cells both induced Pim-1 by three hours after stimulation. WT T cells were able to sustain appreciable levels of Pim-1 up to 12 hours. However, MUT T cells lost Pim-1 expression after 6 hours of OVA stimulation and did not recover it at later time points (Figure 4-11B). Remarkably, Pim-1 expression in  $\beta$ TMDmut T cells was maintained at WT levels in conditions where TCR-dependent NF- $\kappa$ B signaling is recovered in MUT cells (weak TCR stimulation) (Figure 4-11B) (Knudson et al., 2013b). From these results, we conclude that TCR-dependent NF- $\kappa$ B signaling regulates Pim-1 expression.

Next, we investigated how TCR-dependent NF- $\kappa$ B signaling affects Pim-1 expression upon infection. Naïve WT and MUT OT-I T cells were transferred into congenic hosts and challenged with LM-OVA. Pim-1 levels in WT OT-I at the peak of the response were similar to naïve. As the WT T cells underwent contraction and developed into memory, Pim-1 expression gradually increased with the highest levels seen in memory cells (Figure 4-11C). This late increase in Pim-1 expression at memory correlated with a concurrent increase in Eomes expression (Figure 4-5). Strikingly, MUT T cells, correlating with their defect in Eomes expression and memory generation, did not up-regulate Pim-1 expression after the peak of the response. In addition, MUT cells expressed significantly less Pim-1 than WT T cells during contraction (Figure 4-11C). Interestingly, reduced

levels of Pim-1 expression appear in MUT cells at the same time as Eomes expression levels declined at the beginning of the memory phase, suggesting Pim-1 is involved in the regulation of Eomes (Figure 4-5). Together, these results support that TCR-dependent NF- $\kappa$ B signaling regulates Pim-1 expression in CD8 T cells *in vitro* and *in vivo*. Furthermore, our data in the memory defective  $\beta$ TMDmut model strongly suggest that Pim-1 plays an important role in memory generation.

*Pim-1 kinase activity is important for late Eomes expression and memory CD8 T development.*

To investigate whether Pim-1 regulates memory development and Eomes expression during an immune response, OT-I T cells were transduced to overexpress the DN-Pim-1 (Kim et al., 2010) or empty vector control (Figure 4-9). Equal numbers of DN-Pim-1-GFP<sup>+</sup> and EV-GFP<sup>+</sup> OT-I T cells were co-transferred into naïve hosts (Figure 4-12A). They were then infected with LM-OVA. Although the T cells containing DN-Pim-1 expanded similarly to control T cells, impaired Pim-1 kinase activity led to an increased loss of OT-I responders after the peak of the response. This difference was maintained into the memory phase (Figure 4-12B). Next, we determined whether Pim-1 affected Eomes expression *in vivo*. Eomes expression in the EV control T cells was highest around the peak of the immune response and decreased during contraction before levels stabilized around day 18 (Figure 4-12C). Eomes levels increased slightly as the T cells proceeded later into memory (Figure 4-5 and 4-12C).



**Figure 4-12. Pim-1 promotes memory generation and Eomes expression during infection.**

Naïve OT-I T cells were stimulated with 20nM OVA and spin transduced with DN-Pim-1 RV or empty vector RV. 24h after last transduction,  $1 \times 10^6$  EV (CD45.2+CD90.1-) and  $1 \times 10^6$  DN-Pim-1 (CD45.2+CD90.1+) GFP+ OT-I were mixed (A), co-transferred into naïve hosts (CD45.1+CD90.1-), and hosts were challenged with  $7 \times 10^6$  CFU Att-LM-OVA. (B) The kinetics of EV and DN-Pim-1 GFP+ OT-I T cells were determined after challenge. (C) Eomes levels (fold induction over isotype control) of GFP+ OT-I in the blood were determined by flow cytometry. All graphs show mean $\pm$ SD. Data representative of 1 independent experiment; n=7 mice. \*p  $\leq$  0.05, \*\*p  $\leq$  0.005, \*\*\*p  $\leq$  0.001.

DN-Pim-1-transduced OT-I T cells had a similar Eomes expression during the peak of the response and early in contraction as compared to EV-transduced T cells. Strikingly, as Eomes levels stabilized in EV control cells, they continued to decrease in T cells with impaired Pim-1 kinase activity (Figure 4-12C). This indicates that Pim-1 is important for the late expression of Eomes. All together, these data reveal a novel role for Pim-1 in supporting Eomes expression and T cell memory development.

## **Discussion**

CD8 T cell memory development is governed by signals provided by antigenic peptide-MHC-TCR interaction, costimulation, and inflammation (Williams and Bevan, 2007). However, it is largely unknown how these signals individually or together support the expression of transcription factors important in memory generation, such as Eomes. Here, we demonstrate that NF- $\kappa$ B signaling is a key pathway for the regulation of CD8 T cell memory. We found that TCR-dependent NF- $\kappa$ B signaling triggers a program to regulate late Eomes expression, which is necessary for the generation of memory T cells. Furthermore, we show that NF- $\kappa$ B is required for memory survival and responses. We also identified Pim-1 as a novel regulator of Eomes expression that links TCR signals and NF- $\kappa$ B with CD8 T cell memory.

NF- $\kappa$ B signaling is important in thymic development, mature T cell survival, and the early activation of T cells (Boothby et al., 1997; Hettmann et al., 1999; Jimi et al., 2008; Kingeter and Schaefer, 2008; Krishna et al., 2012;

Schmidt-Supprian et al., 2003). Previous studies have implicated several NF- $\kappa$ B signaling components, such as IKK $\beta$  and I $\kappa$ B $\alpha$ , in T cell memory generation (Hettmann et al., 2003; Schmidt-Supprian et al., 2003). However, due to the differential requirement of NF- $\kappa$ B signaling in thymic development of CD8 and CD4 T cells (CD8 T cells are more dependent on NF- $\kappa$ B signaling for their progress through thymic selection (Jimi et al., 2008)), many of these studies were performed with CD4 T cells. Furthermore, some reports only considered CD44<sup>hi</sup> expression on T cells as a readout of memory generation and did not examine memory development during the context of infection (Schmidt-Supprian et al., 2003). NF- $\kappa$ B signaling has also been shown to play a role in the regulation of memory recall responses (Lai et al., 2011), but again these studies were performed with CD4 T cells. A recent study established that NIK, a member of the non-canonical NF- $\kappa$ B pathway, is important for the generation of CD8 memory T cells upon infection (Rowe et al., 2013). Interestingly, while memory numbers were significantly reduced with loss of NIK, memory T cell responses were normal (Rowe et al., 2013). Antigenic receptors primarily activate canonical NF- $\kappa$ B signaling (Vallabhapurapu and Karin, 2009), so it was necessary to determine the contribution of canonical NF- $\kappa$ B signaling intermediates in memory CD8 T cell development. In addition, no previous studies have assessed the contribution of TCR-dependent NF- $\kappa$ B signaling in programming memory CD8 T cell differentiation. The data presented here demonstrate that sustained NF- $\kappa$ B signaling is required to promote memory differentiation. Impaired NF- $\kappa$ B signaling leads to loss of IL-7R<sup>hi</sup> memory precursor development and memory generation.

The role we describe for NF- $\kappa$ B in memory CD8 T cell differentiation is independent of its impact on thymic selection, as NF- $\kappa$ B transcriptional activity was altered after T cell priming during infection. In addition, use of the  $\beta$ TMDmut T cells, which undergo normal thymic selection but have impaired activation of peripheral TCR-dependent NF- $\kappa$ B signaling and loss of memory generation, also supports this conclusion. Thus, our data demonstrate, for the first time, that proper NF- $\kappa$ B signaling is required for CD8 T cell memory differentiation.

Eomes is an important transcription factor in memory CD8 T cell development and function (Banerjee et al., 2010; Intlekofer et al., 2007; Intlekofer et al., 2005). Genetic loss of *Eomes* in CD8 T cells leads to decreased memory T cell numbers during infection and skewing of the remaining memory T cells toward an effector memory phenotype (Banerjee et al., 2010). mTOR and Wnt/TCF-1 signaling are main signaling pathways described in the regulation of Eomes expression in CD8 T cells (Rao et al., 2010; Zhou et al., 2010). Interestingly, MUT T cells, which have a consistent decrease in Eomes expression throughout the immune response, do not have altered mTOR signaling or TCF-1 expression (data not shown). In fact, the only defect described in MUT TCR-dependent signaling is in the induction of NF- $\kappa$ B signaling. Thus, we propose that NF- $\kappa$ B signaling is the dominant regulator of Eomes expression in CD8 T cells. Supporting this conclusion, alteration of upstream NF- $\kappa$ B-dependent signaling molecules PKC $\theta$  and IKK $\beta$  affect Eomes expression. Furthermore, impaired NF- $\kappa$ B transcriptional activity prevents proper Eomes expression both *in vitro* and *in vivo*. This observation is in agreement with

previous studies that show that, in cooperation with Notch, NF- $\kappa$ B is recruited to the *Eomes* promoter and mediates its transcription (Cho et al., 2009). These studies, however, did not address the independent role for NF- $\kappa$ B in regulating *Eomes* expression or whether NF- $\kappa$ B-dependent control of *Eomes* occurred *in vivo*. Our data distinctly demonstrates that NF- $\kappa$ B signaling promotes *Eomes* expression during infection. Additionally, we also demonstrate that proper NF- $\kappa$ B-dependent control of *Eomes* is required for memory development.

In addition to supporting memory generation, *Eomes* regulates memory CD8 T cell survival (Banerjee et al., 2010). In fact, we and others show that *Eomes* levels in memory CD8 T cells are higher than in naïve T cells (Intlekofer et al., 2007; Paley et al., 2013). The reason for higher expression of *Eomes* in memory is unknown. One potential explanation is to regulate pro-survival genes such as Bcl-2 to promote memory survival. Genetic loss of *Eomes* in memory T cells leads to decreased Bcl-2 expression and impaired memory longevity (Banerjee et al., 2010). Whether this is a direct effect of *Eomes* on Bcl-2 expression has not been investigated. Interestingly, we show that NF- $\kappa$ B inhibition at memory impairs both *Eomes* and Bcl-2 expression in memory T cells. This leads to deficient memory CD8 T cell survival. Bcl-2 is a known NF- $\kappa$ B target, so the regulation of Bcl-2 expression in memory cells may be independently regulated by NF- $\kappa$ B (Zheng et al., 2003). It is also possible, however, that NF- $\kappa$ B-dependent *Eomes* expression maintains proper Bcl-2 levels in memory T cells. Future investigations are required to address this possibility. Another potential reason for maintenance of higher *Eomes* expression in memory

cells is to promote rapid effector function upon antigen encounter. A previous study has shown that inhibition of NF- $\kappa$ B-dependent Eomes expression at memory prevents early production of Granzyme B after infectious challenge (Pearce et al., 2003). Supporting this, we also show that inhibition of NF- $\kappa$ B signaling in memory T cells prevents activation, expansion, and effector molecule production after infectious re-challenge.

Antigenic pMHC-TCR signal is required for activation of T cells and early setup of effector and memory programming. However, this signal is lost after clearance of the pathogen, which typically occurs before the peak of the CD8 T cell response. Thus, cognate antigenic pMHC-TCR signaling is most likely not responsible for the induction of NF- $\kappa$ B signaling and maintenance of Eomes expression during contraction and at memory. This observation begs the question of what maintains NF- $\kappa$ B-dependent Eomes expression during these timepoints. Surprisingly, despite playing important roles in either the development or survival of memory cells (Goldrath and Bevan, 1999; Goldrath et al., 2000; Hendriks et al., 2000; Ku et al., 2000; Lodolce et al., 1998; Schluns et al., 2000), homeostatic cytokines IL-7 and IL-15, self-pMHC-TCR signals, and receptors CD27 and OX40 do not independently support Eomes expression in memory cells. Studies need to be performed to determine if the cooperation between these different signals promote NF- $\kappa$ B-dependent Eomes expression at memory. Regardless, these results suggested that later Eomes expression is programmed early in the immune response. This is a definite possibility as it only requires 48-72 hours of signaling with antigen, costimulation, and inflammation for a naïve T

cell to obtain all the signals required for functional memory development (van Stipdonk et al., 2003).

Pim-1 is a serine-threonine kinase expressed in hematopoietic cells that has been shown to mediate differentiation, survival, and proliferation of multiple cell types (Bachmann and Möröy, 2005; Eichmann et al., 2000). Experimental evidence, demonstrated previously and in the studies presented here, supports that Pim-1 expression is induced by antigenic receptors, is regulated by NF- $\kappa$ B signaling, and can also support sustained NF- $\kappa$ B signaling (Fox et al., 2005; Kim et al., 2010; Nihira et al., 2009). This made Pim-1 an attractive target to investigate in the maintenance of NF- $\kappa$ B-dependent Eomes expression late in the immune response. Interestingly, Pim-1 expression increased as CD8 T cells progressed toward the memory timepoint. This up-regulation of Pim-1 expression correlates with the increased frequency of IL-7R<sup>hi</sup> memory precursors which must maintain high levels of pro-survival factors to progress to memory (Kurtulus et al., 2011). Importantly, impaired Pim-1 kinase activity prevented re-expression of Eomes late in the immune response. This also led to a decreased frequency of memory T cells at the end of the immune response. These data suggest a novel role for Pim-1 in promoting CD8 T cell memory development through the regulation of Eomes.

Pim-1 has been shown to be important in T cell proliferation *in vitro* (Fox et al., 2005). However, inhibition of Pim-1 kinase activity did not affect expansion of CD8 T cells in response to LM-OVA infection. As CD4 T cells genetically deficient in both Pim-1 and Pim-2 do not have a problem in expansion during

infection (Fox et al., 2005), these results suggest that Pim family members are not important in the activation or proliferation of naïve T cells after infectious challenge. This correlates with the lower expression of Pim-1 at the peak versus memory timepoints during infection. After the peak of the response, however, impaired Pim-1 activity led to lower recruitment of T cells into the memory pool as shown by increased contraction. This lower memory generation could be due to either impaired memory programming, as was the case when NF- $\kappa$ B signaling was inhibited (Knudson et al., 2013b), or memory survival. Altered Pim-1 kinase activity did not affect the development of IL-7R<sup>hi</sup> memory precursors at the peak of the response or early in contraction (data not shown). This suggests that while the generation of memory precursors was normal in T cells expressing DN-Pim-1, these precursors could not survive the transition to memory. Pro-survival factor Bcl-2 is important for CD8 T cell survival during contraction and is regulated by both NF- $\kappa$ B signaling and Eomes (Banerjee et al., 2010; Kurtulus et al., 2011; Lilly et al., 1999). As inhibition of Pim-1 kinase activity impairs NF- $\kappa$ B signaling in activated T cells and lowers Eomes expression during late contraction, our results suggest that Pim-1 supports NF- $\kappa$ B- and Eomes-dependent survival of CD8 T cells as they progress to memory. Pim-1 can regulate the expression and activity of pro- and anti-apoptotic factors such as Mcl-1 and Bad (Aho et al., 2004; Peperzak et al., 2010). Both of these molecules have been implicated in T cell survival upon activation (Aho et al., 2004; Peperzak et al., 2010). Whether Pim-1-dependent regulation of these molecules affects memory generation needs to be investigated.

In conclusion, our data identifies a novel cooperation between the TCR signal, NF- $\kappa$ B, and Pim-1 that promotes sustained expression of Eomes and the generation of memory CD8 T cells. TCR-dependent NF- $\kappa$ B signaling promotes early expression of Eomes and programs memory differentiation. Then, Pim-1 supports sustained NF- $\kappa$ B-dependent Eomes expression, aiding in the late development and maintenance of memory CD8 T cells. This information is highly relevant for vaccine development and posits the modulation of NF- $\kappa$ B signaling and Pim-1 as targets for immunotherapies for infectious diseases and cancer.

## **Conclusion**

In this set of studies, we investigated the mechanism by which TCR signals, specifically TCR-dependent NF- $\kappa$ B signaling, regulates memory generation, survival, and responses. Importantly, the results presented here demonstrate that NF- $\kappa$ B signaling is required for memory generation, confirming the hypotheses set forth by previous studies (Knudson et al., 2013b; Teixeira et al., 2009). In addition, NF- $\kappa$ B signaling regulated the expression of memory-associated factor Eomes during the primary immune response. Thus, TCR-dependent NF- $\kappa$ B signaling is required for Eomes expression and memory CD8 T cell development.

In regards to the role of NF- $\kappa$ B signaling at memory, memory CD8 T cells required sustained NF- $\kappa$ B signaling to support Eomes expression. Furthermore, loss of NF- $\kappa$ B signaling impaired both memory survival and memory response to rechallenge. As TCR interaction with cognate antigen dissipates as an immune response progresses, we investigated the contribution of other signals to sustain NF- $\kappa$ B signaling. Surprisingly, the signals required for memory T cell survival, such as IL-7, IL-15, and CD27, did not support NF- $\kappa$ B signaling. This suggested that the continuum of NF- $\kappa$ B signaling is programmed early during the immune response. Indeed, we identified a novel role for Pim-1 kinase in the maintenance of NF- $\kappa$ B signaling and Eomes expression late during infection. Loss of Pim-1 kinase activity impaired memory generation and prevented proper expression of Eomes at the memory timepoint.

These studies uniquely demonstrate that TCR-dependent NF- $\kappa$ B signaling programs memory differentiation and maintenance. Furthermore, we show that early TCR signaling promotes expression of Pim-1, which then sustains NF- $\kappa$ B signaling and Eomes expression for memory generation and survival. Together, these results further demonstrate the importance of proper TCR signaling in CD8 T cell memory generation.

## **Materials and Methods**

### *Mice and Reagents*

C57BL/6, C57BL/6 Rag<sup>-/-</sup>, B6.SJL, OT-1 and  $\beta$ TMDmut (MUT) TCR transgenic mice (Teixeiro et al., 2009b), and B6Rag<sup>-/-</sup>b2m<sup>-/-</sup> (Daniels et al., 2006b) were bred and maintained in accordance with University of Missouri OAR ACUC or University of Minnesota IACUC. OVA and Q4H7 peptides were purchased from New England Peptides. rmlL-7 and rmlL-15 were from Peprotech. IL-2 (X63-IL-2 hybridoma) was used at 50U/ml. Wedelolactone (IKKc inhibitor), Rapamycin, and Pim-1 kinase inhibitor IV (Pim-1Ki) were purchased from EMD/Calbiochem.

### *Antibodies and Flow Cytometry*

Anti-CD8 $\alpha$  (53-6.7), -CD90.1 (OX-7), -IL-7R $\alpha$  (A7R34), -CD62L (MEL-14), -CD28 (37.51), -CD122 (TM $\beta$ 1), -CD43 (1B11), and -Bcl-2 (3F11) were from BD Pharmingen. Anti-CD45.1 (A20), -CD45.2 (I04), -CD27 (LG.7F9), -OX40 (OX-86), and -Eomes (Dan11Mag) were from eBioscience. Anti-T-bet (4B10) and -Pim-1 (12H8 and C20) were from Santa Cruz Biotechnology. Anti-IkBa (5A5 and L35A5), -p65-NF- $\kappa$ B (D14E12), -phospho-p65-NF- $\kappa$ B (S536), -phospho-Bad (S112), and -phospho-mTOR (S2481) were from Cell Signaling. Anti- $\alpha$ -tubulin was purchased from Sigma. Granzyme B and secondary antibodies were from Invitrogen. Flow cytometry was performed on a FACSCalibur or LSRIIFortessa flow cytometer (Becton Dickinson), and the data was analyzed with FlowJo FACS Analysis Software (Tree Star, Inc.).

### *Adoptive Transfer*

Naïve ( $1 \times 10^3$ - $1 \times 10^4$ ) or memory CD8 T cells were purified from the lymph nodes of OT-1, MUT or host mice and transferred intravenously (i.v.) into congenic mice.

### *Bacteria*

Listeria strains were generously provided by M. Bevan (University of Washington) (LM-OVA and LM-Q4H7) and Sing Sing Way (University of Minnesota) (Att-LM-OVA). Att-LM-OVA strains were grown to an  $OD_{600}$  of 0.1 as described in (Teixeiro et al., 2009b) while LM-OVA and LM-Q4H7 strains were grown as described in (Zehn et al., 2010). All infections were performed i.v. at least one day after adoptive transfer of naïve transgenic T cells.

### *Retroviral Transduction*

The generation of retroviral particles was performed using the Platinum-E Retroviral Packaging Cell Line (Cell Biolabs) and Genejammer (Agilent Technologies, Inc.) according to the manufacturer's instructions. Spin transfection was performed two ways. For transduction of in vitro-activated CD8 T cells, 24h after stimulation with 20nM OVA or Q4H7 peptides,  $5 \times 10^5$  OT-I T cells were plated each well of 96-well plates. 100-200ul viral supernatant supplemented with 8µg/ml Polybrene (Millipore) and IL-2 was added to each well, and plates were spun at 1800rpm for 75 minutes at 32°C. After an additional 4h incubation at 32°C, viral supernatant was removed, and cells were resuspended

in media containing 20nM peptide and IL-2 and incubated at 37°C overnight. A second round of spin transduction was performed 24h later. Cells were resuspended in IL-2-containing media after the second spin transduction. For transduction of in vivo-primed CD8 T cells, OT-I T cells were harvested after adoptive transfer and challenge for 4-5 days.  $5 \times 10^5$  OT-I T cells were plated each well of 96-well plates. Two rounds of spin transductions were performed by adding 100-200ul viral supernatant supplemented with 8µg/ml Polybrene (Invitrogen) and 10ng/ml IL-7 was to each well, and plates were spun at 1800rpm for 75 minutes at 32°C. After an additional 4h incubation at 32°C, viral supernatant was removed, and cells were resuspended in media containing 10ng/ml IL-7 and incubated at 37°C overnight. In both conditions, GFP+ T cells were adoptively transferred 24h after last spin transduction.

#### *Immunoblot Analysis*

$2-5 \times 10^6$  T cells were stimulated with OVA- or Q4H7-tetramer and anti-CD28 subjected to immunoblot as described in (Daniels et al., 2006a). Densitometry was determined using ImageJ Software.

#### *Statistical Analysis*

For statistical analysis, two-tail unpaired Student's t test was applied using GraphPad Prism software. Significance was set at  $p < 0.05$ .

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## **V: Discussion and Future Directions**

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CD8 T cell memory development is essential for the long term protection against various immune insults, including viral infection and tumors. It was not fully understood how memory T cells are generated during immune responses. The goal of these studies was to provide further insight into the process of memory CD8 T cell differentiation. We focused on how the T cell receptor signal specifically controls memory development. We demonstrate that different TCR signals can program memory T cell fate. In particular, the pMHC-TCR affinity affects CD8 T cell differentiation as well as its ability to respond at memory. We also define a TCR-dependent mechanism, through induction of NF- $\kappa$ B signaling, that is required for these processes. This is the first demonstration that NF- $\kappa$ B signaling directly regulates memory CD8 T cell generation. While we provide important insight into CD8 T cell memory development, we also uncover many questions that require further investigation.

### **Low-Affinity T Cell Function in Immunity**

The generation of immunological memory protects a host from future infection with the same pathogen. It was originally thought that only T cells with TCRs that could bind pathogen-specific antigens with high-affinity would be recruited into the responding T cell pool and generate memory. This conclusion is attributed to the hypothesis that recruitment of low-affinity clones, which could respond to pMHC in the range of self, would induce autoimmunity (discussed below). In addition, the techniques employed for investigating antigen-specific T cell responses rely on high-affinity pMHC-TCR interaction. Many studies use

monoclonal T cell populations and a pathogen expressing their cognate antigen to investigate memory generation. Due to this, these studies only focus on the response of high-affinity T cell clones. Even when studying polyclonal T cell responses, investigators typically use pMHC-tetramers to distinguish antigen-specific populations. The T cells that are identified by this tool, however, must express TCRs with a relatively high-affinity for the given peptide. These limitations led to gross underestimation of the diversity and affinity of TCRs recruited into a T cell response. Sabatino et al. performed elegant experiments to identify and characterize low-affinity clones during both infectious and autoimmune responses. Using a micropipette-based 2D binding technology, they measured the affinities and frequencies of polyclonal CD4 T cells (Sabatino et al., 2011). They showed that the frequency of low-affinity responders to LCMV epitopes was similar to high-affinity T cells. In addition, low-affinity T cells produced cytokines IFN $\gamma$  and TNF $\alpha$ , indicating that they contribute to the immune response (Sabatino et al., 2011). While memory development by low-affinity clones was not examined, this study demonstrates that low-affinity T cells are activated and function during immune responses.

Recent studies using low-affinity altered peptide ligands as antigens demonstrated that low-affinity-stimulated T cells do develop into functional memory (Zehn et al., 2009). We show here that when re-challenged in the context of their specific cognate antigen, low-affinity-generated memory T cells respond similarly to high-affinity-generated memory T cells. They were able to expand and produce effector molecules Granzyme B and IFN $\gamma$  (Chapter II).

Although bacterial titers were not directly analyzed, I hypothesize that the ability of high and low-affinity memory T cells to clear pathogen is similar due to the observed effector phenotype. Of course, this does not take into account the extreme differences in total number of memory T cells generated at the end of high and low-affinity-primed immune responses. Weak pMHC-TCR interaction during priming leads to lower total memory numbers during infection (Chapter III and (Zehn et al., 2009)), so it is possible that clearance would be slower in these memory responses. Regardless, these studies show that low-affinity pMHC-TCR encounter promotes full memory differentiation. The following question remains: why are low-affinity T cell clones recruited into an immune response and generate memory? There is limited space in the lymphoid compartment, so maintaining a memory population of low-affinity T cells may be unfavorable. This is especially true considering low-affinity memory T cells have not and may never encounter their cognate antigen. I argue that the generation of a low-affinity memory population serves to maintain the diversity of the T cell pool. The generation of naïve T cells decreases as we age and we are increasingly dependent on the memory T cell pool for protective immunity (Thome et al., 2014). If immune responses only generated high-affinity memory T cells, this would select for a small number of TCRs. Due to this, we would be vulnerable to infection with closely related but distinct pathogens, such as viral variants. This has been demonstrated by van Gisbergen et al. In their studies, they showed that low-affinity memory generation was dependent on expression of CD27, a costimulatory molecule described here as having greater expression on low

versus high-affinity-stimulated T cells (Chapter II). Importantly, impaired CD27 signaling during viral infection reduced the diversity of the overall T cell response and memory pool. This was due to specific loss of low-affinity T cell activation and memory differentiation. While this loss did not have an effect on protective immunity with a homologous re-infection, protection was not observed when viral variants were used (Wensveen et al., 2012). Many human pathogens mutate rapidly, such as HIV, or have closely related but distinct subtypes, like influenza (Posnett et al., 2005). Thus, the maintenance of a diverse population of memory T cells is indeed important for long-term protection against the greatest number of potential pathogens (Posnett et al., 2005).

There are some interesting questions that remain with regards to low-affinity memory generation and maintenance. A broader range of antigen potency than expected, but not every low-affinity or self-peptide, induces memory T cell generation (Zehn et al., 2009). There then must be a specific affinity threshold that is overcome for T cell activation and differentiation in response to low-affinity antigens. Determining this relative level could aid in the generation of modified tumor or pathogen-associated antigens to induce specific, broad affinity T cell responses in vaccination. In addition, it is also important to determine whether inflammation impacts this affinity threshold, as it has previously been described that inflammation can affect antigen sensitivity of CD8 T cells (Richer et al., 2013).

Due to the limited space in the immune compartment, the generation of T cell memory against irrelevant, low-affinity antigens could impair future

accumulation or survival of relevant clones. There is evidence, however, that the immune compartment can expand with immunological experience (Vezys et al., 2009). In order to regulate the number of memory T cells in that compartment, and to eliminate irrelevant memory cells, I hypothesize that the maintenance of low-affinity T cell memory is regulated differently than high-affinity memory. However, we have not directly compared the longevity or survival requirements of low and high-affinity memory T cells during steady-state or re-infection. Memory T cells compete for pro-survival factors for their homeostasis (Sprent, 2008), and it is possible that low and high-affinity memory T cells differentially compete or require these factors. Furthermore, it is possible that low and high-affinity memory T cells respond/survive differently during heterologous infection (in the absence of cross-reactive antigens). Both of these mechanisms would allow for a selective removal of memory clones that are less likely to encounter their cognate antigen in the future. This process would allow for the maintenance of protective, relevant memory T cells.

### **Potential of Low-Affinity Memory Cells to Induce Autoimmunity**

Memory CD8 T cells can respond to a lower concentration of antigen and require less time in contact with antigen to become activated than naïve T cells (Pihlgren et al., 1996; Rogers et al., 2000; Slifka and Whitton, 2001; Zimmermann et al., 1999). In addition, memory T cells are less dependent on costimulation for activation (Croft et al., 1994). Thus, while this is a topic of some debate (Mehlhop-Williams and Bevan, 2014), memory T cells are considered to

be more sensitive to antigen than their naïve counterparts. This endows memory T cells with the ability to provide superior protection against an infection versus naïve T cells. However, since memory T cells respond with rapid effector function after stimulation, it is possible that aberrant memory generation against low-affinity, pseudo-self peptides may induce an improper immune response against self, or autoimmunity. Strikingly, our results show that low-affinity-generated memory cells, when re-challenged with a low-affinity antigen in the range of self, do not produce effector molecules Granzyme B and IFN $\gamma$  (Chapter II). Although we could not directly test if low-affinity memory cells induce autoimmunity, we hypothesized that low-affinity memory cells are not able to mount an efficient effector response against self due to the defect in effector function. Recently, Koehli et al. directly tested the potential for low-affinity-primed T cells to induce autoimmunity. To do this, they generated a variation on the rat insulin promoter (RIP)-mOVA mouse, which expresses OVA in the thymus and pancreas (Kurts et al., 1996). When OT-I T cells are transferred into RIP-mOVA mice and challenged with LM-OVA, the mice become diabetic due to infiltration of OT-I T cells into the pancreas and destruction of the pancreatic islets (Koehli et al., 2014; Kurts et al., 1996). However, when OT-I T cells were transferred into RIP-mQ4H7 mice, which express the low-affinity antigen Q4H7, and challenged with LM-Q4H7, the mice did not develop diabetes (Koehli et al., 2014). Thus, corroborating our data, low-affinity-stimulated T cells are unable to induce an effector response against low-affinity, or pseudo-self, antigens.

The question remains, however, of why low-affinity-primed memory CD8 T cells are selectively attenuated in their effector response to low-affinity antigens. Low-affinity stimulated T cells produce IFN $\gamma$  and Granzyme B in response to weak antigen encounter during the primary immune response (Chapter II). However, weak antigen stimulation, as compared to strong antigen, induces low expression of IL-12R and do not respond to pro-inflammatory cytokine IL-12 (Knudson et al., 2013a). IL-12 is known to support sustained expression of IFN $\gamma$  and Granzyme B in activated T cells (Agarwal et al., 2009; Pipkin et al., 2010; Xiao et al., 2009). It is also required for promoting a permissive epigenetic state at the Granzyme B gene locus (Agarwal et al., 2009). Thus, it is possible that low-affinity antigen stimulation and the resulting lower response to IL-12 does not induce the required epigenetic changes for rapid effector molecule production at memory. However, as low-affinity memory T cells can produce these effector molecules in response to high-affinity antigen stimulation (Chapter II), low-affinity memory T cells are not irreversibly defective in effector function. They may require a stronger TCR signal to promote re-expression or production of IFN $\gamma$  and Granzyme B, which would be an efficient mechanism to prevent effector cytokine production in response to self-peptides.

Additionally, low-affinity antigen stimulation may promote susceptibility to negative regulatory mechanisms. Indeed, we show here that stimulation of naïve CD8 T cells with weak antigen prevents down-regulation of TGF $\beta$  receptor (Chapter II). TGF $\beta$  signaling restrains T cell proliferation, activation, and effector function (Li and Flavell, 2008). Deletion of the cytokine or its receptor leads to

severe dysregulation of T cell homeostasis and autoimmunity (Li and Flavell, 2008). Interestingly, TGF $\beta$  is important for regulating naïve T cells responses against low-affinity self-antigens (Johnson and Jameson, 2012; Zhang and Bevan, 2012). TGF $\beta$  does this by increasing the antigen dose threshold required for activation with weak peptides (Zhang and Bevan, 2012). This is most likely due to impaired down regulation of its receptor by weak antigens, as we demonstrated (Chapter II). The impact of TGF $\beta$  signaling on memory responses is less clear. This is due to experimental evidence supporting both positive and negative regulation of memory T cell function by TGF $\beta$  (Filippi et al., 2008; Sanvito et al., 1995; Takai et al., 2013). Interestingly, the discrepancies seen in these studies were attributed, at least in part, to how the T cells were primed. We did not track the expression of TGF $\beta$  receptor on high and low-affinity-stimulated T cells during the immune response or at the memory phase. If low-affinity memory cells do express higher levels of TGF $\beta$  receptor, this could explain why high but not low-affinity stimulation induces effector function. In this case, high-affinity antigens could down-regulate TGF $\beta$  receptor expression and promote memory function. As seen in the periphery, low-affinity antigens would not be able to induce this change (Chapter II), preventing memory responses against self. Thus, future studies need to determine whether TGF $\beta$  signaling regulates low-affinity memory T cell function.

### **Implications for Tumor Adoptive Transfer Therapies**

Tumor antigens are characterized as low-affinity. This is due to the fact that tumor antigens are self-peptides, and autoreactive T cells are deleted through the process of thymic selection (Starr et al., 2003). However, CD8 T cells do mount anti-tumor responses and protect against tumor formation throughout life (Kaplan et al., 1998; Shankaran et al., 2001; Smyth et al., 2000; Street et al., 2001; Street et al., 2002). CD8 T cells are now being used as an immunotherapeutic tool. Adoptive T cell transfer (ACT) utilizes CD8 T cells to mediate anti-tumor responses (Dudley, 2005; Ho et al., 2003). ACT requires harvesting a patient's tumor-specific T cells, expanding them *in vitro* using strong TCR signals (provided by anti-CD3 antibody stimulation) and high levels of IL-2, and reintroducing them to the patient. While there has been some success with ACT (Dudley and Rosenberg, 2003; Dudley et al., 2002), there are also issues that must be resolved. One of the main problems is that, due to the amount of stimulation provided and the number of proliferative cycles the T cells undergo, the expansion protocol can promote terminal differentiation or exhaustion of the T cells. This prevents long-term protection against the tumor due to a lack of CD8 T cell function and memory generation. In addition, the tumor microenvironment itself is also suppressive and can prevent tumor-infiltrating lymphocyte responses. A better ACT immunotherapy protocol would do the following: expand tumor-specific CD8 T cells, maintain and enhance anti-tumor T cell function, prevent suppression in the tumor microenvironment, and promote memory generation for long-term protection. Due to the propensity of low-affinity-stimulated T cells to generate memory and express high Eomes levels, which is

important for anti-tumor function in CD4 T cells (Chapter II, III) (Curran et al., 2013; Qui et al., 2011), I hypothesize that utilizing low-affinity T cell stimulation during ACT will increase its effectiveness. Indeed, preliminary experiments show that the expression of CTLA-4, a known molecule important in T cell inhibition that is now being targeted to improve anti-tumor responses (Dudley and Rosenberg, 2003; McCoy and Le Gros, 1999), is expressed lower in T cells upon weak versus strong antigen stimulation (K. Knudson and N. Goplen, unpublished observation). Of course, significant investigation is required to fully characterize low-affinity T cell responses to tumors. It is important to remember that low-affinity antigen stimulation promotes both qualitative and quantitative differences in CD8 T cell programming (Chapter II, III). Thus, a completely different regulation of anti-tumor responses may be present in low-affinity-stimulated T cells. In addition, as the majority of the T cell population in adults is memory, not naïve, it is also necessary to determine if the anti-tumor function will be seen after memory T cell stimulation/expansion with low-affinity antigens. Due to our observations of impaired low-affinity memory response against weak antigen, this may not occur (Chapter II).

### **Activation of NF- $\kappa$ B by Low-Affinity Ligands**

Altered peptides ligands signal differently through the TCR than cognate antigen. TCR stimulation with APLs leads to partial phosphorylation of the CD3 $\zeta$  ITAMs and lower phosphorylation of ZAP70 (Kersh et al., 1998; Madrenas et al., 1995; Sloan-Lancaster et al., 1994). Stefanova et al. showed that stimulation with

lower affinity antigens also promoted a negative feedback loop, involving activation of phosphatase SHP-1, which inhibited LCK (Stefanová et al., 2003). This led to decreased ERK activation (Stefanová et al., 2003), which supports the idea that APLs and low-affinity antigens have weak signaling capacity. In contrast to this, we show that NF- $\kappa$ B activation, on a per cell basis, is higher after low versus high-affinity antigen stimulation (Chapter III). Altered NF- $\kappa$ B activation could be due to changes upstream and/or downstream of NF- $\kappa$ B subunits. However, due to the early timepoints where higher NF- $\kappa$ B activation was observed with low-affinity antigen stimulation (~30 minutes), upstream regulation of NF- $\kappa$ B specific signaling will be focused on here.

TCR signaling is connected to NF- $\kappa$ B through PKC $\theta$  (Coudronniere et al., 2000). Activation and translocation of PKC $\theta$  is required for induction of NF- $\kappa$ B signaling (Coudronniere et al., 2000; Sun et al., 2000; Wang et al., 2003). After TCR engagement with high-affinity pMHC, PKC $\theta$  and the TCR are translocated to the immunological synapse (IS) (Kong et al., 2011; Wang et al., 2003). The IS is important for activation of both TCR signaling and PKC $\theta$  activity (Grakoui et al., 1999; Lee et al., 2003; Wang et al., 2003). In fact,  $\beta$ TMDmut T cells, which have defective TCR-dependent NF- $\kappa$ B signaling, have impaired TCR clustering, PKC $\theta$  recruitment to the IS, and activation/phosphorylation of PKC $\theta$  upon strong antigen stimulation (Teixeiro et al., 2009) (data not shown). Low-affinity APL-TCR interaction, on the other hand, does not promote the formation of a typical IS (Cemerski et al., 2007). As proper IS formation aids in the down-regulation of TCR and cessation of signaling (Lee et al., 2003), some processes in APL-

stimulated T cells are actually induced better with weak versus cognate antigen stimulation (Cemerski et al., 2007). How IS formation with low-affinity antigen stimulation affects PKC $\theta$  activation has not been investigated. It is possible that low-affinity antigens are able to effectively induce PKC $\theta$  membrane translocation in the absence of IS formation. Furthermore, PKC $\theta$  activation may be more efficient by this method due to lack of negative regulation in the IS. This would promote the increased NF- $\kappa$ B signaling seen with weak antigen stimulation. Also, if low-affinity antigens induce efficient activation of PKC $\theta$  without IS formation, this could explain the reversion of the defect in  $\beta$ TMDmut TCR-dependent NF- $\kappa$ B signaling upon weak TCR signaling.

Differential control of NF- $\kappa$ B negative regulators could be responsible for the increased NF- $\kappa$ B signaling with weak antigen stimulation. Of particular interest is how CYLD, a deubiquinating ligase (DUB) and known negative regulator of NF- $\kappa$ B signaling, functions upon weak TCR stimulation. DUB CYLD mediates regulation of NF- $\kappa$ B signaling upstream of NF- $\kappa$ B subunits (Scheidereit, 2006). CYLD deubiquinates IKK $\gamma$ /NEMO and TAK1, which inhibits NF- $\kappa$ B signaling (Kovalenko et al., 2003; Reiley et al., 2007). CYLD activity is induced upon TCR signaling in thymocytes and can affect the activity of proximal TCR signaling molecule LCK (Lineberry and Fathman, 2006). Very interestingly, loss of CYLD promotes hyperactive naïve and memory T cell responses to TCR signaling (Reiley et al., 2007). The regulation of CYLD by TCR affinity has not been explored.

## Regulation of Eomes Expression by Low-Affinity Antigens

Memory differentiation and maintenance is programmed through the temporal and relative expression of various transcription factors. While Zehn *et al.* showed that low-affinity antigens promoted full memory development, the transcriptional program induced by high and low-affinity antigens was not explored (Zehn *et al.*, 2009). Our studies report that stimulation with low-affinity antigens induces high expression of memory-associated factor Eomes. The difference in Eomes expression between high and low-affinity-stimulated T cells is due to a qualitative difference in TCR signaling (Chapter II). However, we did not fully elucidate how low-affinity antigens are able to induce these levels of Eomes. Weak antigens may distinctly promote increased activation of signaling pathways that support Eomes expression. One of these pathways, as already discussed, is NF- $\kappa$ B signaling. We demonstrate that NF- $\kappa$ B signaling does support Eomes expression (Chapter IV). There are other pathways, however, that may regulate Eomes levels differentially depending on TCR signal strength. The Wnt/TCF-1 pathway positively regulates Eomes expression (Zhou *et al.*, 2010). Genetic loss of TCF-1 prevents full expression of Eomes in T cells (Zhou *et al.*, 2010). Furthermore, the Wnt/TCF-1 pathway has been implicated in central memory differentiation (Boudousquié *et al.*, 2014; Gattinoni *et al.*, 2009), which we show is the preferential memory phenotype of low-affinity stimulated T cells during infection. The ratio of stimulatory to inhibitory TCF-1 isoform expression is altered upon TCR stimulation, and the level of Wnt/TCF-1 signaling may induce effector versus memory differentiation (Boudousquié *et al.*, 2014; Gattinoni *et al.*,

2009). Thus, it is necessary to determine whether the Wnt/TCF-1 pathway is differentially by TCR affinity. In sum, whether TCR affinity affects Wnt signaling, and thus Eomes expression, in T cells should be evaluated.

Conversely, low-affinity antigen stimulation may prevent inhibition of Eomes expression. mTOR is a known negative regulator of Eomes (Li et al., 2011; Rao et al., 2010). While strong TCR signals promote robust activation of mTOR signaling, weak TCR signaling does not (data not shown). Interestingly, low-affinity antigen stimulation does not support high T-bet expression, and T-bet is positively regulated by mTOR (Li et al., 2011; Rao et al., 2010). Thus, it is a distinct possibility that weak TCR signaling does not induce negative regulation of Eomes expression by mTOR signaling. This, paired with an increase in NF- $\kappa$ B signaling, may induce the high levels of Eomes seen in CD8 T cells after low-affinity antigen stimulation. The interplay between mTOR and NF- $\kappa$ B signaling in the regulation of memory-associated factor Eomes needs to be explored in the future.

### **Regulation of Eomes and T-bet Expression by NF- $\kappa$ B**

In the studies presented here, we demonstrate that NF- $\kappa$ B signaling regulates Eomes expression in activated T cells (Chapter IV). We also show that NF- $\kappa$ B signaling is required for Eomes expression in memory T cells (Chapter IV). One question we do not address is whether Eomes expression is directly or indirectly regulated by NF- $\kappa$ B-dependent transcription. Cho *et al.* has demonstrated that NF- $\kappa$ B directly binds to the Eomes promoter in *in vitro*

activated T cells (Cho et al., 2009). However, the studies were not performed during infection on effector or memory CD8 T cells. It is possible that the regulation of Eomes by NF- $\kappa$ B signaling changes depending on the differentiation state of the T cell, as well. It is highly likely that NF- $\kappa$ B directly binds to the Eomes promoter and directly regulates its expression. Nevertheless, due to the intricate crosstalk between NF- $\kappa$ B with other signaling pathways, this needs to be directly tested.

Due to the similarities in homology and function between Eomes and T-bet, we also investigated the control of T-bet expression in effector and memory CD8 T cells. T-bet is known to be regulated by mTOR signaling, and both T-bet and mTOR are major mediators of effector differentiation in CD8 T cells (Araki et al., 2009; Intlekofer et al., 2007; Intlekofer et al., 2005; Joshi et al., 2007; Rao et al., 2010). NF- $\kappa$ B signaling also possibly controls T-bet expression through mTOR signaling, as IKK $\beta$  activation is known to inhibit mTOR negative regulators TSC1/2 (Lee et al., 2007; Tee et al., 2002). However, in contrast to Eomes, our data demonstrate that altered NF- $\kappa$ B signaling, even constitutive activation of IKK $\beta$ , does not affect T-bet expression in activated T cells (Chapter IV). This result was further supported by the observation that MUT T cells, which have impaired TCR-dependent NF- $\kappa$ B signaling, expressed normal T-bet levels and effector differentiation. While we are confident that T-bet is not regulated by NF- $\kappa$ B signaling in effector CD8 T cells, in Chapter IV, we do present data that is in direct contrast with this conclusion. Activation of naïve CD8 T cells in the presence of the NF- $\kappa$ B chemical inhibitor Wedelolactone completely abrogates

expression of T-bet. This could be due to greater inhibition of IKK $\beta$  activity and NF- $\kappa$ B in the presence of the chemical inhibitor. However, I propose that this effect was due to improper activation of the T cells upon Wedelolactone treatment. The T cells were unable to up-regulate activation-associated markers CD69 and CD25 (both are NF- $\kappa$ B targets (Fu et al., 2013; Pahl, 1999)) upon stimulation in the presence of the NF- $\kappa$ B inhibitor. This lack of T cell activation can explain why T-bet was not expressed in these experiments. Importantly, when OT-I CD8 T cell were primed *in vivo* with LM-OVA infection, the addition of Wedelolactone at the peak of the response (after T cell activation and effector differentiation) did not affect T-bet expression. However, Eomes expression, which we demonstrate is a NF- $\kappa$ B target in effector T cells (Chapter IV), was lost under these conditions (K. Knudson, unpublished observations).

While T-bet function does not seem to be specifically required for memory CD8 T cell generation, it does play a role in memory effector function. Genetic loss of T-bet prevents IFN $\gamma$  production by memory T cells during secondary infection (Intlekofer et al., 2007) . However, the regulation of T-bet expression has not been studied after the peak of the response. It is not known whether mTOR signaling supports T-bet expression at memory, which is expressed higher in memory versus naïve T cells (Intlekofer et al., 2007). Surprisingly, our data show that mTOR inhibition did not affect T-bet expression, or any of its known targets, at the memory timepoint (Chapter IV). On the other hand, pharmacological inhibition of NF- $\kappa$ B signaling completely abrogated T-bet levels in resting memory cells and prevented re-expression upon infection (Chapter IV).

These results support the conclusion that regulation of T-bet expression depends on the differentiation state of the T cell. In particular, while NF- $\kappa$ B signaling does not regulate T-bet expression in effector T cells, it is important to support T-bet levels at memory. Similarly to NF- $\kappa$ B regulation of Eomes expression, we did not investigate whether T-bet is a direct NF- $\kappa$ B target. Due to the disparity seen in the regulation of T-bet between effector and memory T cells, it is possible that potential NF- $\kappa$ B binding sites in the T-bet promoter are differentially accessible at those timepoints. Alternatively, NF- $\kappa$ B may regulate another molecule that promotes T-bet expression at the memory but not the effector stage. Regardless, further investigation is required to determine the complex regulation of T-bet expression and its role in effector and memory T cells.

### **Signal Quality Model of T Cell Programming**

There are numerous models that seek to explain how effector and memory generation are concurrently programmed during an immune response. The signal quality model states that the collaboration between antigenic pMHC-TCR signal, costimulation, and inflammation provided during priming determines this diversity and eventual T cell fate (Kaech and Cui, 2012). In brief, less signaling during priming promotes more memory generation. Using a unique model that alters TCR signaling by mutating the  $\beta$ TMD of the TCR, we have previously demonstrated that early TCR signaling distinctly supports effector and memory generation (Teixeiro et al., 2009). In addition, the work presented here shows that TCR signaling induces programming which regulates this fate

decision. Specifically, TCR-dependent NF- $\kappa$ B signaling sets up a signaling axis that supports the expression of Eomes both early and late during the immune response (Chapter IV). Interestingly, MUT T cells have a defect in TCR-dependent induction of NF- $\kappa$ B signaling, Eomes expression, and memory development after priming with high-affinity antigens. This defect is recovered, independently of the presence of an inflammatory signal, when  $\beta$ TMDmut T cells are stimulated with a low-affinity antigen (Chapter III) (Knudson et al., 2013b; Teixeira et al., 2009). These results further indicate that TCR signaling distinctly regulates memory generation. In these experiments, as only the TCR was mutated, the only identified signal altered during the immune response in MUT T cells was early TCR signaling. Pro-survival signals and the input of inflammatory signals were comparable to WT (Chapter III). Thus, our data supports the signal quality model of T cell differentiation.

In conclusion, the studies presented in this thesis further support that (1) the TCR signal controls CD8 T cell fate during infection. We uncovered evidence that (2) TCR signal strength differentially regulates effector and memory programming. These results provide insight into the potential use of different affinity antigens for vaccination or generation of immunotherapies. In addition, we determined that (3) NF- $\kappa$ B signaling is a required pathway for memory differentiation. Thus, manipulation of NF- $\kappa$ B signaling in antigen-specific T cells could be used to enhance memory generation during vaccination or inhibit the persistence of unwanted memory CD8 T cells.

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## VITA

Karin Marie Knudson was born on June 7, 1986 and grew up in the mountains of Colorado. Her parents, Wendy Koenig and Lyle Knudson, fostered her love of unsolved questions and puzzles. She attended Colorado State University, where she became interested in microbiology after attending an introduction to microbiology course with Dr. Erica Suchmann. After graduating *Cum Laude* with a Bachelor's in Biological Sciences in 2008, Karin spent a year exploring the effects of sand fly saliva on immune responses in the laboratory of Dr. Richard Titus. This research fostered her interest in immunology, and in 2009, Karin began her doctorate in Molecular Microbiology and Immunology at the University of Missouri as Life Sciences Fellow. She performed her research on T cell immunological memory under the guidance of Dr. Emma Teixeira.

Karin will next be investigating tumor vaccines at the National Cancer Institute in Bethesda, Maryland.