Effects of Dysregulated Diacylglycerol–Mediated Signaling on T Cell Function

by

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Qi-Jing Li

Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Immunology in the Graduate School of Duke University

2013
ABSTRACT

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**Abstract**

Diacylglycerol (DAG), a lipid messenger generated upon T cell receptor (TCR) engagement, mediates signaling through the IKK/NF-κB and Ras/ERK pathways. Further downstream of the Ras/ERK pathway are the kinase mammalian target of rapamycin (mTOR) and the MAP kinase signal integrating kinases Mnk1 and Mnk2. While mTOR acts as a critical regulator of T cell metabolism, homeostasis and function, Mnk1 and Mnk2 phosphorylate the initiation factor eIF4E that plays an important role in cap-dependent mRNA translation. Diacylglycerol kinases (DGKs) terminate DAG-mediated signals by phosphorylating DAG into phosphatidic acid. T cells that lack both α and ζ isoforms of DGK accumulate excess DAG upon activation, resulting in hyper-activation of the IKK/NF-κB, Ras/ERK and mTOR pathways. Such T cells are hypersensitive to TCR stimulation and resistant to the induction of anergy. Here, we sought to dissect the mechanisms by which dysregulated DAG-mediated signaling affects T cell function. To this end, we studied the effects of hyper-activating individual DAG-mediated pathways (IKK/NF-κB and TSC/mTOR) on T cell function. We also examined the role of ERK-activated kinases Mnk1 and Mnk2 in T cell function.

Using mice with T cell-specific expression of a constitutively active form of IKKβ (‘IKK’ mice), we found that uncontrolled IKKβ/NF-κB signaling attenuates responsiveness to TCR stimulation and promotes T cell apoptosis. Increased FasL
expression contributed to enhanced IKK T cell apoptosis via the extrinsic pathway. Impaired IKK T cell activation and proliferation were associated with defects in TCR signaling, and upregulation of the cell surface inhibitory receptor PD1. Notably, expression of the transcriptional repressor Blimp1 (a regulator of T cell exhaustion) was increased in IKK T cells, and conditionally deleting Blimp1 was able to largely restore responsiveness to TCR stimulation. *In vivo*, IKK T cells mounted compromised antigen-specific CD8 responses with curtailed expansion and exaggerated contraction phases. Our results suggest that uncontrolled IKKβ/NF-κB signaling is sufficient to impair both T cell function and survival.

Investigating Mnk1/2 double knockout (DKO) mice, we found that Mnk1 and Mnk2 are dispensable for T cell development and function, but important for the pathogenesis of experimental autoimmune encephalomyelitis (EAE). TCR engagement activated Mnk1/2 in a Ras/ERK-dependent manner and was inhibited by DGK α and ζ. Mnk1/2 deficiency did not affect the development of conventional αβ T cells, regulatory T cells, or invariant NKT cells. Mature T cells from DKO mice showed normal activation, proliferation and CD4 T\textsubscript{H} differentiation *ex vivo*, but DKO mice developed lower clinical scores than wild type counterparts in an EAE model, correlating with a smaller pool of antigen-specific IL-17-producing and IFN\textgamma-producing CD4 cells. These results suggest that Mnk1/2 may play a minimal role in T cell development and function but may control non-T cell lineages to regulate T\textsubscript{H}1 and T\textsubscript{H}17 differentiation *in vivo*. 
To determine the effect of constitutive mTOR complex 1 activity on anti-bacterial CD8 responses, we investigated mice with T cell-specific deletion of TSC1, a natural suppressor of mTOR complex 1 activity. Using an established model system of transgenic CD8 cell adoptive transfer and challenge with *Listeria monocytogenes* expressing a cognate antigen, we found that TSC1 deficiency impairs antigen-specific CD8 responses, resulting in a weak expansion phase, exaggerated contraction phase and poor memory generation. Weak expansion of TSC1-deficient cells correlated with defects in survival and proliferation *in vivo*, while exaggerated contraction was associated with an increased ratio of SLECs to MPECs in the effector cell population. This perturbation in effector-memory differentiation was concomitant with enhanced T-bet expression and decreased Eomes expression among activated TSC1 KO cells. Upon competitive adoptive transfer with wild type counterparts and antigen re-challenge, TSC1-deficient memory cells showed moderate defects in expansion but not cytokine production. Taken together, these findings provide direct evidence of a CD8 cell-intrinsic role for TSC1 in regulating antigen-specific primary and memory responses.

In sum, findings from these studies provide deeper insight into the regulation of T cell function by DAG-mediated pathways, and may have implications for the design of immune-modulation strategies during vaccination and immunotherapy.
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<tr>
<td>APC</td>
<td>antigen presenting cell</td>
</tr>
<tr>
<td>Bcl10</td>
<td>B cell lymphoma 10</td>
</tr>
<tr>
<td>Blimp1</td>
<td>B lymphocyte-induced maturation protein 1</td>
</tr>
<tr>
<td>caIKKβ</td>
<td>constitutively active form of inhibitor of NF-κB kinase, subunit β</td>
</tr>
<tr>
<td>CARMA1</td>
<td>CARD-domain containing MAGUK protein 1</td>
</tr>
<tr>
<td>CD4SP</td>
<td>CD4 single positive (CD4⁺ CD8⁻)</td>
</tr>
<tr>
<td>CD8SP</td>
<td>CD8 single positive (CD4⁻ CD8⁺)</td>
</tr>
<tr>
<td>CTL</td>
<td>cytotoxic T lymphocyte</td>
</tr>
<tr>
<td>DAG</td>
<td>diacylglycerol</td>
</tr>
<tr>
<td>DGK</td>
<td>DAG kinase</td>
</tr>
<tr>
<td>DKO</td>
<td>double knockout</td>
</tr>
<tr>
<td>DN</td>
<td>double negative (CD4⁺ CD8⁻)</td>
</tr>
<tr>
<td>DP</td>
<td>double positive (CD4⁺ CD8⁺)</td>
</tr>
<tr>
<td>EAE</td>
<td>experimental autoimmune encephalomyelitis</td>
</tr>
<tr>
<td>eIF</td>
<td>eukaryotic translation initiation factor</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FasL</td>
<td>Fas ligand</td>
</tr>
<tr>
<td>α-GalCer</td>
<td>α-galactosyl ceramide</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase activating protein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>iNKT</td>
<td>invariant NKT</td>
</tr>
<tr>
<td>IKK</td>
<td>inhibitor of NF-κB kinase</td>
</tr>
<tr>
<td>IP3</td>
<td>inositol triphosphate</td>
</tr>
<tr>
<td>KO</td>
<td>knockout</td>
</tr>
<tr>
<td>LAT</td>
<td>linker for activation of T cells</td>
</tr>
<tr>
<td>Lm-OVA</td>
<td><em>Listeria monocytogenes</em> expressing rOVA</td>
</tr>
<tr>
<td>LN</td>
<td>lymph node</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MAPKAPK</td>
<td>MAPK-activated protein kinase</td>
</tr>
<tr>
<td>MARCKS</td>
<td>myristoylated alanine-rich C-kinase substrate</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>Mnk1/2</td>
<td>MAPK-interacting kinase 1 and 2</td>
</tr>
<tr>
<td>MOG</td>
<td>myelin oligodendrocyte glycoprotein</td>
</tr>
<tr>
<td>MPEC</td>
<td>memory precursor effector cell</td>
</tr>
<tr>
<td>mTOR</td>
<td>mammalian target of rapamycin</td>
</tr>
<tr>
<td>mTORc1</td>
<td>mammalian target of rapamycin, complex 1</td>
</tr>
<tr>
<td>mTORc2</td>
<td>mammalian target of rapamycin, complex 2</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor κB</td>
</tr>
<tr>
<td>PA</td>
<td>phosphatidic acid</td>
</tr>
<tr>
<td>PC</td>
<td>phosphatidyl choline</td>
</tr>
</tbody>
</table>
**PIP₂**  phosphatidyl inositol bisphosphate

**PKCθ**  protein kinase C θ

**PKD**  protein kinase D

**PLC**  phospholipase C

**PMA**  phorbol myristate acetate

**qPCR**  quantitative PCR

**RasGRP1**  ras guanyl nucleotide releasing protein 1

**RHD**  rel homology domain

**Rsk**  ribosomal S6 kinase

**SLEC**  short-lived effector cell

**Sos**  son of sevenless

**TCR**  T cell receptor

**TCM**  T central memory

**TEM**  T effector memory

**Treg**  regulatory T cell

**TSC**  tuberous sclerosis complex

**WT**  wild type
Acknowledgements

As I begin to write this section, I realize that the kindness, generosity and support of many have been instrumental in helping me earn my doctoral degree at Duke. First of all, I would like to thank my advisor, Dr. Xiao-Ping Zhong, for giving me the opportunity to work on some very interesting projects, and for being a patient and enthusiastic mentor. Through all the ups and downs of my research work, he has been very supportive and ready to provide helpful advice. I would also like to thank my committee members, Drs. Weiguo Zhang, Garnett Kelsoe, Soman Abraham and Qi-Jing Li, for their constructive criticism and for always pointing my research work in the right direction. I have grown to be good friends with many members of the Zhong lab (particularly Edwin Wan, Tommy O’Brien, Li Xu, and Jinwook Shin), and I am grateful to them for fostering a positive work environment. I would also like to give a special shout-out to my wonderful classmates Claudia OuYang, Elizabeth Chan, Mingxiao He and Jia Li, with whom I’ve forged a strong bond over the years. Girls’ lunches with the Zhang lab will count among some of my fondest memories of graduate school, and I would like to thank everyone in the Duke Immunology community, including the flow facility staff (Mike Cook, Nancy Martin and Lynn Martinek) and Jennifer Goins, for being so generous with their help, guidance and reagents!

No single person has supported and motivated me more through the challenges of graduate school than my husband, Krishna Monian. With their contagious optimism,
Krishna and his mother Veena Monian have been a tremendous source of positive energy in my life, and have always pushed me to be the best version of myself. This section would be grossly incomplete without a special note of thanks to my parents and brother, Saranya, Rajan and Srashta Srivatsan, whose tremendous encouragement and moral support have been instrumental throughout my life. My mother’s prayers and my father’s sage advice have helped me find my way out of some difficult situations over these past few years, and I am grateful for their unconditional love and kindness. Lastly, I would like to thank my dearest friends Sathya Gopalakrishnan, Malavika Murugan, Sujatha Jagannathan, Sulochana Dhar, Radhika Das and Sabarni Palit, who have almost been like sisters to me. Graduate school would not have been half as much fun, if not for these wonderful ladies.

I feel very fortunate to be a Blue Devil, and as I strive towards my professional and personal goals, I do so knowing that “If I have seen further it is by standing on the shoulders of giants.”
1. Introduction

1.1 T cell development and function

The elimination of pathogens and cancer cells by the adaptive immune system requires the orchestrated action of several types of cells that perform distinct functions (Dempsey et al., 2003; Litman et al., 2010). One such cell type is the thymus-derived T lymphocyte, which plays a critical role in cell-mediated immunity (Cooper et al., 1965; Mosmann and Sad, 1996; Wong and Pamer, 2003). Each T cell expresses a specific T cell receptor (TCR) on its surface that allows for the recognition of a particular foreign peptide presented on self-MHC molecules (Davis and Bjorkman, 1988). Based on their expression of CD4 or CD8 co-receptors, T cells are classified into CD4+ T helper cells and CD8+ cytotoxic T cells. As their names suggest, CD4 cells secrete cytokines that aid the function of CD8 cells and B cells, and CD8 cells are capable of becoming cytotoxic effectors that kill infected or cancerous target cells. In this section, we present a concise overview of T cell development and function.

1.1.1 Thymocyte development

Like all hematopoietic cells, T cells are derived from bone marrow-resident stem cells. Lymphoid progenitor cells generated from these stem cells migrate to the thymus, where they travel through the cortex and medulla, developing into mature T cells (Takahama, 2006; Weerkamp et al., 2006). The thymus itself does not contain self-renewing progenitors that can sustain long-term thymopoiesis, and studies point
towards the existence of multiple types of thymus-seeding progenitors that have undergone varying degrees of T lineage commitment (Bhandoola et al., 2007; Ciofani and Zuniga-Pflucker, 2007; Donskoy and Goldschneider, 1992).

Successive developmental stages of a thymocyte can be distinguished by the combination of CD4 and CD8 co-receptors expressed on its surface (Ellmeier et al., 1999). Early committed T cells do not express TCR, CD4, or CD8 on the cell surface and are called CD4^-CD8^- double-negative (DN) cells. These DN progenitors can be divided further into four stages from DN1 to DN4 based on the surface expression of CD44, CD25 and CD117 (c-kit) (Godfrey et al., 1993). Upon rearrangement of V, D, and J gene segments at the TCRβ locus, DN3 cells express a pre-TCR that consists of the TCRβ protein and a pre-Ta chain (von Boehmer and Fehling, 1997). Cells that express a functional pre-TCR pass through the so-called “β-selection” checkpoint, while others apoptose (Michie and Zuniga-Pflucker, 2002). The β-selected DN cells undergo six to eight rounds of proliferation, maturing into CD4^+CD8^- double-positive (DP) cells through a brief, intermediate immature CD8 single positive stage (Miyazaki, 1997). DP cells constitute about 90% of all thymocytes, and rearrange V and J gene segments at the TCRα locus, leading to the expression of a unique TCR on the cell surface.

Following TCR expression, DP thymocytes are subjected to processes called positive and negative selection (Starr et al., 2003; Wiegers et al., 2011), which ensure the generation of a functional, non self-reactive T cell repertoire. In order to be positively
selected, DP cells must express a TCR that is able to recognize self-peptide-MHC complexes expressed by thymic epithelial cells or bone-marrow-derived dendritic cells in the thymus (Dervovic and Zuniga-Pflucker, 2010; Jameson et al., 1995). In general, a vast majority of DP cells fail to receive survival signals and undergo ‘death by neglect’ at this stage. On the other hand, DP cells with TCRs that recognize self-peptide-MHC with high affinity also undergo apoptosis, a process referred to as negative selection (Sprent and Kishimoto, 2002; von Boehmer and Melchers, 2010). Thus, only DP cells with TCRs that recognize self-peptide-MHC molecules with low affinity survive positive and negative selection, developing further into mature CD4+ CD8- single positive (CD4SP) or CD4- CD8+ single positive (CD8SP) cells that exit the thymus.

CD4 versus CD8 lineage fate is determined by the MHC-restriction specificity of the TCR (Singer et al., 2008; Teh et al., 1988). DP thymocytes that recognize MHC class II-associated peptides differentiate into CD4SP cells, while those recognizing MHC class I-associated peptides develop into CD8SP cells. Several models have been proposed to explain how a DP cell ‘chooses’ to terminate transcription of CD4 or CD8. Such models include stochastic selection, strength-of-signal instruction, duration-of-signal instruction, and more recently, kinetic signaling (Singer et al., 2008). A number of transcription factors such as Th-POK, Runx3, and GATA3 also play a vital role in CD4/CD8 lineage choice (Taniuchi and Ellmeier, 2011). A simplified schematic of T cell development is presented below (Fig. 1).
Figure 1. DN cells undergo VDJ recombination at the TCRβ locus to express a pre-TCR on the cell surface. Expression of a functional pre-TCR enables the DN cell to pass through the β-selection checkpoint and mature into a DP cell. DP cells rearrange V and J gene segments at the TCRα locus, and are subsequently subjected to positive and negative selection processes that ensure the generation of a functional but non-self-reactive T cell repertoire. DP cells that survive these selection processes mature into CD4SP cells or CD8SP cells. Please see text for more details.
1.1.2 T cell activation and anergy

A T cell becomes activated when it recognizes cognate peptide-MHC complexes and co-stimulatory molecules on an antigen-presenting cell (APC) in an appropriate milieu of cytokines (Smith-Garvin et al., 2009). Engagement of TCR and co-stimulatory receptors triggers a number of biochemical events within the T cell, which are discussed in detail in section 1.2. Together, these pathways activate a transcriptional program that allows for the transformation of a quiescent T cell into a functionally and metabolically active lymphoblast.

T cell activation proceeds sequentially through phases of initial activation, proliferation and differentiation (Abbas et al., 2000). Early activation of T cells is associated with increased cell surface expression of the C-type lectin receptor CD69, the glycoprotein CD44, and the IL-2R α chain CD25 (Baaten et al., 2010; Ziegler et al., 1994). Upregulation of CD69 expression is dependent upon the transcription factor AP-1 (Castellanos et al., 1997), and CD69 engagement plays an immuno-regulatory role during anti-tumor responses and autoimmunity (Sancho et al., 2005). Recent studies have identified that CD69 engagement may promote Stat5 signaling to inhibit Th17 type CD4 responses (Martin and Sanchez-Madrid, 2011). While resting T cells constitutively express β and γ chains of the IL-2R that bind IL-2 with moderate affinity, activation-induced expression of CD25 allows the formation of a high affinity hetero-trimeric receptor. Binding of CD44 to its ligand hyaluronan on vascular endothelial cells is
important for lymphocyte adhesion and extravasation at sites of inflammation (Siegelman et al., 1999). Two hallmarks of T cell activation are proliferation and cytokine production. IL-2 acts on T cells via both autocrine and paracrine mechanisms, promoting cell cycle progression and inhibiting apoptosis (Liao et al., 2013). Activated CD4 cells can differentiate into a variety of Th subsets (Th1, Th2, Th17, Treg) depending on the type of cytokines present in the extracellular milieu (Zhu et al., 2010a). CD8 cells, on the other hand, differentiate into cytotoxic T lymphocytes as discussed in section 1.1.3.

Proper immune function is critically dependent on the ability of the immune system to distinguish between self and non-self antigens. While mounting effective immune responses to foreign pathogens is important for host defense, retaining tolerance to self-antigens is necessary to prevent autoimmunity. In the thymus, negative selection serves as the first checkpoint where thymocytes bearing self-reactive TCRs are eliminated to enforce self-tolerance (Sprent and Kishimoto, 2001). Apart from this mechanism of central tolerance, peripheral tolerance is maintained by the action of regulatory T cells and by the induction of clonal anergy (Xing and Hogquist, 2012).

Anergy refers to a state of acquired cell-intrinsic functional unresponsiveness in which T cells are refractory to antigenic stimulation (Chappert and Schwartz, 2010; Schwartz, 2003). According to the two-signal model of T cell activation (Baxter and Hodgkin, 2002), binding of TCR to cognate peptide-MHC must be accompanied by co-stimulation (for instance via the CD28 receptor) to fully trigger all TCR-coupled
signaling pathways and result in full-fledged T cell activation. In the absence of co-stimulation, TCR engagement has been shown to selectively activate a subset of signaling pathways that culminate in the expression of a transcriptional program that enforces unresponsiveness (Macian et al., 2002; Zheng et al., 2008). Anergized T cells fail to proliferate or produce IL-2, even when re-activated in the presence of co-stimulatory signals. However, signals through the IL-2 receptor have been shown to prevent and reverse T cell anergy (Boussiotis et al., 1994; Dure and Macian, 2009).

1.1.3 Antigen-specific CD8 T cell responses

CD8 T cells play a critical role in clearing several types of microbial infections by mounting robust cytotoxic T lymphocyte (CTL) responses that kill infected cells. A typical CTL response is characterized by the presence of an expansion phase, contraction phase and memory maintenance phase (Fig. 2) (Williams and Bevan, 2007; Zhang and Bevan, 2011). Before infection, the frequency of any particular CD8 cell clone is thought to be close to 1 in $10^5$ (Blattman et al., 2002). When activated by APCs in a milieu of pro-inflammatory cytokines, these naïve precursors undergo massive clonal expansion in secondary lymphoid organs. Studies suggest that CD8 cells can undergo up to 15-20 cycles of cell division, and increase in numbers by up to 50,000-fold during the expansion phase (Butz and Bevan, 1998a; Butz and Bevan, 1998b).

At the peak of response, the population of CD8 effectors is heterogeneous and consists of a majority of short-lived effector cells (SLECs) and a small pool (5-15%) of
memory-precursor effector cells (MPECs) (Joshi et al., 2007; Kaech et al., 2003). SLEC
are identifiable by high expression of the NK cell receptor KLRG1 and low expression of
the IL7R α chain (CD127) on the cell surface, and have been shown to have very little
potential to give rise to long-lived memory cells. Upon antigen clearance, 90-95% of total
CD8 effectors undergo apoptosis during the contraction phase (Badovinac et al., 2002;
Porter and Harty, 2006). While most SLEC are eliminated at this stage, MPECs
(KLRG1lo IL7Rαhi) preferentially contribute to the formation of a small but stable
population of long-lived memory cells. The presence of inflammatory signals (such as
IL-12) during T cell priming has been shown to drive the graded expression of
transcription factors that regulate effector CD8 cell fate determination (Joshi et al., 2007).
The T-box transcription factors T-bet and Eomes play a critical role in this process, with
high IL-12 levels promoting increased T-bet expression and the generation of SLEC.
Others have shown that the levels of T-bet and Eomes are inversely regulated by IL-12
(Takemoto et al., 2006), with the expression of Eomes enabling CD8 cells to compete for
the memory cell niche (Banerjee et al., 2010).

Memory cells are capable of long-term persistence in the absence of antigen
because of their ability to self-renew in response to homeostatic cytokines such as IL-15
(Cox et al., 2013). Memory cells respond more rapidly than naïve cells to antigen re-
exposure, and are further classified into CD44hi CD62Llo CCR7lo effector memory (TEM)
and CD44hi CD62Lhi CCR7hi central memory (TCM) cells that have distinct circulation
patterns and functional characteristics (Sallusto et al., 1999). While both populations are found in the blood and spleen, high expression of CD62L and CCR7 allows T<sub>CM</sub> cells to home predominantly to lymph nodes. Low expression of these molecules drives the localization of T<sub>EM</sub> cells to non-lymphoid tissues. Upon activation, T<sub>CM</sub> cells show low cytotoxicity, but produce high levels of IL-2 and have a high capacity to proliferate and give rise to effector cells. On the other hand, T<sub>EM</sub> cells produce high levels of IFNγ and show pronounced cytotoxicity, but produce very little IL-2 and have limited proliferation capacity.

A number of studies have aimed to understand the lineage relationship between T<sub>CM</sub> and T<sub>EM</sub> cells. Results from some have suggested that effector cells may first become replication-incompetent T<sub>EM</sub> cells and subsequently regain replicative function to become T<sub>CM</sub> cells (Huster et al., 2006; Wherry et al., 2003), giving rise to a ‘linear differentiation’ model. However, others have found that asymmetric division of a single naïve CD8 cell upon antigen-stimulation can give rise to daughter cells that are differentially fated towards effector and memory lineages (Chang et al., 2007). The relationship between T<sub>CM</sub> and T<sub>EM</sub> subsets remains controversial (Ahmed et al., 2009) and actively investigated.
Figure 2. When exposed to cognate peptide-MHC complexes in an inflammatory environment, CD8 cells undergo rapid clonal expansion reaching peak numbers by around 7 days after infection. Upon resolution of infection, up to 90-95% of effector cells undergo apoptosis. This leaves behind a small self-renewing pool of memory cells that are capable of responding robustly to a subsequent re-challenge.
1.2 DAG-mediated pathways of TCR signaling

Lipids are small hydrophobic molecules that perform a variety of functions within the cell. Though best known for their roles in maintaining cell structure and storing energy, lipids have gained in importance over the past few decades as signaling mediators (Eyster, 2007; Fernandis and Wenk, 2007). While lipids that participate in signaling are thought to be much less abundant in the cell as compared to structural lipids, their levels vary dynamically in response to external signals.

Diacylglycerols (DAGs), esters of glycerol in which two of its hydroxyl groups are esterified with long-chain fatty acids, have been identified as essential signaling intermediates in T cells (Huang and Sauer, 2010). Several enzymes contribute towards DAG production upon receptor stimulation in immune cells (Zhong et al., 2008). Phosphatidylinositol-dependent phospholipases hydrolyze membrane phosphatidylinositol bisphosphate (PIP$_2$) to DAG and inositol triphosphate (IP$_3$) (Rhee, 2001), phosphatidylcholine (PC)-dependent phospholipases hydrolyze PC to DAG and phosphoryl choline (Kambayashi et al., 2002), and sphingomyelin synthase generates DAG and sphingomyelin from PC and ceramide (Villani et al., 2008). On the other hand, DAG is primarily removed by DAG kinases (DGKs), which catalyze its phosphorylation to phosphatidic acid (PA) (Rincon et al., 2012).
1.2.1 DAG-binding effectors: An overview

Multiple signaling pathways are triggered in T cells upon DAG production, as a variety of downstream effector molecules including protein kinase Cθ (PKCθ), Ras guanyl nucleotide releasing protein 1 (RasGRP1), protein kinase D (PKD) and chimaerins are recruited to the plasma membrane through their cysteine-rich DAG-binding C1 domains (Colon-Gonzalez and Kazanietz, 2006).

The PKC family of serine/threonine kinases consists of ten isozymes that are activated by a number of distinct mechanisms (Breitkreutz et al., 2007; Gould and Newton, 2008). Upon engagement of the TCR, production of DAG by activated phospholipase Cγ1 (PLCγ1) recruits PKCθ to the plasma membrane. Activation of PKCθ is indispensable for TCR-mediated nuclear factor κB (NF-κB) activation in mature T cells (Isakov and Altman, 2002; Sun et al., 2000). A role for PKCθ also identified in an array of key processes (Hayashi and Altman, 2007) including T cell survival (Manicassamy et al., 2006), IL-2 production (Werlen et al., 1998), Th2 responses (Marsland et al., 2004), and Th17 responses (Kwon et al., 2012). Thus, by recruiting PKCθ, DAG regulates multiple aspects of T cell function.

Another important protein that is brought to the plasma membrane by DAG upon TCR stimulation is RasGRP1 (Carrasco and Merida, 2004). RasGRP1 is a member of the RasGRP family of factors that help activate Ras by exchanging bound GDP for GTP (Roose et al., 2005). It is selectively expressed in T cells and a few other cell types
(Ebinu et al., 2000). RasGRP1 plays an essential role in thymocyte development (Dower et al., 2000), and is particularly required for the selection of thymocytes that express weakly selecting TCRs (Priatel et al., 2002). Other studies have shown that RasGRP1 may play a role in promoting antigen-induced CD8 cell expansion by lowering the threshold of T cell activation (Priatel et al., 2010). RasGRP1 is therefore a key effector downstream of DAG that plays a number of critical roles in T cell development and function.

PKD is a DAG effector that has been identified more recently (Wang, 2006). A unique characteristic of PKD is that it is both a direct target of DAG and a target of DAG-activated PKCs (Rozengurt et al., 2005). PKDs are thought to be activated by a multi-step mechanism. Upon T cell stimulation, inactive PKD translocates from the cytosol to the plasma membrane in response to membrane DAG production, where it is then activated by novel PKCs that are also recruited to the membrane by DAG. During T cell development, PKD has been shown to exert different effects on VDJ recombination at the TCRβ locus and on CD4 and CD8 expression, based on its localization at the cytosol or plasma membrane (Marklund et al., 2003). Subsequent work has revealed that regulation of thymocyte development by membrane-localized PKD, but not cytosol-localized PKD, is dependent on the GTPase RhoA (Mullin et al., 2006). Bringing PKD to the plasma membrane therefore represents another important mechanism by which DAG signals regulate T cell development.
Chimaerins, a family of proteins that possess Rac-specific GTPase activating protein (GAP) activity, contain C1 domains that bear about 40% homology to those present in PKCs (Caloca et al., 1999; Yang and Kazanietz, 2007). Chimaerin isoforms α2 and β2 are expressed at different levels in T cells and have been shown to participate in TCR signaling (Caloca et al., 2008). These chimaerin isoforms translocate to the immunological synapse upon T cell activation, but in a manner that is independent of canonical DAG-binding by the C1 domains. Other studies have delineated a role for β2 chimaerin in mediating DAG-dependent changes in T cell adhesion and chemotaxis (Siliceo et al., 2006). Though these studies have established a role for chimaerins as important DAG effectors in T cells, further work is required to dissect aspects of their function that are dependent on and independent of DAG-binding.

1.2.2 The IKK-NF-κB and Ras-ERK pathways

As stated in the previous section, DAG recruits PKCθ to the plasma membrane upon TCR engagement. Translocation of PKCθ to membrane rafts is essential for T cell activation, and occurs in a lymphocyte-specific protein tyrosine kinase (Lck)-dependent manner (Bi et al., 2001). Membrane-localization and activation of PKCθ initiates signaling via the inhibitor of NF-κB kinase (IKK)-NF-κB pathway, which plays a critical role in multiple aspects of T cell development and function (Schulze-Luehrmann and Ghosh, 2006; Vallabhapurapu and Karin, 2009). A complex of three proteins - CARD-containing MAGUK protein 1 (CARMA1), B-cell lymphoma 10 (Bcl10) and mucosa
associated lymphoid tissue lymphoma translocation gene 1 (MALT1) - called the CBM signalosome, couples PKCθ activation to that of its downstream mediator IKK (Rosebeck et al., 2011).

Bcl10 and MALT1 were originally identified as targets of chromosomal translocation in human mucosa associated lymphoid tissue lymphomas, but the subsequent generation of knockout mice revealed an essential role for both proteins in TCR-induced NF-κB activation (Ruland et al., 2001; Xue et al., 2003). While Bcl10 is a small protein consisting of a single caspase recruitment domain (CARD), MALT1 contains several structural features including an amino-terminal death domain, a protease domain and a ubiquitin ligase-binding domain (Thome et al., 2010). A role for the Bcl10-interacting scaffolding protein CARMA1 in TCR-induced NF-κB activation was identified by multiple groups (Blonska and Lin, 2009; Egawa et al., 2003; Gaide et al., 2002; Hara et al., 2003; Pomerantz et al., 2002; Wang et al., 2002). Some studies suggest that CARMA1 can recruit Bcl10, IKKβ and PKCθ to the immunological synapse upon TCR/CD28 engagement (Wang et al., 2004), while others have identified a key role for the kinase 3-phosphoinositide dependent protein kinase-1 (PDK1) in nucleating this signaling complex (Lee et al., 2005).

The IKK complex consists of the catalytic kinases IKKα and IKKβ, and the regulatory scaffolding protein IKKγ/ NF-κB essential modulator (NEMO) (Mercurio et al., 1997). Both recognition of K63-linked poly-ubiquitin chains on Bcl10 by NEMO (Wu
and Ashwell, 2008) and K63-linked poly-ubiquitination of NEMO via the CBM complex (Zhou et al., 2004) are critical for TCR-induced NF-κB activation. Phosphorylation of key serine residues in the activation loop is known to be critical for activating both IKKα (S176 and S180) and IKKβ (S177 and S181) (Mercurio et al., 1997), and studies have identified that the E3 ubiquitin ligase TNF receptor associated factor 6 (TRAF6) and the kinase TGF-β-activated kinase 1 (TAK1) mediate IKK activation by the CBM complex in T cells (Liu et al., 2012; Sun et al., 2004). The phosphorylation of inhibitor of NF-κB α (IκBα) by active IKK at S32 and S36 targets it for K48-ubiquitination (at K21 and K22) and subsequent proteasomal degradation (Chen et al., 1995; DiDonato et al., 1996; Scherer et al., 1995). TCR signal-induced degradation of IκBα disrupts NF-κB-IκB complexes, allowing the released NF-κB dimers to translocate to the nucleus.

In mammals, the NF-κB family of transcription factors consists of five proteins – p65 (RelA), RelB, cRel, NF-κB1 (p105/p50) and NF-κB2 (p100/p52) (Oeckinghaus and Ghosh, 2009) which share a conserved Rel homology domain (RHD) (Baldwin, 1996; Ghosh et al., 1998). The RHD contains sequences responsible for dimerization, interaction with IκB and nuclear translocation, and also mediates DNA-binding to the NF-κB consensus sequence (Chen et al., 1998; Muller et al., 1995). p65, RelB and cRel also contain a trans-activation domain. NF-κB-driven transcriptional programs are required for T cell development, activation, proliferation, differentiation, and effector function (Hayden and Ghosh, 2011).
In T cells, two Ras guanine nucleotide exchange factors - son of sevenless (Sos) and RasGRP1 - facilitate Ras activation downstream of TCR engagement. While Sos is brought to the immunological synapse via growth factor receptor-bound protein 2 (Grb2) and linker for activation of T cells (LAT), RasGRP1 is recruited through its DAG-binding C1 domain upon T cell activation (Tomlinson et al., 2000). Studies have shown that Sos-Ras-ERK signaling cannot compensate for defective RasGRP1-Ras-ERK signaling, suggesting that these signals might be functionally unique (Roose et al., 2005). Membrane-localized RasGRP1 is activated by PKCθ-mediated phosphorylation at T184 (Roose et al., 2005), and subsequently converts inactive Ras-GDP to active Ras-GTP.

Ras is a 21-kDa guanine nucleotide-binding protein that is anchored to the plasma membrane by lipid modifications (Roose and Weiss, 2000). The active GTP-bound form of Ras is converted to an inactive GDP-bound form by Ras’ intrinsic GTPase activity. Cycling of Ras between its active and inactive forms is regulated by the activity of GAPs (GTPase activating proteins, which enhance Ras’ GTPase activity) and GEFs (guanine nucleotide exchange factors, which promote dissociation of GDP from Ras, allowing Ras to associate again with GTP). When GEF activity of RasGRP1 converts Ras to its active GTP-bound form, active Ras in turn binds to activates the serine/threonine kinase RAF1 through a multi-stage process (Roskoski, 2010). Members of the RAF family share three conserved structural regions – the first region contains a Ras-binding domain and a zinc-binding cysteine rich domain, the second has a serine/threonine rich domain,
and the third has a kinase domain (Wellbrock et al., 2004). Active RAF1 phosphorylates and activates mitogen-activated protein kinase (MAPK) kinases MEK1 and MEK2, which subsequently phosphorylate and activate extracellular-signal-regulated kinases ERK1 and ERK2.

MEK1/2 are serine/threonine kinases, structurally composed of a tri-functional N-terminal sequence and a kinase domain (Roskoski, 2012b). The N-terminal sequence contains an ERK binding site, nuclear export sequence and inhibitory segment. Activation of MEK1 requires phosphorylation at S218 and S222 in the activation segment of the kinase domain (Zheng and Guan, 1994). Kinase suppressor of Ras (KSR) proteins serve an essential scaffolding function in facilitating the assembly of RAF-MEK-ERK complexes (Brown and Sacks, 2009). ERK1/2 are ubiquitously expressed serine/threonine kinases that, unlike RAFs and MEKs, are able to phosphorylate a wide range of substrates. Both ERK1 and ERK2 are activated in response to a variety of mitogenic and other stimuli, and their activation requires MEK1/2-catalyzed phosphorylation of two residues within the activation segment (Roskoski, 2012a).

Dimers of c-Fos and c-Jun DNA-binding proteins constitute the transcriptional regulator AP-1 (Chiu et al., 1988; Zenz et al., 2008). Phosphorylation of c-Fos by ERK1/2 at S374 (and by RSK1 at S362) is thought to be critical for its stability (Chen et al., 1996; Monje et al., 2003), and others have shown that ERK activity can increase the expression of c-Fos mRNA by enhancing the transcriptional activity of Elk1 (Gille et al., 1995).
While the MAPK c-Jun N-terminal kinase (JNK) is thought to be predominantly responsible for c-Jun phosphorylation at S63 and S73, studies with fibroblasts have revealed that these residues can also be phosphorylated by ERK1/2 (Morton et al., 2003). Like NF-κB, AP-1-driven gene expression is critical for a plethora of key processes including T cell development, activation, cytokine production and survival (Foletta et al., 1998; Macian et al., 2001). By directly activating these essential signaling pathways, DAG plays an important role in the life of a T cell.

1.2.3 The TSC1-mTOR pathway and Mnk proteins

Recent studies have identified a role for TCR-induced DAG as a positive regulator of mammalian/mechanistic target of rapamycin (mTOR) activity (Gorentla et al., 2011; Salmond et al., 2009). mTOR is a 289-kDa serine/threonine kinase that acts as a signal integrator in T cells, integrating a variety of extracellular cues including the presence of nutrients, growth factors, cytokines, antigen and co-stimulation (O’Brien and Zhong, 2012; Waickman and Powell, 2012). Within the cell, mTOR exists as a part of two multi-protein complexes called mTOR complex 1 (mTORc1) and mTOR complex 2 (mTORc2) that differ from each other in substrate specificity and sensitivity to the antifungal macrolide rapamycin. In complex 1, mTOR associates with the scaffolding protein regulatory-associated protein of mTOR (raptor), and with mammalian lethal with Sec13 protein 8 (mLST8), proline-rich Akt substrate 40 kDa (PRAS40) and DEP-domain-containing mTOR-interacting protein (deptor). mTORc2 consists of mTOR,
raptor-independent companion of TOR (rictor), mLST8, mSin1 and protein observed with rictor (protor) (Laplante and Sabatini, 2009). mTORC1-mediated phosphorylation of ribosomal protein S6 kinase 1 (S6K1) and eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1 (4E-PB1) promotes translation, while phosphorylation of protein kinase B (PKB/Akt) at S473 by mTORC2 is thought to contribute to complete Akt activation (Alessi et al., 1996; Bhaskar and Hay, 2007).

The tuberous sclerosis complex (TSC), a dimer of the tumor suppressor proteins TSC1 and TSC2, serves as an upstream negative regulator of mTORC1 (Inoki et al., 2002; Tee et al., 2002). While TSC2 possesses GAP activity, TSC1 is required to stabilize TSC2 and prevent its ubiquitin-mediated degradation (Benvenuto et al., 2000; Chong-Kopera et al., 2006). Under resting conditions, GAP activity of the TSC complex maintains the Ras family protein Ras-homolog enriched in brain (Rheb) (Manning and Cantley, 2003) in an inactive GDP-bound form, precluding mTORC1 activation. In T cells, signaling via CD28 or receptors for IL-2, IL-4, IL-7 or IFN-γ can activate phosphatidylinositol 3-kinase (PI3K). Active PI3K in turn phosphorylates PDK1, which phosphorylates Akt at T308. When TSC2 is inactivated by Akt-mediated phosphorylation, active GTP-bound Rheb enhances mTORC1 activity. Although some studies have shown that Rheb directly binds mTOR (Long et al., 2005) and that Rheb can promote the phosphorylation of mTOR at S1261 in response to insulin (Acosta-Jaquez et al., 2009), the exact mechanisms by which Rheb activates mTORC1 in T cells remain incompletely understood. The PI3K-
PDK1-Akt axis represents one pathway by which the TSC1-mTORc1 pathway is activated in T cells.

Results from recent studies have shown that TCR engagement and DAG can activate mTORc1 and mTORc2 via the Ras-ERK pathway in thymocytes (Gorentla et al., 2011). Deletion of RasGRP1 or inhibition of MEK1/2 was sufficient to decrease mTOR activity, while a constitutively active form of Ras or MEK1 could promote mTOR activation. Further, combined genetic deletion of DGKα and ζ enhanced mTORc1 and mTORc2 activity, while over-expression of DGKζ inhibited the activity of the mTOR complexes in thymocytes. Others have demonstrated that maximal activation of the ribosomal protein S6 (a substrate of S6K1) in CD8 cells requires the activation of both PI3K-Akt and Ras-ERK pathways (Salmond et al., 2009). Together, these recent studies have established a novel role for the DAG-Ras-ERK axis as a mediator of TCR-induced mTOR activation. Optimal mTOR activity is essential for normal T cell trafficking and activation, CD4 T H differentiation and CD8 effector/memory differentiation (Powell et al., 2012).

A quiescent T cell that recognizes its cognate antigen faces a sudden need for rapid protein synthesis, which it meets by increasing both gene transcription and mRNA translation. Previous work has identified that the expression of a subset of genes is regulated at the translational level during T cell activation (Garcia-Sanz et al., 1998). In addition, others have shown that poor mitogen-induced proliferation of T cells from
patients with systemic lupus erythematosus is attributable in part to defects in translation initiation (Grolleau et al., 2000). The initiation factor eIF4E plays an important role in mRNA translation by binding to the 5’ cap structure (m’GpppX) of mRNAs and facilitating the recruitment of other translation factors to the mRNA (Sonenberg, 2008). Phosphorylation of 4E-BP1 by active mTORc1 disrupts the eIF4E-4E-BP1 complex, allowing eIF4E to participate in translation initiation. On the other hand, MAPK signal-integrating kinases (Mnks) 1 and 2 bind to eIF4G within the eIF4F complex and phosphorylate eIF4E at S209. Mnks are serine/threonine kinases that are phosphorylated and activated by ERK and p38 kinases (Fukunaga and Hunter, 1997; Waskiewicz et al., 1997). The functional consequences of Mnk-mediated eIF4E phosphorylation are unclear but thought to favor the translation of certain mRNAs such as those with complex 5’ UTRs (Scheper and Proud, 2002; Scheper et al., 2002). Together, these data suggest that DAG-initiated signals converge on the eIF4E-4E-BP1 complex along two pathways – the Ras-ERK-mTORc1 pathway that phosphorylates 4E-BP1, and the Ras-ERK-Mnk pathway that phosphorylates eIF4E – to promote translation. DAG-mediated pathways of TCR signaling discussed in sections 1.2.2 and 1.2.3 are summarized in Fig. 3.
Figure 3: DAG-mediated pathways of TCR signaling
Figure 3. Following TCR engagement, orchestrated action of proximal tyrosine kinases and adaptor molecules results in the activation of PLCγ1, which hydrolyzes membrane PIP2 into second messengers IP3 and DAG. While IP3 triggers the Ca2+-calcineurin-NFAT pathway, DAG recruits multiple effectors through their C1 domains. DAG-mediated recruitment of PKCθ initiates signaling via the PKCθ-IKK-NF-κB pathway, and recruitment of RasGRP1 activates the RasGRP1-Ras-ERK pathway. CD28 signaling plays a critical role in activating both IKK-NF-κB and PI3K-Akt-mTORc1 pathways. DAG-mediated activation of the Ras-ERK pathway contributes to mTORc1 and mTORc2 (not shown in this figure) activation upon TCR stimulation. While mTORc1 activity promotes translation by phosphorylating 4E-BP1, Mnk1/2 kinases function downstream of ERK1/2 and JNK (not show in this figure) to phosphorylate eIF4E. DGKs remove DAG by converting it to PA. Please refer to the text for a more detailed explanation of these pathways.
### 1.3 Role of DGKs in T cell development and function

As stated previously, DGKs are a family of enzymes that catalyze the conversion of DAG to PA. Work from several groups, including ours, has shown that DGKs serve as a braking mechanism in immune cell signaling, dampening DAG levels after receptor stimulation and preventing hyper-activation of immune cells (Rincon et al., 2012; Wattenberg and Raben, 2007; Zhong et al., 2008; Zhong et al., 2011). Notably, both the substrate and product of the DGK-catalyzed reaction, DAG and PA, are bioactive lipids that can act as second messengers (Cai et al., 2009; Huang and Sauer, 2010; Stace and Ktistakis, 2006; Wang et al., 2006). While DAG recruits RasGRP1 and PKC\(\theta\) to the cell membrane, PA can bind to signaling molecules such as mTOR, Src homology region 2 domain-containing phosphatase-1 (SHP-1), Ras GTPase activating protein (RasGAP), Sos, and p47(phox) (Cai et al., 2009; Fang et al., 2001; Frank et al., 1999; Karathanassis et al., 2002; Shin and Loewen, 2011; Tsai et al., 1991; Waite et al., 1997; Zhao et al., 2007). DGK activity therefore serves as a switch that simultaneously dampens DAG-mediated signals and boosts PA-mediated signals.

Ten DGK isoforms have been identified in mammals, many of which are expressed in cells of the immune system. All isoforms contain a catalytic kinase domain, consisting of a conserved motif and an accessory domain, and at least two cysteine-rich DAG-binding C1 domains. However, DGKs also possess other distinct structural domains, based on which they are classified into five types. Two DGK isoforms, the
type-I α isoform and the type-IV ζ isoform, are highly expressed in T cells (Sanjuan et al., 2001; Zhong et al., 2002). Several DGK isoforms, including ζ, undergo alternative splicing (Ding et al., 1997; Sakane et al., 2007). Due to their distinct structural domains, different types of DGKs tend to localize to specific subcellular compartments and are regulated by unique cues in the intracellular milieu (Kobayashi et al., 2007). Both DGKα and DGKζ have been observed to reside in or move to the nucleus upon stimulation in different cell types (Raben and Tu-Sekine, 2008).

1.3.1 Role of DGKs in thymocyte development

PLCγ1 deficiency in thymocytes impairs both positive and negative selection processes, suggesting a potential role for DAG-mediated signals in T cell development (Fu et al., 2010). Lending further credence to this notion, numerous studies have shown that defects in DAG-effector pathways profoundly impact thymocyte development. For instance, thymocytes deficient in RasGRP1 display severely impaired positive selection, with a marked paucity of mature single positive cells (Dower et al., 2000). Also, though deficiency of PKCθ or IKKβ does not appear to affect conventional αβ T cell maturation (Schmidt-Supprian et al., 2003; Sun et al., 2000), a recent study has revealed a differential role for NF-κB activity in the selection and survival of CD4 and CD8 thymocytes (Jimi et al., 2008). The importance of DAG-triggered Ras-ERK and PKCθ-NF-κB pathways in thymocyte selection processes thus suggests that tight regulation of DAG levels by DGKs may be critical for normal T cell development.
While genetic deficiency of either DGKα or ζ does not obviously alter thymocyte populations (Olenchock et al., 2006; Zhong et al., 2003), more recent work from our group has provided genetic evidence that DGKα and DGKζ synergistically regulate T cell development (Guo et al., 2008). Combined deficiency of the DGKα and ζ isoforms led to a severe block in murine thymocyte development at the DP stage, with a dramatic reduction in the number of mature CD4SP and CD8SP cells. Crossing with HY TCR transgenic mice revealed that combined DGKαζ deficiency was associated with impaired positive selection, but not negative selection. Reduced DGK activity in DGKαζ double knockout (DGKαζDKO) thymocytes was associated with increased levels of intracellular DAG after TCR stimulation, and enhanced signaling via the Ras-ERK pathway. However, the developmental blockade was partially overcome by PA treatment, suggesting that DGKs play a critical role in thymocyte development not only by terminating DAG-mediated signaling but also by initiating PA-mediated signals. A novel role for DGKs in regulating mTOR activity in thymocytes has also emerged recently (Gorentla et al., 2011), as discussed in section 1.2.3.

1.3.2 Role of DGKs in T cell activation and anergy

Mice deficient in DGKζ have slightly fewer T cells in the periphery than WT counterparts (Zhong et al., 2003). DGKζ−/− T cells show selective perturbations in DAG-mediated signaling including enhanced Ras-ERK activation and reduced PA production upon TCR stimulation. However, DAG-independent events including TCR-induced
calcium mobilization remain unaffected. Upon TCR cross-linking with anti-CD3 antibodies, a greater proportion of DGKζ-/- T cells upregulate surface markers of activation, such as CD69 and CD25, as compared to DGKζ-sufficient counterparts. In addition, T cells deficient in DGKζ proliferate more readily and rapidly than WT T cells upon ex vivo stimulation with anti-CD3 or transfer to lymphopenic hosts. T cell numbers in the spleens and lymph nodes of DGKα-/- mice are comparable to those of WT littermates (Olenchock et al., 2006). DGKα-/- T cells resemble DGKζ-/- counterparts in showing enhanced activation of the Ras-ERK pathway and increased proliferation in response to TCR stimulation. Taken together, studies with DGKα-/- and DGKζ-/- mice establish important and non-redundant roles for these isoforms in limiting T cell activation and proliferation in response to TCR stimulation.

Given the equimolar production of DAG and IP₃ following TCR engagement, it stands to reason that DGKs may play a role in anergy induction by selectively dampening DAG-mediated signals in the absence of co-stimulation. Studies have revealed a critical role for DGK isoforms, particularly DGKα, in the induction and enforcement of T cell anergy. In primary T cells, both DGKα and ζ are expressed at higher levels in the anergic state than in the activated state (Olenchock et al., 2006). Similarly, anergic CD4 (T₉₁ clone) cells express five-fold to ten-fold more DGKα and two-fold more DGKζ than control CD4 cells (Zha et al., 2006). Overexpression of DGKα in T₉₁ cells resulted in an anergy-like state, characterized by suppressed Ras-ERK
activation and reduced IL-2 transcription in response to stimulation with anti-CD3 and anti-CD28. DGKα overexpression also produced an anergy-like state in 2C TCR transgenic CD8 cells, as seen by impaired recruitment of RasGRP1 to the plasma membrane. Pharmacological inhibition of DGK activity led to a dose-dependent recovery of IL-2 production by anergic Th1 cells \textit{ex vivo}, and anergic 2C cells \textit{in vivo}.

In an \textit{in vivo} model of anergy induction with staphylococcal enterotoxin B (SEB), T cells from DGKα\textsuperscript{−/−} mice (in contrast to WT counterparts) were resistant to the induction of anergy and retained the ability to produce IL-2 and proliferate when re-stimulated with SEB \textit{ex vivo}, providing direct genetic evidence of the role of DGKα in enforcing T cell anergy (Olenchock et al., 2006). When CD8-depleted splenocytes were stimulated under anergy-inducing conditions (anti-CD3 and CTLA4-Ig) \textit{ex vivo}, very few surviving WT cells divided in 48 hours. In contrast, DGKα\textsuperscript{−/−} and DGKζ\textsuperscript{−/−} T cells were relatively resistant to anergy induction and underwent two to three rounds of cell division. When DGKζ\textsuperscript{−/−} cells were stimulated in a similar fashion, but in the presence of a DGKα inhibitor, they showed growth and division comparable to WT cells receiving anti-CD3 and anti-CD28 stimulation. Taken together, results from these studies reveal a key role for DGK activity in the induction of T cell anergy.

\textbf{1.3.3 Regulation of CD8 T cell responses by DGKs}

In an early study, DGKζ\textsuperscript{−/−} mice showed a greater increase in CD8 splenocyte numbers upon infection with lymphocytic choriomeningitis virus (LCMV), as compared
to WT counterparts (Zhong et al., 2003). In addition, a higher percentage of CD8 cells in DGKζ−/− mice showed an activated phenotype, as evidenced by up-regulation of CD44 and down-regulation of CD62L markers on the cell surface. A recent study investigated in further detail the effect of DGK deficiency on CD8 T cell responses to LCMV (Shin et al., 2012). DGKα−/− and DGKζ−/− mice showed increased CD8 T cell expansion upon infection with LCMV, and more DGK-deficient CD8 cells produced IFNγ than WT counterparts. These changes were determined to be CD8 cell intrinsic in DGKζ−/−, but not DGKα−/− mice, by adoptive transfer experiments. Fewer memory cells were generated/maintained in the absence of either DGK isoform. When equal numbers of WT or DGK-deficient LCMV-specific CD8 memory cells were transferred into WT recipients and re-challenged with LCMV, DGK-deficient memory cells showed impaired expansion but normal cytokine production. Taken together, studies with the LCMV model reveal that DGK activity may differentially regulate primary and memory CD8 immune responses.

Apart from their role in responding to pathogens, CD8 cells play a critical role in defending against tumors (Ochsenbein, 2002). Recent experiments have shown that DGKζ−/− mice develop smaller tumors than WT mice upon implantation with EL4 lymphoma cells expressing ovalbumin (Riese et al., 2011). An increased proportion of CD44hi CD62Llo TEM type CD8 cells was found in the spleens of DGKζ−/− mice, and a greater proportion of tumor-infiltrating CD8 cells was proliferating in DGKζ−/− mice than
WT counterparts. Adoptive transfer of congenically marked WT OT1 or DGKζ+ OT1 cells into WT mice that were subsequently injected with EL4-Ova cells produced similar results, arguing for a CD8 cell-intrinsic role of DGKζ deficiency in enhancing anti-tumor responses.

A higher expression of DGKα was also found in tumor-infiltrating CD8 cells from renal cell carcinoma patients, as compared to non-tumor kidney-infiltrating cells, in another recently published study (Prinz et al., 2012). While the tumor-infiltrating cells showed normal TCR proximal signaling, distal events such as phosphorylation of ERK, JNK, Akt, and IκB were impaired. No such defects were observed in CD8 cells residing outside tumors. The signaling defects in tumor-infiltrating cells also correlated with functional impairment in lytic activity and cytokine production. Treatment of tumor-infiltrating CD8 cells with a DGK inhibitor or with low-dose IL-2 was found to enhance ERK phosphorylation and lytic granule exocytosis, suggesting that enhancement of DGK expression/activity may be a possible mechanism by which infiltrating T cells are rendered less potent by the tumor micro-environment. Taken together, the studies discussed in this section suggest that DGK activity plays an important role in restraining CD8 responses to pathogens and tumors. The functions of DGK discussed in sections 1.3.1 to 1.3.3 (and other functions in iNKT cell development and T cell microtubule-organizing center polarization) are summarized in Fig. 4.
Figure 4. DGKs in T cell development and function

DGKs α and ζ are the predominant isoforms expressed in T cells. These isoforms synergistically regulate the development of αβ T cells and iNKT cells in the thymus. In the periphery, absence of DGKs is associated with TCR-induced hyper-activation and resistance to anergy induction. DGK activity differentially regulates primary and memory CD8 responses, as it dampens the former and promotes the latter (in a model of viral infection). Localized accumulation of DAG also plays an important role in T cell microtubule-organizing center polarization towards the immunological synapse, and DGKs are thought to play an essential role in this process.
1.4 Thesis motivation and rationale

Based on the findings discussed thus far, it stands to reason that tight regulation of DAG-mediated signaling by DGKα and DGKζ is essential for normal T cell development and function. Unpublished data from our lab suggests that DGKαζDKO T cells show hyper-activation of the Ras-ERK, NF-κB and mTORc1 pathways and hyper-proliferate in response to TCR-mediated stimulation. DGKαζDKO mice are also significantly more susceptible than WT counterparts to the development of autoimmune hepatitis. While multiple DAG-mediated pathways are hyper-activated upon DGKαζ deficiency, the contribution of dysregulated signaling via each pathway to the phenotype observed in DGKαζDKO T cells and mice remains unknown. Here, we aim to dissect the mechanistic contribution of hyper-activation of individual DAG-mediated pathways to the functional abnormalities observed in DGKαζDKO T cells. In Chapter 3, we examine the effects of chronic activation of the IKK-NF-κB pathway on T cell survival and function. While others in the laboratory have examined the effect of constitutive Ras-ERK activation on T cell function, in Chapter 4, we move one step further downstream of ERK to investigate the role of Mnk1/2 (which are phosphorylated by ERK and p38) in T cell function. In Chapter 5, we study the effects of hyperactive TSC1-mTORc1 signaling on antigen-specific CD8 responses. Taken together, the results from these studies provide insight into the mechanisms underlying T cell dysfunction in the absence of DGKαζ activity.
2. Materials and Methods

2.1 Mice

C57BL6/J, R26STOP\textsuperscript{FL}IKK2\textsuperscript{ca} (Sasaki et al., 2006), OT1-TCR transgenic, and TSC1\textsuperscript{FL/FL} (Kwiatkowski et al., 2002) mice were obtained from The Jackson Laboratory. Mice expressing CD4Cre (C57BL6/J background) were obtained from Taconic Farms and Mnk1/2DKO (Ueda et al., 2004) mice were provided by Rikiro Fukunaga. DGKαζDKO mice were reported previously (Guo et al., 2008). All animals were housed in specific-pathogen free conditions and used in accordance with National Institutes of Health guidelines. Experiments described were approved by the Institutional Animal Care and Use Committee of Duke University.

2.2 Flow cytometry

Cells from the thymus, spleen, and lymph nodes (LN)s were isolated in IMDM containing 10% FBS and antibiotics using standard protocols and treated with ACK buffer to lyse RBCs. Liver mononuclear cells were isolated as described previously (Shen et al., 2011a). Staining with fluorescently labeled antibodies was performed in PBS containing 2% FBS, samples were collected on a BD FACSCanto II cytometer, and data were analyzed using FlowJo software. Fluorochrome-conjugated antibodies against CD3\textepsilon, CD4, CD8α, CD11b, CD62L, CD44, CD25, CD69, Thy1.1, Thy1.2, Fas, FasL, Vα2, IL-2, IL-17A, IFN-γ, NK1.1 and PD1; isotype controls; and anti-FasL blocking Ab were obtained from BioLegend. CD1d-tetramers were provided by the National Institutes of
Health Tetramer Facility. Anti-CD3ε and anti-CD28 antibodies for T cell stimulation, and BrdU flow kits, were obtained from BD Biosciences. Annexin V- allophycocyanin (BD Biosciences) staining was performed using the recommended annexin binding buffer, and cell viability was assessed using the Invitrogen Live/Dead Fixable Violet stain or 7AAD. Anti-FITC Alexa Fluor 488 was obtained from Invitrogen.

2.3 Immunoblot analysis

Experiments with IKK mice - Purified T cells were used directly to make cell lysates in 1% Nonidet P-40 buffer containing protease and phosphatase inhibitors and subjected to immunoblot analysis for total IκBα, cleaved caspases, and Bcl-xL. Additionally, purified T cells were rested in PBS at 37°C for 30 min and then left unstimulated or stimulated with an anti-CD3ε antibodies (5 μg/ml; 500A2) for 2 or 15 minutes. These cells were subsequently lysed in a 1% Nonidet P-40 buffer containing protease and phosphatase inhibitors. Proteins were resolved by SDS-PAGE, transferred to a nitro-cellulose membrane (Bio-Rad), and subjected to immunoblot analysis for phospho-ZAP70 (Y493), total ZAP70, and p-ERK 1/2. Membranes were stripped and reprobed with anti-β-actin antibodies for loading control. All antibodies for immunoblotting were purchased from Cell Signaling Technology.

Experiments with Mnk1/2DKO mice - Thymocytes or splenocytes were washed with PBS. Cell stimulation, lysate preparation, protein resolution and transfers to nitrocellulose membranes were performed as described above. To examine protein
phosphorylation, the membranes were incubated overnight with antibodies specific for phospho-ERK1/2, phospho-p38, phospho-4E-BP1 (T37/46), phospho-eIF4E (S209), phospho-Mnk1 (T197/202), total Mnk1 (Cell Signaling Technology), total Mnk2, and β-actin (Santa Cruz Biotechnology). Later, the membranes were incubated with the appropriate secondary, peroxidase-conjugated antibodies. The blots were developed using the ECL System from Perkin-Elmer. The same blots were stripped and reprobed using control antibodies.

2.4 Isolation of nuclear and cytoplasmic fractions

Purified T cells (20 million) were left unstimulated or stimulated with plate-bound anti-CD3ε and anti-CD28 for 6 h and then washed twice with PBS. The cell pellets were resuspended in 0.8 ml buffer A (10 mM HEPES [pH 7.9], 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, and 0.5 mM PMSF) and left on ice for 15 min. Nonidet P-40 was added to 0.5%, and samples were vortexed for 10 s and then centrifuged at 15,000 rpm for 30 s. The supernatants collected at this point constituted the cytosolic fraction. The residual pellets were resuspended with 0.1 ml buffer C (20 mM HEPES [pH 7.9], 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 0.5 mM PMSF) for 10 min on ice. Samples were centrifuged at 15,000 rpm for 10 min at 4°C. The supernatants collected at this point constituted the nuclear fraction. Protein concentrations were determined using Bio-Rad reagent. Collected samples were subjected to SDS-PAGE, followed by immunoblot, as described above.
2.5 T cell activation, anergy and proliferation assays

Splenocytes were left unstimulated or stimulated with anti-CD3ε (1 μg/ml; 145-2C11) overnight in the presence or absence of either anti-CD28 (0.5 μg/ml) or CTLA4-Ig (10 μg/ml; BioXcell) to assess the upregulation of early activation markers by FACS. For proliferation assays, splenocytes were labeled with 10 μM CFSE for 9 min at room temperature and plated at a density of 1.5–2 × 10⁶ cells/well in 48-well plates. These cells were either left unstimulated or stimulated with 1 μg/ml anti-CD3ε Ab (145-2C11) for 65–72 h at 37°C. After staining for CD4 and CD8, cells were subsequently subjected to FACS analysis. Blocking Abs against FasL (BioLegend) were used in culture at a final concentration of 10 μg/ml. Human IL-2 was used at a final concentration of 100 U/ml, and anti-CD28 antibody was used at 0.5 μg/ml.

In some experiments, CGP57380 (TOCRIS Bioscience, a Mnk1/2 inhibitor) was added to the culture at the indicated concentrations. To examine T cell anergy, splenocytes were stimulated with anti-CD3ε in the presence of either anti-CD28 (0.5 μg/ml) or CTLA4-Ig (10 μg/ml) at 37°C for 48 h. Cells were then washed three times and rested in IMDM at 37°C for 24 h. Live cells enriched after Lympholyte (Cedarlane) gradient separation were restimulated with plate-bound anti-CD3ε (1 μg/ml) and soluble anti-CD28 (0.5 μg/ml) in the presence of 5 μM GolgiPlug (BD Biosciences) at 37°C for 24 h. Cells were surface-stained for CD4 and CD8 and intracellularly stained for IFN-γ before flow cytometric analysis.
2.6 Calcium influx assay

LN cells, at a density of $10^7$ cells/ml in loading buffer (1% FBS and 10 mM HEPES in HBSS without phenol red), were loaded with intracellular calcium indicator dye Indo-1 (2 μg/ml; Molecular Probes) in the presence of FBS and Pluronic at 30°C for 30 min. Cells were washed with loading buffer and subsequently stained with fluorochrome-conjugated antibodies against CD4 and CD8. Flow cytometric analysis was performed on a BD FACStar Plus cytometer. Cells were incubated for 2–3 min at 37°C with a mixture of biotin-conjugated antibodies against CD3, CD4, and CD8 (10, 5, and 5 μg/ml, respectively); baseline fluorescence (ratio of 450/510 nm) was measured; and streptavidin (12 μg/ml) was added to cross-link TCR and co-receptors. Once the ratio returned to baseline levels, ionomycin (1 μg/ml) was added to ensure that samples were properly loaded with Indo-1 and to rule out defects in calcium buffering.

2.7 Adoptive transfer and Listeria monocytogenes expressing rOVA infection

Naive OT1 cells (Vα2+CD8+ 7AAD−CD44−) were sorted from spleen and LN cells of donor mice. A total of $10^4$ (Mnk and TSC experiments) or $10^5$ (IKK experiments) sorted cells in 200 μl serum-free IMDM were adoptively transferred by retro-orbital injection into sex-matched WT Thy1.1−Thy1.2+ recipients. After 24 h, recipient mice were injected i.v. with $10^4$ CFU of *Listeria monocytogenes* expressing recombinant OVA (Lm-OVA) (Pope et al., 2001; Shen et al., 1995). Peripheral blood samples collected (in PBS with 5 mM EDTA) at 1, 2, 4, 7 or 12 weeks post-infection were treated to lyse RBCs, labeled
with fluorochrome-conjugated antibodies, and analyzed by flow cytometry. For BrdU incorporation, mice received 1.5 mg of BrdU i.p. on day 5 post-infection, and were sacrificed after 16 hours.

**2.8 Quantification of gene expression by real-time PCR**

Total RNA was isolated from cells using a TRIzol (Invitrogen)-based protocol and reverse transcribed using Superscript II reverse transcriptase (Invitrogen). Transcripts encoding Fas, FasL, PD1/Pdcd1, Blimp1/Prdm1, CCL3, CCL5, CCL28 and β-actin were quantified by SYBR Green real-time PCR (Eppendorf Mastercycler ep Realplex²), using the following primers: Fas F: 5'-ATGCACACTCTGCGATGAAG-3', Fas R: 5'-CAGTGTTCACACGCCAGGAGA-3', FasL F: 5'-ACTCCGTGAGTTCACCAACC-3', FasL R: 5'-ATTCCAGAGGGATGGACCTT-3', PD1/Pdcd1 F: 5'-CTGGAAGCAAGGACGACACT-3', PD1/Pdcd1 R: 5'-TGTTGGCATATTCTGTGTGC-3', Blimp1/Prdm1 F: 5'-TGTTATTGTCCGGACTTTTG-3', Blimp1/Prdm1 R: 5'-TGCCCTTGCTGTCTTCTCTCTCT-3', CCL3 F: 5'-TGCGGACACTCTTTGGGTAG-3', CCL3 R: 5'-GATGAATTGGCGTGGAATCT-3', CCL5 F: 5'-GTGCCCAACGTAAGGAGAT-3', CCL5 R: 5'-CCACTTTCTTCTCTGGGTGG-3', CCL28 F: 5'-GAGTTTCATGCAGCATCCAGA-3', CCL28 R: 5'-AGGCTCTCATCCACTGCTTC-3', β-actin F: 5'-TGTCACCTTCCAGCAGATG-3', and β-actin R: 5'-AGGCTCAGTAAAAGCTCCGCTAG-3'. The transcript levels of other genes were normalized with respect to those of β-actin using the ΔΔCt method.
2.9 Immunofluorescence microscopy

Liver pieces were frozen in Optimal Cutting Temperature medium and cryostat sectioned (10 μm). Sections were fixed in a 1:1 mixture of acetone/methanol and subsequently stored at −20°C. For staining, sections were hydrated in wash buffer (PBS with 1% BSA) for 20 min and blocked in PBS with 10% BSA for 20 min. They were then incubated with primary antibodies (CD3ε-FITC, CD4-PE, or CD11b-FITC; 1:200 dilution) and Hoechst nuclear stain (1:1000 dilution of 10 mg/ml solution) in 3% BSA for 20 min at room temperature. After extensive washing, sections were incubated with anti-FITC Alexa Fluor 488 antibody (1:400 dilution in 3% BSA) for 20 min at room temperature. Sections were washed extensively, cover-slipped with Fluoromount G, and imaged on a Zeiss ApoTome microscope at 200× magnification using AxioVision 4.0 software. Images were processed and colocalization images were prepared using Adobe Photoshop CS4.

2.10 In vitro stimulation of iNKT cells

Thymocytes were cultured in vitro in 10% FBS-complete IMDM with or without α-galactosylceramide (α-GalCer; 125 ng/ml) stimulation at 37°C for 72 h. During the last 5 h of stimulation, PMA (phorbol 12-myristate 13-acetate, 50 ng/ml), ionomycin (500 ng/ml, Sigma) and GolgiPlug were added. After surface staining with anti–TCR-β antibody and the PBS-57-loaded mouse CD1d tetramer (CD1d-Tet), cells were intracellularly stained for IFN-γ and IL-17 followed by FACS analysis. iNKT cell proliferation was similarly assessed except that thymocytes were labeled with CFSE and
PMA and ionomycin stimulation was not added.

**2.11 T helper differentiation assay**

Naive CD4 T cells were purified from LN cells and cultured with plate-bound anti-CD3ε (2C11) 5 µg/ml, soluble anti-CD28 (1 µg/ml) and indicated skewing conditions. Skewing conditions were as follows: Th1, IL-12 (5 ng/ml), IFN-γ (100 ng/ml) and anti–IL-4 (100 µg/ml), with IL-2 (100 U/ml) during the rest period; Th2, IL-4 (1 ng/ml), anti–IL-12 (100 µg/ml), and anti–IFN-γ (100 µg/ml), with IL-2 (100 U/ml) during the rest period; Th17, TGF-β (10 ng/ml), IL-6 (10 ng/ml), anti–IFN-γ (100 µg/ml) and anti–IL-4 (100 µg/ml); Th9, IL-4 (20 ng/ml) anti–IL-12 (10 µg/ml), and TGF-β (2 ng/ml) during rest period; Th0, IL-2 (100 U/ml) during the rest period. After culturing for 5 d, cells were stimulated with PMA and ionomycin in the presence of GolgiPlug at 37°C for 5 h. Following surface staining, cells were intracellularly stained for indicated cytokines, followed by FACS analysis.

**2.12 LCMV infection**

LCMV Armstrong stocks were propagated on BHK-21 cells and quantitated as described previously (Ahmed et al., 1984). LCMV infection and assessment of virus-specific CD8 T cell responses were performed as described previously (Shin et al., 2012). Mice were infected with $2 \times 10^5$ PFU of virus i.p. and monitored by serial bleeding and tetramer staining. For memory experiments, viable CD8+ CD44+ memory cells were sorted from donor mice 8 weeks after LCMV infection. While donor mice were
Thy1.1-Thy1.2+, recipients were Thy1.1-Thy1.2+. Frequency of T cells recognizing H-2D^b loaded with LCMV gp33-41 peptide (TetG-positive cells) in the sorted population was determined by flow cytometry, and an appropriate number of total memory cells was transferred such that each recipient mouse received 5000 TetG^+ memory cells. Recipients were infected with 2 × 10^6 PFU of LCMV i.p. the next day and taken 7 d later to assess the memory response.

2.13 Induction and scoring of experimental autoimmune encephalomyelitis

Experimental autoimmune encephalomyelitis (EAE) was induced in 6–10-week-old female mice by s.c. injection of myelin oligodendrocyte glycoprotein (MOG_{35-55}) peptide (100 μg/mouse) emulsified in CFA containing 2 mg/ml *Mycobacterium tuberculosis* (100 μl/mouse). Mice were also injected i.p. with 200 ng of pertussis toxin on day 0 (day of immunization) and day 2. Mice were monitored for ∼40 d to assess the development of a clinical score based on the following criteria: 1 = tail limpness; 2 = impaired righting reflex; 3 = hind limb paralysis; 4 = complete paralysis; 5 = death. Some mice were sacrificed on day 7 to assess CD4 T cell differentiation in response to the immunization. Draining LN cells were stimulated with MOG_{35-55} peptide for 3 d to expand the pool of antigen-specific CD4 T cells, followed by stimulation for 5 h with PMA (50 ng/ml) and ionomycin (500 ng/ml) in the presence of GolgiPlug. After stimulation, cells were stained for cell surface CD4 and intracellularly stained for IL-17A and IFNγ.
3. Chronic Activation of the Kinase IKKβ Impairs T Cell Function and Survival

3.1 Introduction

NF-κB is a ubiquitously expressed transcription factor that plays an important role in the survival and function of various immune cells (Gerondakis and Siebenlist, 2010; Hayden et al., 2006). Engagement of the costimulatory receptor CD28 cooperates with TCR-derived signals to cause robust NF-κB activation in T cells (Huang et al., 2002; Tuosto, 2011). Please refer to section 1.2.2 for a detailed description of signaling via the PKCθ-IKK-NF-κB pathway.

The role of NF-κB signaling in T cell survival, development, activation and differentiation has been investigated using various genetic models (Li and Verma, 2002). Mature T cells deficient in PKCθ fail to activate NF-κB upon TCR triggering, resulting in impaired activation, proliferation (Sun et al., 2000), and Th2 differentiation (Cannons et al., 2010; Marsland et al., 2004; Salek-Ardakani et al., 2004). PKCθ−/− mice also show defects in IL-17 production, as well as resistance to the induction of experimental autoimmune encephalomyelitis (Hayashi and Altman, 2007; Tan et al., 2006). Similarly, Bcl10−/− and MALT1−/− T cells fail to upregulate activation markers, produce IL-2, and proliferate in response to anti-CD3 stimulation (Ruefli-Brasse et al., 2003; Ruland et al., 2001; Ruland et al., 2003). Targeted deletion of IKKγ partially blocks T cell development in the thymus but completely abolishes the peripheral T cell pool, indicating a critical role for IKKγ in mature T cell maintenance. In contrast, conditional deletion shows that
T cells deficient in IKKβ maintain residual NF-κB activity by forming non-canonical IKKγ/IKKα complexes (Schmidt-Supprian et al., 2003). Preventing NF-κB activation in T cells by transgenic expression of a dominant degradation-resistant form of IκBα was also shown to impair survival, proliferation, and production of cytokines, such as IL-4 and IL-10 (Ferreira et al., 1999). Therefore, NF-κB signaling is critically involved in several aspects of T cell function.

Although a deficiency in PKCθ–IKK–NF-κB signaling is often detrimental to T cell function, the consequences of its uncontrolled activation remain less studied and understood. Thymocyte development is unperturbed in IκBα−/− fetal liver chimeras, but mature T cells from these mice fail to proliferate in response to TCR cross-linking ex vivo (Chen et al., 2000). Chimeras that lack both IκBα and IκBe show severe thymic atrophy and a dramatically reduced peripheral T cell pool, suggesting that elevated NF-κB signaling may adversely affect thymocyte and/or mature T cell survival (Goudeau et al., 2003). However, given the pleiotropic roles played by NF-κB in different cell types, results drawn from fetal liver chimeras that show constitutive NF-κB activity in all hematopoietic cells prove difficult to interpret. A more recent study probed the effect of constitutive T lineage-specific IKKβ activation on thymocyte development and revealed that uncontrolled NF-κB signaling results in the negative selection of developing CD4SP thymocytes (Jimi et al., 2008). Taken together, the findings from these studies motivate the hypothesis that NF-κB activity must be tightly regulated to maintain normal T cell
homeostasis and function. Emerging evidence suggests that chronic viral pathogens, such as HIV-1 and human T cell leukemia virus 1, often trigger NF-κB signaling in infected cells (Hiscott et al., 2001; Santoro et al., 2003). An improved understanding of the effects of persistent NF-κB signaling on peripheral T cell function may therefore reveal mechanisms that can be targeted to promote desirable outcomes during chronic disease.

To study the functional and mechanistic consequences of T cell-specific IKK–NF-κB hyperactivation, we expressed a conditional constitutively active allele of IKKβ (caIKKβ) (Sasaki et al., 2006) in the presence of Cre recombinase driven by the CD4 promoter (caIKKβCD4Cre or ‘IKK’ mice). We report that uncontrolled IKKβ activation promotes T cell apoptosis and attenuates responsiveness to TCR stimulation, in part, by increasing the expression of Fas ligand (FasL) and B lymphocyte-induced maturation protein 1 (Blimp1), respectively. Diminished IL-2 production also contributed to defects in survival and proliferation. In vivo, IKK T cells mounted a compromised antigen-specific T cell response to bacterial infection. Interestingly, only certain T cell functions were selectively impaired by chronic IKKβ signaling: IKK T cells readily produced proinflammatory cytokines, and mice developed multiorgan inflammation. Our findings suggest that persistent IKKβ activity in T cells adversely affects function and survival, compromises antigen-specific responses, and promotes autoimmunity.
3.2 Results

3.2.1 Constitutive IKKβ signaling impairs T cell survival

We first sought to determine whether caIKKβf/+ CD4Cre (IKK) mice demonstrated defects in thymocyte development similar to those reported previously in a transgenic model of constitutive NF-κB signaling (Jimi et al., 2008). In that model, a constitutively active IKKβ transgene was expressed under the control of a proximal Lck promoter. Although total thymocyte numbers in IKK mice were not significantly different from WT counterparts, FACS analysis showed that T cell development was partially blocked at the DP stage, with a reduction in the percentage and absolute numbers of CD4SP and CD8SP cells (Fig. 5A, 5B). Therefore, the developmental phenotype of our IKK thymocytes is consistent with previously reported data. Staining with the Live/Dead marker also revealed an increased frequency of dead cells among CD4SP and CD8SP IKK thymocytes, although the difference was not statistically significant (Fig. 6A, 6B). Consistent with decreased thymic output, we observed that CD4 and CD8 T cell percentages and numbers were significantly diminished in IKK spleens and LNs (Fig. 5C, 5D, data not shown).
Figure 5: Constitutive IKKβ signaling impairs mature T cell survival
Figure 5. (A) Representative FACS plots of CD4 and CD8 expression on total thymocytes from WT and IKK mice. (B) Absolute numbers of total thymocytes and DN, DP, CD4SP and CD8SP thymocytes from WT and IKK mice. Mean ± SEM are calculated from four independent experiments. (C) Representative FACS plots of CD4 and CD8 expression on total splenocytes from WT and IKK mice. (D) Percentages and absolute numbers of CD4 and CD8 T cells in WT and IKK spleens. Mean ± SEM are calculated from four independent experiments. (E) Representative photograph of spleens from 9-week-old WT and IKK mice (left panel). Spleen weights (middle panel) and absolute splenocyte numbers (right panel) of mice 7 weeks or older are also shown. Each symbol represents data from an individual mouse, and horizontal bars indicate the mean ± SEM. (F) Representative FACS plots of splenocytes stained with Invitrogen Live/Dead marker (left panel). Percentages of Live/Dead+ cells from WT and IKK spleens (right panel). Mean ± SEM are calculated from six independent experiments. *p < 0.05, **p < 0.01 (Student t test)
Figure 6: Cell death in the IKK thymus and changes in splenic composition and architecture in IKK mice with age
Figure 6. (A) Representative FACS plots of Live/Dead staining in the DP, CD4SP and CD8SP subsets of WT and IKK thymocytes. (B) Percentage of dead cells in above thymocyte subsets. Data shown are mean ± SEM calculated from three independent experiments. (C) Representative histograms of B220 expression on total splenocytes from 5 week, 9 week, and 6 month old WT and IKK mice. (D) Representative FACS plots of CD93 and B220 expression on total spleen and bone marrow cells from 9 week old WT and IKK mice. (E) Hematoxylin and eosin staining of WT and IKK spleens from 6-month old mice. (F) Representative FACS plots of Ter119 and CD45 expression on total (RBC-lysed) splenocytes from 9 week old WT and IKK mice. All histograms and photographs shown are representative of at least three independent experiments. (G) Percentage of TCRβ+ cells, B220+ cells, CD11b+ cells, and CD45- cells in spleens of 4-6 month old WT and IKK mice. n=2 for WT mice and n=6 for IKK mice.
IKK mice that were ≥7 weeks of age demonstrated a consistent and substantial increase in spleen size and weight (Fig. 5E), although the time of onset of splenomegaly varied among mice. Interestingly, splenomegaly was not accompanied by a statistically significant increase in viable splenocyte numbers (Fig. 5E). This discrepancy between spleen size and splenocyte numbers is consistent with the progressive loss of splenic B cells that we observed in IKK mice (Fig. 6C). Flow cytometric analysis revealed a loss of CD93⁺ B220⁺ immature/transitional B cells and CD93⁻ B220⁻ mature B cells in both the bone marrow and spleen of older IKK mice (Fig. 6D). This suggests that older IKK mice may suffer from defects in B cell development in the bone marrow, leading to a loss of mature B cells with age. Histological analysis of enlarged spleens revealed a dramatic disruption of the red pulp/white pulp architecture (Fig. 6E). A marked increase in the frequency of CD45⁻ Ter119⁺ erythroid cells in the spleen indicated that splenomegaly may be associated with extramedullary erythropoiesis (Fig. 6F). In addition, the frequency of CD11b⁺ myeloid cells was increased in the spleens of older IKK mice (Fig. 6G). The changes in T cells, B cells, erythroid cells, and myeloid cells may lead to variations in total splenocyte numbers in caIKKβ mice. Therefore, constitutive IKKβ activity in T cells appears to cause multiple secondary changes in splenocyte composition and spleen structure with increasing age, prompting us to perform most experiments using mice younger than 2 months of age.
In several cell types, NF-κB signaling can either prevent or promote apoptosis, depending upon the cellular context in which the pathway is activated (Kucharczak et al., 2003; Perkins and Gilmore, 2006). In T cells, NF-κB is known to play a pro-survival role during TCR-mediated activation and a pro-apoptotic role during the process of activation-induced T cell death (Gerondakis and Siebenlist, 2010). FACS analysis of freshly isolated splenocytes stained with Live/Dead marker revealed a significant increase in cell death in both CD4 and CD8 T cell compartments of IKK splenocytes compared with WT controls (Fig. 5F). Therefore, constitutive IKKβ activity adversely affects peripheral T cell survival.

### 3.2.2 Increased FasL expression promotes IKK T cell apoptosis via the extrinsic pathway

We asked whether the increased cell death observed in IKK T cells was due to activation of apoptotic pathways. Upon staining of freshly isolated splenocytes, an increased proportion of Live/Dead− IKK T cells showed annexin V binding compared with WT cells (Fig. 7A), suggesting that enhanced apoptosis may indeed underlie the defect in cell survival. Western blot analysis of purified WT and IKK T cells showed decreased IκBα levels in IKK T cells, consistent with increased IKKβ activity in these cells (Fig. 7B). In addition, cleaved caspase-3 (downstream effector caspase) was markedly increased in IKK T cells, indicating enhanced apoptosis.
Figure 7: Increased FasL expression contributes to enhanced IKK T cell death via the extrinsic pathway.
Figure 7. (A) Representative FACS plots of Live/Dead and annexin V staining of CD4 and CD8 T cells from WT and IKK spleens. (B) Representative immunoblots of lysates prepared from purified WT and IKK T cells and probed for the indicated proteins. (C) qPCR of Fas and FasL mRNA in CD4 and CD8 cells from WT and IKK spleen. Data shown are representative of two independent experiments. (D) Representative graphs of Fas and FasL expression on CD4 and CD8 T cells from WT and IKK spleen. (E and F) WT and IKK splenocytes were left unstimulated or stimulated with anti-CD3 (2C11) antibody for 65 h in the presence or absence of 10 μg/ml anti-FasL-blocking Ab, followed by CD4, CD8, and Live/Dead staining and FACS analysis. (E) Representative FACS plots. (F) Bar graphs of mean ± SEM from a single experiment in triplicate. Data shown are representative of three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001 (Student t test)
To further investigate whether caspase-3 was activated downstream of the intrinsic (mitochondrial) or extrinsic (death receptor) apoptosis pathways, we probed for the presence of cleaved initiator caspases-9 and -8, respectively. IKK T cells contained more cleaved caspase-8 than did WT T cells, but levels of cleaved caspase-9 were not increased. Expression of Bcl-xL, a Bcl-2 family member that promotes mitochondrial integrity and cell survival, was not decreased in IKK T cells. Taken together, these observations suggest that the extrinsic death pathway is activated in IKK T cells and that signaling downstream of death receptor engagement may be responsible for enhanced IKK T cell apoptosis.

Several death ligand/receptor interactions are known to play a role in T cell apoptosis (Wilson et al., 2009). However, given that FasL was shown to be a direct transcriptional target of NF-κB (Kasibhatla et al., 1999; Matsui et al., 1998), we investigated the role of Fas/FasL interactions in promoting IKK T cell apoptosis. First, we examined the expression of Fas and FasL transcripts in IKK T cells by quantitative PCR (qPCR) and found that IKK CD4 T cells expressed a higher level of Fas mRNA than did WT CD4 T cells, and both CD4 and CD8 IKK T cells expressed more FasL mRNA than did their WT counterparts (Fig. 7C). This was accompanied by a slight, but noticeable, increase in surface expression of FasL, but not Fas, as detected by flow cytometry (Fig. 7D). To understand whether increased FasL expression plays a role in promoting IKK T cell apoptosis, we cultured WT and IKK splenocytes for 65 h in the
presence of anti-CD3 stimulation, with or without a FasL-blocking antibody. In the absence of FasL blocking, IKK CD4 and CD8 T cells showed greatly diminished survival upon TCR stimulation compared with WT counterparts (Fig. 7E, 7F). Blocking Fas/FasL interactions increased IKK T cell survival, indicating a causal role for increased FasL expression in enhanced IKK T cell apoptosis. However, the presence of FasL-blocking antibody was unable to restore T cell survival to WT levels, suggesting that multiple mechanisms may contribute to the survival defect resulting from constitutive IKKβ activation.

3.2.3 TCR proximal-signaling defects inhibit IKK T cell activation

Multiple signaling pathways, including the PKCθ–IKK–NF-κB axis, are triggered when the TCR binds to a cognate peptide–MHC complex. Although it is appreciated that the PKCθ–IKK–NF-κB pathway plays a critical role in IL-2 production and T cell activation, the effects of persistent signaling from this pathway on T cell function remain unknown. To examine how constitutively active IKK may affect T cell activation, we cultured splenocytes from WT and IKK mice overnight in the presence or absence of an anti-CD3 antibody and examined the upregulation of T cell activation markers, such as CD69 and CD25. In the absence of TCR cross-linking, we observed that IKK CD4 cells showed increased CD25 and CD69 expression compared with WT cells (Fig. 8A), suggesting that constitutively active IKKβ may result in increased basal state activation of CD4 T cells.
Figure 8: Defects in TCR-proximal signaling impair IKK T cell activation
Figure 8. (A) Representative graphs of CD25 and CD69 expression on WT and IKK splenocytes stimulated overnight with anti-CD3 antibody. (B) Representative graphs showing CFSE dilution in WT and IKK splenocytes stimulated for 72 h with anti-CD3 antibody. (C) Representative immunoblots of lysates prepared from purified WT and IKK T cells stimulated for the indicated times with anti-CD3 (500A2) antibody and probed for the indicated TCR-signaling proteins. (D) Representative FACS plots of calcium influx (monitored as a ratio of fluorescence at 405/510 nm) into Indo-1–loaded WT and IKK LN cells after TCR and CD4/CD8 cross-linking at the indicated times. (E) Representative immunoblots of nuclear and cytoplasmic fractions from purified WT and IKK T cells left unstimulated or stimulated for 6 h with anti-CD3 (500A2) and anti-CD28 antibodies. Data shown are representative of at least three independent experiments.
However, in the presence of anti-CD3 stimulation, IKK T cells showed significantly impaired upregulation of both markers (Fig. 8A), with an increase in defect severity from the CD8 to CD4 compartment. We also examined the ability of IKK T cells to proliferate in response to TCR engagement, by culturing CFSE-labeled WT and IKK splenocytes in the presence or absence of the anti-CD3 antibody for 72 h. FACS analysis of CFSE dilution, a measure of cell division, at this time point revealed proliferation defects in both CD4 and CD8 IKK T cell compartments (Fig. 8B). Impairment of proliferation was more pronounced in CD4 cells than in CD8 cells, consistent with the trend observed during activation marker analysis. Taken together, these data suggest that constitutively active IKKβ impairs the ability of T cells to undergo activation and proliferation upon TCR engagement ex vivo.

Based on these defects in activation and proliferation, we hypothesized that constitutive IKKβ activity may impair TCR signaling. To test this hypothesis, we examined TCR-induced ZAP70 phosphorylation, an early TCR signaling event. As shown in Fig. 8C, ZAP70 phosphorylation was more transient in IKK T cells than in WT counterparts following TCR engagement. TCR-induced Ca\(^{2+}\) influx, an event dependent on PLC\(\gamma\)1-derived inositol trisphosphates, was obviously decreased in IKK CD4 T cells but largely intact in IKK CD8 T cells (Fig. 8D). However, treatment with ionomycin was able to induce equivalent Ca\(^{2+}\) influx into both WT and IKK CD4 T cells (data not shown), ruling out the possibility that the defects observed in IKK CD4 T cells may be
due to improper Indo-1 loading. ERK1/2 phosphorylation, a signaling event downstream of PLCγ1-derived diacylglycerol, was also decreased in IKK T cells following TCR stimulation (Fig. 8C). Together, these observations suggest that constitutive IKKβ activity leads to TCR proximal-signaling defects that may attenuate the responsiveness of IKK T cells to TCR stimulation.

Downstream of TCR engagement, the PKCθ–IKK pathway leads to degradation of IκB and nuclear translocation of NF-κB. Having observed a reduction in IκBα levels in IKK T cells, we sought to better understand the degree of activation of the NF-κB pathway in IKK cells under resting and activating conditions. Cytosolic and nuclear fractions were obtained from purified WT and IKK T cells that were either left unstimulated or stimulated with plate-bound anti-CD3 and anti-CD28 for 6 h. Under resting conditions, increased levels of NF-κB were found in the nuclear fraction of IKK T cells compared with their WT counterparts, suggesting that the IKKβ–NF-κB axis is constitutively activated in these cells (Fig. 8E). Previous studies showed that IKKβ directly phosphorylates NF-κB p65 at S536 and that this phosphorylation can control the kinetics of NF-κB nuclear import (Mattioli et al., 2004). NF-κB phosphorylation at S536 was clearly increased in resting IKK T cells, providing additional evidence of constitutive IKKβ activity in these cells. Surprisingly, we did not observe increased phosphorylation of IκB by IKKβ at S32/36. However, total IκB levels were lower in resting IKK T cells, suggestive of increased degradation. Upon activation with anti-CD3
and anti-CD28, we observed a robust increase in NF-κB p65 levels in the nuclear fraction of WT T cells. However, consistent with the TCR proximal-signaling defects, activation of IKK T cells did not induce additional translocation of NF-κB p65 into the nucleus, which may contribute to the impairment of activation and proliferation in these cells.

### 3.2.4 Defective IL-2 production contributes to impaired IKK T cell survival and proliferation

To uncover other mechanisms that may underlie the defects in IKK T cell survival and proliferation, we first tested the ability of IKK T cells to produce IL-2 upon stimulation. Freshly isolated WT and IKK splenocytes were either left unstimulated or stimulated with anti-CD3 or anti-CD3 and anti-CD28. When IL-2 production was subsequently assessed by intracellular staining and flow cytometry, fewer CD4 and CD8 IKK cells were found to produce IL-2 upon stimulation (Fig. 9A). Treatment with PMA (a diacylglycerol analog) and ionomycin was unable to restore IL-2 production by IKK T cells to WT levels, suggesting that the TCR proximal-signaling defects seen in IKK cells may be accompanied by additional defects downstream of diacylglycerol and calcium influx.

We then asked whether defective IL-2 production contributed to the impairment of IKK T cell survival and proliferation. To test this, we cultured WT and IKK splenocytes for 65 h in the presence of anti-CD3, with or without the addition of exogenous IL-2. We observed that the addition of exogenous IL-2 substantially increased IKK CD8 cell survival (Fig. 9B).
Figure 9: Defective IL-2 production contributes to impairment of IKK T cell survival and proliferation
Figure 9. (A) Representative FACS plots of IL-2 production by WT and IKK splenic T cells left unstimulated or stimulated as indicated in the presence of Golgi Plug for 4–6 h. Percentage of viable CD4 and CD8 cells among WT and IKK splenocytes stimulated with anti-CD3ε (2C11) antibody for 72 h in the presence or absence of 100 U/ml exogenous IL-2 (B) or 0.5 μg/ml anti-CD28 Ab (C). Data shown are mean ± SEM from a single experiment in triplicate and are representative of three independent experiments. (D) Representative graphs showing CFSE dilution in WT and IKK splenocytes stimulated for 65 h with anti-CD3ε antibody, in the presence or absence of 100 U/ml exogenous IL-2 or 0.5 μg/ml anti-CD28 Ab. All FACS plots are representative of three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001 (Student t test)
IKK CD4 cell survival showed only a slight increase, consistent with the increased severity of signaling defects observed in this compartment (Fig. 8D). IKK CD8 cell survival was lower than that of the WT counterparts, even in the presence of exogenous IL-2, suggesting that other factors (including increased FasL/Fas interaction) may contribute to increased apoptosis. Analysis of proliferation by CFSE dilution revealed that the addition of exogenous IL-2 enhanced IKK T cell proliferation in both CD4 and CD8 compartments (Fig. 9D). Similar to the addition of exogenous IL-2, costimulation via CD28 enhanced IKK T cell survival and proliferation (Fig. 5C, 5D). Taken together, these results suggest that a defect in IL-2 production contributes to diminished IKK T cell proliferation and survival.

### 3.2.5 Impaired antigen-specific IKK T cell responses *in vivo*

Based on the functional defects observed *ex vivo*, we sought to determine whether constitutive IKKβ activity was detrimental to physiological T cell function *in vivo*. We adoptively transferred WT (Thy1.1⁺) or IKK (Thy1.2⁺) Vα2⁺ CD8 T cells bearing the OT1 OVA-specific transgenic TCR into WT Thy1.1⁺ Thy1.2⁺ recipient mice. These recipients were subsequently infected with Lm-OVA (Fig. 10A). When the antigen-specific immune response was monitored by serial bleeding and staining for Vα2⁺ CD8 cells in the peripheral blood (Fig. 10B, 10C), IKK OT1 T cells had expanded less than had WT OT1 cells by day 7 (Fig. 10B–D).
Figure 10: Impaired antigen-specific IKK T cell responses \textit{in vivo}
Figure 10. (A) Schematic representation of experimental design showing individual (solid lines) and competitive (dotted lines) adoptive-transfer models. (B–D) Individual adoptive transfers of sorted WT (Thy1.1⁺ Vα2⁺ CD8⁺) OT1 and IKK (Thy1.2⁺ Vα2⁺ CD8⁺) OT1 cells into WT Thy1.1⁺ Th1.2⁺ recipients. Representative FACS analysis of peripheral blood samples to detect Vα2⁺ CD8 T cells (B) and congenic markers within the gated Vα2⁺ CD8 T cell population (C) at the indicated times postinfection. (D) Percentage of WT (Thy1.1⁺) and IKK (Thy1.2⁺) cells among total CD8⁺ cells in the peripheral blood. Mean ± SEM are calculated for three mice per group. Data shown are representative of two independent experiments. (E and F) Competitive adoptive transfers. Sorted WT and IKK OT1 T cells were mixed in a 1:1 ratio, transferred into recipients, and analyzed as in (B–D). Mean ± SEM are calculated for three mice per group. Data shown are representative of two independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001 (Student t test)
Notably, IKK cells also showed dramatically enhanced contraction and became less frequent than endogenous Vα2⁺CD8 cells by day 14. Remarkably fewer IKK memory T cells persisted at 3 months post-infection compared with their WT counterparts. When co-injected with an equal number of WT OT1 cells in competitive adoptive-transfer experiments (Fig. 6A, dotted lines), IKK OT1 cells expanded 6-fold less than did WT OT1 cells in response to antigenic challenge (Fig. 10E, 10F). Correlating with observations from individual transfers, the IKK immune response was again characterized by exaggerated contraction and a marked paucity of memory cells. Therefore, consistent with the defects in function and survival observed ex vivo, chronic IKKβ activation appears to impair antigen-specific immune responses in this model by limiting T cell expansion and promoting excessive contraction.

3.2.6 The transcription factor Blimp1 plays a critical role in enforcing IKK T cell unresponsiveness to TCR stimulation

We asked whether caIKKβ activity could attenuate TCR signaling indirectly by increasing the expression of inhibitory co-receptors on the cell surface. PD1 (or Pdcd1) is an inhibitory co-receptor of the CD28 family that is highly expressed on exhausted T cells generated during chronic infection (Barber et al., 2006; Trautmann et al., 2006; Urbani et al., 2006). Given that the NF-κB pathway is activated by many of these viral pathogens, we first tested the expression of PD1 in IKK T cells. qPCR and flow cytometric analysis revealed increased mRNA and surface protein levels of PD1 in both IKK CD4 and CD8 T cells (Fig. 11A), suggesting that chronic IKKβ activity is sufficient to
promote increased PD1 expression on the T cell surface. Recent studies in a chronic viral infection model identified Blimp1 as an important transcriptional regulator of CD8 T cell exhaustion (Shin et al., 2009). Increased Blimp1 expression in these exhausted T cells was found to promote the expression of inhibitory receptors and the development of clonal exhaustion. Blimp1 is also known to directly repress IL-2 and Fos transcription to attenuate T cell survival and proliferation (Martins et al., 2008). Although a role for NF-κB in inducing Blimp1 expression in T cells has not been investigated, NF-κB has been identified as a key mediator of Blimp1 expression in murine B cells, and chromatin immunoprecipitation experiments using murine B lymphoma cells revealed that NF-κB dimers can directly bind to the Blimp1 promoter (Morgan et al., 2009). In addition, Rel-B was shown to induce Blimp1 expression in human breast cancer cells (Wang et al., 2009). These observations prompted us to examine the expression of Blimp1 in IKK T cells. qPCR analysis revealed enhanced Blimp1 mRNA expression in both CD4 and CD8 IKK compartments (Fig. 11B).

To investigate whether the impairment of T cell function upon constitutive IKKβ activation is dependent on the presence of Blimp1, we generated IKKβ Blimp1kitCD4Cre− (IKK Blimp knockout [KO]) mice. Blimp1-deficient IKK T cells showed improved activation and proliferation compared with their Blimp1-sufficient IKK counterparts (Fig. 11C, 11D), suggesting an essential role for Blimp1 in the attenuation of T cell activation and proliferation caused by constitutively active IKKβ.
Figure 11: Blimp1 deficiency partially restores IKK T cell responsiveness to TCR stimulation
Figure 11. (A) qPCR and representative FACS analysis of PD1 mRNA and protein expression in WT and IKK CD4 and CD8 splenic T cells. (B) qPCR analysis of Blimp1/Prdm1 mRNA expression in WT and IKK CD4 and CD8 splenic T cells. Mean ± SEM for qPCR analyses are calculated from two independent experiments. (C) Representative FACS plots of CD25 and CD69 expression on WT, IKK, and IKK Blimp KO CD4 and CD8 splenic T cells left unstimulated or stimulated overnight with anti-CD3 antibody. (D) Representative graphs showing CFSE dilution in WT, IKK, and IKK Blimp KO CD4 and CD8 splenic T cells, with or without anti-CD3 stimulation for 72 h. Graphs are representative of at least two independent experiments. *p < 0.05, **p < 0.01 (Student t test)
However, activation and proliferation in IKK Blimp KO T cells were not completely restored to WT levels, suggesting that mechanisms other than Blimp1 induction may be involved in rendering IKK T cells less responsive to TCR stimulation. Future studies are required to investigate these mechanisms.

3.2.7 Constitutive IKKβ signaling in T cells promotes autoimmunity in mice

Based on the adverse effects of persistent IKKβ activation on TCR responsiveness and T cell survival, we asked whether other aspects of T cell function (such as effector cytokine production) are compromised as well. We first examined the proportion of CD44hiCD62Llo T_EM cells in the spleen and LNs, a subset known for its ability to rapidly produce cytokines upon stimulation. FACS analysis of freshly isolated splenocytes and LN cells revealed an increased proportion of T_EM cells in IKK mice, with a concomitant decrease in naive cells (Fig. 12A, data not shown). We then directly examined the ability of IKK T cells to produce cytokines by stimulating freshly isolated splenocytes with PMA and ionomycin in the presence of Golgi Plug. Intracellular staining and FACS analysis showed that an increased proportion of IKK CD4 and CD8 cells readily secreted cytokines, such as IFNγ and IL-17, compared with their WT counterparts (Fig. 12B). Therefore, IKK T cells, although impaired in their ability to get activated through the TCR and proliferate, appear to be skewed toward a T_EM phenotype and primed for cytokine production.
Figure 12: Enhanced cytokine production and multiorgan inflammation in IKK mice
Figure 12. (A) FACS plots of CD44 and CD62L expression on WT and IKK CD4 and CD8 splenic T cells. (B) FACS plots showing intracellular staining for IFN-γ and IL-17A in WT and IKK CD4 and CD8 T cells left unstimulated or stimulated for 6 h in the presence of PMA, ionomycin, and Golgi plug. (C) H&E staining of lung, liver, and pancreas sections from 6-month-old WT and IKK mice. Photographs were taken using a Fisher Scientific Micromaster microscope and Westover Scientific Micron software (original magnification ×400). Arrows indicate areas of mononuclear cell infiltration. (D) Immunofluorescence staining of liver sections from 6-month-old WT and IKK mice. Photographs were taken using a Zeiss ApoTome Microscope and AxioVision software (original magnification ×200). Arrow indicates area of CD4 T cell infiltration. (E) qPCR analysis of mRNA levels of indicated chemokines in purified WT and IKK CD4 and CD8 splenic T cells. Data shown are representative of at least four (A) or three (B–E) independent experiments.
Consistent with this increased propensity for cytokine production, IKK mice developed multiorgan autoimmune disease characterized by mononuclear cell infiltration of the lungs, liver, and pancreas (Fig. 12C). Immunofluorescence microscopy of liver sections revealed the presence of CD4 T cells and CD11b+ myeloid cells in such infiltrates (Fig. 12D).

Regulatory T cells (T_{reg}), characterized by the expression of the transcription factor Foxp3, are known to play an important role in the maintenance of self-tolerance (Josefowicz et al., 2012; Wing and Sakaguchi, 2010). Previous work has shown that NF-κB can regulate T_{reg} development by directly binding to the Foxp3 enhancer region and promoting the expression of Foxp3 (Long et al., 2009). Though this suggests that IKK mice might have increased T_{reg} numbers and intact self-tolerance, we examined if changes in T_{reg} development or function could contribute to autoimmunity in IKK mice. In contrast to observations from previous studies, the frequency of Foxp3+ T_{reg} cells was lower among mature CD4SP IKK thymocytes compared with their WT counterparts, and T_{reg} frequency was comparable between WT and IKK CD4 splenocytes (Fig. 13A). T_{reg}-specific expression of caIKKβ led neither to a reduction in T_{reg} numbers nor to the development of autoimmune disease in mice (data not shown). Moreover, caIKKβ T_{reg} cells displayed enhanced in vitro suppressive activity (Fig. 13B) in a contact-inhibition assay. Thus, our data suggest that aberrant conventional T cell function may play a dominant role in the development of autoimmunity in IKK mice.
Figure 13: Constitutive IKKβ activity does not cause obvious defects in T\textsubscript{reg} frequency or function

Figure 13. (A) Representative FACS plots of Foxp3 and GITR expression on pre-gated CD4SP thymocytes and CD4 splenocytes from WT and IKK mice. Percentage of Foxp3\textsuperscript{+} cells among CD4 splenocytes in WT and IKK mice. Data shown are mean ± SEM calculated from four independent experiments. (B) Representative histograms showing CFSE dilution in conventional Foxp3\textsuperscript{+} CD4 cells upon culture under indicated conditions in a contact inhibition assay. Data shown are representative of at least two independent experiments.
Quantitative PCR analysis of IKK splenic T cells revealed markedly increased mRNA levels of CCL3 (MIP-1α) and CCL5 (RANTES), chemokines known to promote inflammation by attracting monocytes, macrophages, and granulocytes (Fig. 12E). However, levels of CCL28 (MEC), a chemokine not generally expressed by T cells, were comparable in WT and IKK T cells (Fig. 12E), arguing against a global upregulation of nonspecific chemokines in the presence of constitutive IKKβ activity. Taken together, these data suggest that persistent IKKβ activity in T cells may drive the development of autoimmunity via enhanced production of proinflammatory cytokines and chemokines.

3.3 Discussion

In this study, we demonstrated that persistent IKKβ activation renders T cells less responsive to stimulation through the TCR and promotes cell death. In vivo, IKK T cells mount an ineffective antigen-specific T cell response to bacterial infection, with poor expansion and dramatically enhanced contraction. A search for the mechanisms that impair T cell survival and function revealed that increased expression of FasL plays a role in promoting IKK T cell death, whereas upregulation of Blimp1 contributes to the TCR unresponsiveness of IKK T cells. Defects in IL-2 production also impair IKK T cell survival and proliferation (Fig. 14). Although IKK T cells are hyporesponsive to TCR-induced activation, they display a low-grade spontaneous-activation phenotype, and mice develop multiorgan autoimmune disease. Our results provide direct evidence that
chronic IKKβ activation, as observed in certain chronic viral infections, is sufficient to attenuate T cell function and survival and promote autoimmunity.

Figure 14: Model of caIKKβ-mediated impairment of T cell function and survival

Members of the NF-κB family have long been established to play a critical role in preventing apoptosis in response to death-inducing signals and promoting cell survival in several cell types, including T cells. However, studies have also demonstrated that their activity can instead serve to promote apoptosis in certain cellular contexts, suggesting a more complex role for NF-κB in regulating cell death decisions (Kucharczak et al., 2003).
Early after TCR and CD28 engagement, active NF-κB dimers promote cell survival by inducing the expression of anti-apoptotic genes (such as Bcl-2, Bcl-xL, and XIAP) and pro-survival cytokines (IL-2 and GM-CSF). In contrast, NF-κB also plays a key role in facilitating the process of activation-induced T cell death by increasing the transcription of death receptors and their ligands (such as Fas, FasL, DR4, and TRAIL), pro-apoptotic transcription factors (like p53 and c-myc), and Bcl-xS. Currently, the nature of factors that determine whether NF-κB activation prevents or promotes apoptosis remains largely unknown and actively investigated.

A previous study that infected dividing CD4 cells with retrovirus expressing a constitutively active form of IKKβ found that this was sufficient to promote activated T cell survival *ex vivo* in the absence of further TCR stimulation (Zheng et al., 2003). On the contrary, our findings suggest that constitutive IKKβ activation promotes T cell apoptosis by increasing the expression of FasL and activation of caspase-8. This apparent discrepancy suggests the possibility that acute and chronic IKKβ activation may exert different effects on T cell survival. IKKβ activation may also function differently in the pre-activated and quiescent T cells used in these studies. Further investigation of the basis behind these seemingly contradictory results may shed new light on mechanisms that interact with or influence the NF-κB pathway to drive divergent cell-survival outcomes in differing cellular contexts.
In addition to cell survival, the PKCθ–IKK–NF-κB pathway regulates other critical processes, including proliferation and differentiation. In this study, we found that constitutively active IKKβ signaling adversely affects T cell activation and proliferation upon *ex vivo* TCR engagement. The impairment of T cell activation and proliferation is consistent with our observation that TCR-induced NF-κB nuclear translocation is defective in IKK T cells. Activation of the NF-κB pathway is known to set in motion multiple negative-feedback mechanisms that ensure its eventual termination. For instance, NF-κB was shown to increase transcription of IκB genes (Sun et al., 1993), and active IKKβ phosphorylates BcI10, targeting it for degradation (Lobry et al., 2007). However, the decreased ZAP70 phosphorylation and impaired calcium influx that we observed in IKK T cells upon TCR cross-linking suggests the presence of a novel regulatory mechanism by which IKKβ activity can attenuate TCR proximal-signaling events upstream of PLCγ1. Given that NF-κB is a versatile transcription factor with numerous targets, some of which are transcription factors themselves, it is possible that one or more of these targets may inhibit the TCR-signaling machinery via direct or indirect mechanisms.

We observed that constitutively active IKKβ promotes upregulation of Blimp1, a transcriptional repressor associated with increased expression of inhibitory co-receptors (such as PD1) and T cell exhaustion (Shin et al., 2009). Studies in mouse B cells identified NF-κB as a key mediator of Blimp1 expression in response to LPS stimulation (Morgan
et al., 2009), suggesting that Blimp1 may be a transcriptional target of NF-κB in T cells as well. Conditional deletion of Blimp1 in IKK T cells was able to partially restore T cell activation and proliferation, indicating that high Blimp1 levels contribute to the unresponsiveness of IKK T cells. Further studies are required to fully characterize other negative-feedback mechanisms that allow NF-κB activity to dampen TCR-proximal signaling.

In peripheral lymphoid organs, T cell anergy serves to enforce self-tolerance, whereas exhaustion prevents protracted immune responses to chronic infection. Although both processes produce a similar state of unresponsiveness to further stimulation, global gene-expression profiling in an LCMV model of T cell exhaustion suggests that anergy and exhaustion are distinct metabolic states enforced by mostly non-overlapping molecular mechanisms (Wherry et al., 2007). Our preliminary analysis suggests that IKK T cells do not upregulate anergy-associated genes, such as Egr2, Egr3, DGKα, Itch, and Cbl-b (data not shown). In contrast, IKK cells resemble exhausted cells in their high expression of inhibitory co-receptor PD1 and the transcription factor Blimp1 but differ from them in their ability to readily produce cytokines. Therefore, our findings suggest that T cells with constitutive IKKβ activity share certain characteristics with exhausted cells but fail to fit completely into an anergic or exhausted profile.

Viral infection of a host cell triggers multiple signaling pathways. As a key regulator of several critical cellular processes, the IKK–NF-κB pathway is thought to be
an attractive target for co-option by viral pathogens. For instance, human pathogens, such as HIV-1 and human T cell leukemia virus 1, use multiple strategies that converge on IKK activation to modulate NF-κB activity in infected cells (Santoro et al., 2003). Activation of NF-κB by these viruses may serve a number of functions that include increasing transcription of viral genes and preventing infection-induced apoptosis (Hiscott et al., 2001). Additionally, the impairment of ligand-induced NF-κB nuclear translocation caused by over-activation of IKKβ could prevent mounting of an effective immune response to the viral pathogens.

Admittedly, constitutively activating IKKβ does not mimic the whole range of signaling events associated with chronic viral infection. However, it is likely that understanding the effects of constitutive IKKβ activity on host cell function may provide key insights into a subset of cellular changes associated with chronic infection. Our results suggest the possibility that inhibiting excessive IKK activity may serve as a strategy to reverse T cell unresponsiveness during chronic infection. In conclusion, although signaling via the PKCθ–IKK–NF-κB pathway is critical for T cell function, our study suggests that chronic IKKβ activation promotes T cell dysfunction and apoptosis, dampening antigen-specific responses and promoting autoimmunity.
4. **Mnk1 and Mnk2 are dispensable for T cell development and activation but important for the pathogenesis of experimental autoimmune encephalomyelitis.**

Experiments in this chapter were performed in collaboration with Dr. Balachandra Gorentla. SK performed *in vivo* experiments, BG performed immunoblotting, and both SK and BG performed *ex vivo* assays.

**4.1 Introduction**

Signals from the TCR, via multiple intracellular signaling pathways such as the RasGRP1-Ras-ERK1/2-AP1, PKC0-NF-κB, PI3K-Akt, and Ca++-calcineurin-NFAT pathways, play critical roles in T cell maturation and activation (Smith-Garvin et al., 2009; Weiss, 2010; Zhong et al., 2011). In addition to TCR engagement, various other extracellular stimuli such as growth factors, cytokines, and stress can induce activation of MAPKs in T cells. Based on the signals that trigger their activation, MAPKs are categorized as ERKs (ERK1/2), p38 kinases, and C-Jun N-terminal kinase/stress-activated protein kinases (JNKs) (Chen et al., 2001). MAPKs control a wide range of functions including proliferation, differentiation, survival, and apoptosis through direct phosphorylation and activation of substrates (Kyriakis and Avruch, 2001). These substrates, called MAPK-activated protein kinases (MAPKAPKs), are divided into four families based on the type of phosphorylating MAPK (Cargnello and Roux, 2011; Gaestel, 2008). For example, the p90 ribosomal S6 kinase (Rsk) family includes Rsk1, Rsk2, and Rsk3 that are specifically phosphorylated and activated by ERKs (Anjum and
MAPKAPKs such as MK2/3 and MK5 are activated by JNKs (Gaestel, 2006); and mitogen- and stress-activated kinases (MSKs) including MSK1 and MSK2 are phosphorylated by p38 MAPKs (Arthur, 2008).

Different from these MAPKAPKs, MAPK-interacting kinases 1 and 2 (Mnk1/2) are serine/threonine kinases and are phosphorylated by both ERKs and p38 kinases (Buxade et al., 2008). Murine Mnk1/2 are phosphorylated at threonine 197 and 202 (T197 and T202) or T244 and T249 respectively, which leads to their activation (Fukunaga and Hunter, 1997; Morley and McKendrick, 1997; Ueda et al., 2004; Wang et al., 1998; Waskiewicz et al., 1997). Activated Mnk1/2 directly phosphorylates the eukaryotic translation initiation factor 4E (eIF4E) at S209 downstream of growth factor receptors (Ueda et al., 2004). eIF4E binds to 5' methyl guanosine (m7GpppN) cap structure found in all eukaryotic mRNAs, and this binding is obligatory for the initiation of cap-dependent translation (Gingras et al., 1999; Pause et al., 1994). Cap-dependent translation is the primary mode of eukaryotic translation by which 95% of total cellular mRNAs are translated (Merrick, 2004). It has been hypothesized that Mnk1/2 are key protein kinases that can promote cap-dependent translation through eIF4E phosphorylation (Pyronnet et al., 1999).

The roles of Mnk1/2 were originally studied in Drosophila, whose Mnk ortholog is called LK6. Deficiency of this gene was found to impair growth and development, leading to a shortened life span (Arquier et al., 2005). However, in mice, Mnk1/2 double
deficiency did not grossly affect development and growth, although eIF4E phosphorylation at S209 was abolished (Ueda et al., 2004). While dispensable for murine development, Mnk1 and Mnk2 have been demonstrated to play an oncogenic role in mice, and their deficiency delays tumor development in a murine tumor model (Ueda et al., 2010). Studies using pharmacologic inhibitors and eIF4E phosphorylation mutants have shown that eIF4E phosphorylation has an important role in cell survival and cancer progression (Mavrakis et al., 2008).

Using chemical inhibitors, several previous studies have reported that Mnk1/2 could have an important role in immune cells. For example, chemical inhibition of Mnk1/2 was found to decrease the translation of IL-17 in CD4 cells (Noubade et al., 2011), IFN-γ and IL-4 in iNKT cells (Nagaleekar et al., 2011), and inflammatory cytokines in macrophages (Rowlett et al., 2008). Although these studies provide preliminary evidence that Mnk1/2 activity could have a critical role in immune cell function, possible off-target effects of the chemical inhibitors used cannot be overlooked.

In this report, we demonstrate that TCR engagement induces activation of Mnk1/2 and phosphorylation of eIF4E, which is enhanced by Ras signaling, and inhibited by DGK α and ζ (Zhong et al., 2008). By using mice deficient in Mnk1/2 (Mnk1/2DKO), we show that Mnk1 and Mnk2 are essential for TCR-induced phosphorylation of eIF4E. However, deficiency of both Mnk1 and Mnk2 does not affect gross T cell development, activation, proliferation, or cytokine production. Furthermore, Mnk1/2 activities are dispensable
during CD8 T cell–mediated immune responses against *Listeria monocytogenes* and LCMV, and for iNKT cell development and cytokine production.

**4.2 Results**

**4.2.1 Regulation of Mnk1/2 activation and expression in T cells**

TCR signaling has been shown to trigger several intracellular signaling pathways leading to phosphorylation and activation of p38 and ERK1/2. We assessed anti-CD3–induced Mnk1 activation in freshly isolated thymocytes or splenic T cells from WT mice. As shown in Fig. 15A, TCR engagement induced Mnk1 and eIF4E phosphorylation in both thymocytes and splenic T cells, correlated with ERK1/2 and p38 activation. In T cells, DAG binds to and activates RasGRP1, which in turn activates the Ras-MEK1/2-ERK1/2 pathway. In the presence of a constitutively active form of Ras (kRas), TCR-induced ERK1/2, Mnk1, and eIF4E phosphorylation were significantly increased, indicating that Ras signaling promotes Mnk1/2 activation in T cells (Fig. 15B). We have previously demonstrated that DGKα and ζ inhibit the activation of the Ras-ERK1/2 signaling (Olenchock et al., 2006; Zhong et al., 2008; Zhong et al., 2003; Zhong et al., 2002). In DGKαζDKO thymocytes, both ERK1/2 and eIF4E phosphorylation were enhanced (Fig. 15C). Furthermore, this phosphorylation was greatly inhibited by U0126 (U0), a MEK1/2 inhibitor. Together, these observations indicate that TCR engagement induces Mnk1/2 activation, and that such activation is mediated by the Ras-ERK1/2 pathway and is inhibited by DGK activity.
Figure 15: Regulation of Mnk1/2 activation in T cells
Figure 15. Thymocytes and splenocytes of indicated genotypes were rested in PBS at 37°C for 30 min and were then left unstimulated or stimulated with 5 μg/ml anti-CD3ε (500A2) for the indicated times. Lysates were subjected to immunoblot analysis with the indicated antibodies. (A) TCR engagement activates Mnk1/2 in WT thymocytes (left) and splenocytes (right). (B) Constitutively active kRas promotes Mnk1/2 activation. Thymocytes from WT and ca.kRas-CD4Cre mice were subjected to similar analysis as in (A). (C) DGKα and ζ inhibit TCR-induced Mnk1/2 activation in a MEK1/2–dependent manner. WT and DGKαζDKO (azDKO) thymocytes were examined as in (A) with the addition of groups treated with the MEK1/2 inhibitor U0126 (10 μM). (D and E) Differential expression of Mnk1/2 in naive and activated T cells. Mnk1 and Mnk2 mRNA (D) and protein (E) levels in sorted WT naive and in vitro–activated CD4 and CD8 T cells were examined by real-time quantitative PCR and Western blotting analysis, respectively. *p < 0.05, **p < 0.01, ***p < 0.001. (Student t test)
Although Mnk1 and Mnk2 are ubiquitously expressed, their expression is varied in different tissues (Waskiewicz et al., 1997). Using real-time quantitative PCR, we assessed Mnk1/2 mRNA levels in naive and activated CD4 and CD8 T cells. Both Mnk1 and Mnk2 mRNA levels were expressed at higher levels in naive T cells than in activated T cells. Mnk1 was decreased to 45% and 30%, whereas Mnk2 expression was reduced to 50% and 20% in activated CD4 and CD8 T cells, respectively, compared with naive T cells (Fig. 15D). The decreased expression of Mnk1/2 proteins in activated T cells was further confirmed by immunoblotting analysis (Fig. 15E). Naive and activated T cells are drastically different in metabolism and in protein synthesis. Given the proposed role of Mnk1/2 in cytokine production, it is intriguing that Mnk1/2 expression is decreased in activated T cells, in which proteins including cytokines are actively translated.

4.2.2 Effect of combined Mnk1/2 deficiency on T cell development

To investigate the role of Mnk1/2 in T cells, we analyzed mice with germline deletion of these two genes. Because individual deficiency of Mnk1 or Mnk2 did not affect T cell development or activation (data not shown), we examined Mnk1/2DKO (‘Mnk DKO’) mice. The percentages and absolute numbers of CD4 and CD8 subsets in thymi and spleens from Mnk DKO mice were similar to those from WT control mice (Fig. 16, A-D). The overall thymic and splenic cellularity in Mnk DKO mice were also comparable to WT mice (Fig. 16E).
Figure 16: T cell development in Mnk1/2 DKO mice
Figure 16. (A and B) CD4 and CD8 expression in WT and Mnk DKO thymocytes (A) and splenocytes (B). Representative dot-plots of CD4 and CD8 staining are shown. (C and D) Absolute numbers of thymic (C) and splenic (D) T cell populations in WT and Mnk DKO mice ($n = 6$). (E) Total thymic and splenic cellularities in WT and Mnk DKO mice. (F) Absolute numbers of thymic T cell populations in WT-OT1 and Mnk DKO-OT1 mice ($n = 3$). (G) Regulatory T cell staining in the thymus and spleen. CD25 and Foxp3 staining in CD4 T cells are shown. (H) CD44 and CD62L staining of gated WT and Mnk DKO CD4 and CD8 T cells. (I) Mean ± SEM presentation of cell numbers of indicated T cell populations ($n = 5$). Data shown are representative of at least three experiments.
To examine the role of Mnk1/2 in T cell development, we generated Mnk DKO mice carrying the OT1 TCR transgene, which directs CD8 T cell development. As shown in Fig. 16F, thymocyte numbers were not obviously different between Mnk DKO OT1 mice and WT OT1, supporting a minimal role for Mnk1/2 in intrathymic T cell development. Mnk DKO mice did not display an obvious alteration in natural regulatory T cell numbers as compared with WT controls (Fig. 16G). Furthermore, CD44 and CD62L staining showed similar naive and effector T cell populations in WT and Mnk DKO mice (Fig. 16, H and I). These observations indicate that Mnk1/2 double deficiency does not cause obvious defects in T cell development or homeostasis.

4.2.3 Mnk1/2 are required for TCR induced eIF4E phosphorylation

As mentioned above, TCR engagement induced eIF4E phosphorylation at S209. To determine whether such phosphorylation is dependent on Mnk1/2, we compared TCR-induced eIF4E phosphorylation in WT and Mnk DKO T cells. Although eIF4E total protein was similar between WT and Mnk DKO T cells, TCR-induced eIF4E phosphorylation was virtually abolished in Mnk DKO T cells (Fig. 17A). On the contrary, TCR-induced phosphorylation of ERK1/2, Rsk1 (ERK1/2 substrate), and p38 were not affected by Mnk1/2 deficiency (Fig. 17B), suggesting that Mnk1/2 deficiency does not cause global signaling defects, and that there is no obvious negative feedback regulation of ERK1/2 and p38 by Mnk1/2 in T cells.
Figure 17: Effect of Mnk1/2 deficiency on TCR-induced signaling

Figure 17. WT and Mnk DKO thymocytes and splenocytes were similarly stimulated and analyzed by immunoblot as in Fig. 15A. (A) Mnk1/2 are critical for TCR-induced eIF4E phosphorylation at S209. (B) Mnk1/2 deficiency does not alter ERK1/2 and p38 phosphorylation. (C) Mnk1/2 deficiency does not impair mTOR-mediated 4E-BP1 phosphorylation. Data shown are representative of three experiments.
Binding of eIF4E to mRNA is inhibited by its association with 4E-BP1. mTOR phosphorylates 4E-BP1, leading to the release of eIF4E from 4E-BP1 to initiate translation (Richter and Sonenberg, 2005). Neither 4E-BP1 protein levels nor its phosphorylation was altered in Mnk1/2-deficient T cells compared with WT T cells (Fig. 17C), suggesting that Mnk1/2–mediated eIF4E phosphorylation does not affect 4E-BP1–mediated suppression of eIF4E or mTOR activity. These results suggest that TCR-induced eIF4E phosphorylation is mediated by Mnk1/2.

4.2.4 Normal in vitro T cell activation in the absence of Mnk1/2

To investigate whether Mnk1/2 deficiency affects T cell activation, we first examined the upregulation of early activation markers CD69 and CD25 following overnight anti-CD3 stimulation in the presence or absence of CD28-mediated costimulation. Mnk DKO T cells upregulated CD69 and CD25 similarly to WT controls in response to a wide range of anti-CD3 stimulation in the presence of anti-CD28 (Fig. 18A). The presence of CTLA4-Ig to block CD28-mediated costimulation decreased CD25 and CD69 upregulation in WT T cells. However, the absence of Mnk1/2 did not cause further reduction of CD25 and CD69 expression in T cells.

Mnk DKO T cells also showed comparable proliferation to WT T cells following anti-CD3 stimulation for 72 h as demonstrated by a CFSE dilution assay (Fig. 18B).
Figure 18: Mnk1 and Mnk2 are not essential for *in vitro* T cell activation
Figure 18. (A) Upregulation of early activation markers in Mnk DKO T cells. WT and Mnk DKO splenocytes were left unstimulated or stimulated overnight with an anti-CD3 antibody (2C11) at the indicated concentrations in the presence or absence of an anti-CD28 antibody (37.51, 0.5 μg/ml) or CTLA4-Ig (10 μg/ml). Overlaid histograms show CD69 and CD25 expression on gated CD4 and CD8 cells. (B) Mnk1/2 deficiency does not affect T cell proliferation. CFSE-labeled WT and Mnk DKO splenocytes were unstimulated or stimulated with an anti-CD3 antibody for 72 h. Cultured cells were stained for CD4 and CD8 and analyzed by flow cytometry. Histograms show CFSE intensity on CD4 and CD8 cells. (C) Effect of Mnk1/2 deficiency on cytokine production by T cells. Splenocytes from WT or Mnk DKO mice were left unstimulated (top) or stimulated with an anti-CD3 Ab (bottom) for 48 h, followed by PMA (50 ng/ml) and ionomycin (500 ng/ml) stimulation in the presence of a GolgiPlug for 5 h. Cells were stained for surface CD4 and CD8 and intracellular cytokines followed by FACS analysis. IFNγ and TNFα expression in gated CD4 and CD8 T cells are shown. (D) Mnk1/2 deficiency does not affect OT1 T cell proliferation. Splenocytes from WT OT1 and Mnk DKO OT1 mice were either labeled or not labeled with CFSE, and then treated with SIINFEKL peptide at indicated concentrations for 18 or 72 h to assess early T cell activation and proliferation, respectively. Overlaid histograms show CD25 and CD69, and CFSE intensity on live-gated CD8^Vα2^ T cells. (E and F) WT and Mnk DKO splenocytes were stimulated with anti-CD3 in the presence or either anti-CD28 (0.5
μg/ml) or CTLA4-Ig (10 μg/ml) at 37°C for 48 h. After resting for 24 h, live T cells were restimulated with plate-bound anti-CD3 (1 μg/ml) and soluble anti-CD28 (0.5 μg/ml) in the presence of 5 μM GolgiPlug at 37°C for 24 h. Cells were surface stained for CD4 and CD8 and intracellularly stained for IFNγ. FACS plots show IFNγ expression in live-gated CD4 and CD8 T cells (E). Bar graph is mean ± SEM presentation of percentages of IFNγ+ cells in the indicated populations of cells (n = 3). Data shown are representative of three experiments.

Moreover, Mnk DKO CD4 and CD8 T cells produced similar levels of IFNγ and TNFα after stimulation for 48 h, compared with WT controls, based on intracellular staining (Fig. 18C). To study the effect of Mnk1/2 deficiency on Ag-specific T cell activation, we used OT1 T cells, which express the Vα2′Vβ5′ TCR and recognize the OVA257-264 (SIINFEKL) epitope of ovalbumin presented on H-2Kb. When stimulated with different concentrations of OVA257-264 peptide, Mnk DKO OT1 T cells upregulated CD25 and CD69, and proliferated similarly to WT OT1 T cells (Fig. 18D). These data indicate that Mnk1/2 double deficiency does not obviously affect T cell activation in vitro.

As mentioned earlier, mTORC1 phosphorylates 4E-BP1 to promote eIF4E-mediated translation initiation. Decreased mTORC1 activity causes T cell anergy while enhanced mTORC1 activity leads to resistance to anergy (Xie et al., 2012; Zheng et al., 2007; Zhong, 2012). We asked further whether Mnk1/2-mediated phosphorylation of
eIF4E has a role in T cell anergy. We stimulated WT and Mnk DKO splenocytes with anti-CD3 in the presence of CTLA4-Ig to block CD28-mediated costimulation for 48 h. After an additional 24 h of resting, live T cells were restimulated by plate bound anti-CD3 and soluble anti-CD28 overnight, followed by intracellular staining for IFNγ. As shown in Fig. 18, E and F, similar IFNγ levels were detected in WT and Mnk DKO T cells under anergic conditions, suggesting that Mnk1/2 deficiency does not obviously affect T cell sensitivity to anergy induction *in vitro*.

### 4.2.5 Minimal effect of Mnk1/2 deficiency on Th differentiation *in vitro*

Using a Mnk1/2 inhibitor, a recent study has implicated Mnk1/2 in IL-17 production by T cells (Noubade et al., 2011). We examined whether deficiency of Mnk1/2 affected Th differentiation. Sorted naive WT and Mnk DKO CD4 T cells were subjected to *in vitro* Th1, Th2, Th17, and Th9 differentiation. Intracellular staining was used to assess the production of IFNγ, IL-17, and IL-9 under different skewing conditions, while IL-4 levels were measured by ELISA. As shown in Fig. 19, no obvious difference was observed between WT and Mnk DKO T cells in the production of these cytokines. These observations suggest that Mnk1/2 and Mnk1/2-mediated eIF4E phosphorylation are dispensable for Th differentiation *in vitro*. 
Figure 19: Combined Mnk1/2 deficiency does not affect TH differentiation *in vitro*

Figure 19. CD44−CD62L+ naive CD4 T cells sorted from WT and Mnk DKO spleen and LNs were subjected to *in vitro* TH skewing conditions. PMA, ionomycin, and Golgi Plug were added to the culture during the last 5 h of differentiation. At the end of culture, cells were stained with anti-CD4, Live/Dead, and antibodies against indicated cytokines. Dot plots show (A) IFNγ and IL-17 staining of gated live CD4 T cells under TH1 or TH17 skewing conditions, and (B) IL-9 staining under TH0 and TH9 skewing conditions. (C) IL-4 production by CD4 cells that were cultured under TH2 skewing conditions was determined by ELISA of culture supernatants. All data shown are representative of at least three independent experiments.
4.2.6 Mnk 1/2 deficiency may impair in vivo TH1 and TH17 differentiation in an EAE model

To determine whether Mnk1/2 deficiency could affect CD4 cell differentiation in vivo, we used a model of experimental autoimmune encephalomyelitis (EAE) because the differentiation of CD4 cells into TH1 and TH17 cells is known to have an important role in the pathogenesis of the disease (Pierson et al., 2012). Spleens and draining LNs from WT and Mnk DKO mice immunized with MOG35–55 peptide emulsified in CFA showed comparable total cellularity and frequency of CD4 cells 1 week after immunization (Fig. 20A). When antigen-specific cells were expanded by ex vivo stimulation of LN cells with MOG35–55 for 3 d, stimulation with PMA and ionomycin revealed a marked reduction in the frequency of Mnk DKO CD4 T cells that were able to produce IL-17A or IFNγ (Fig. 20B). Bearing in mind our previous results that Mnk1/2-deficient T cells survive and proliferate similar to WT counterparts (Fig. 18), the reduction in the IFNγ–producing and IL-17A–producing pools of antigen-specific cells suggests that the absence of Mnk1/2 might somehow impair the differentiation of CD4 T cells into TH1 and TH17 cells in response to antigen stimulation in vivo. Correlating with the smaller pool of IFNγ–producing and IL-17A–producing cells, mice deficient in Mnk1/2 developed milder EAE disease scores than WT counterparts (Fig. 20C). These results suggest that Mnk1/2 deficiency impairs TH1 and TH17 differentiation in vivo to perturb disease development in the EAE model.
Figure 20: Mnk1/2 deficiency can impair Th1 and Th17 differentiation in vivo
Figure 20. (A) Total cell numbers in the spleen and LNs and percentages of CD4 T cells in these organs in WT and Mnk DKO mice 7 d after immunization with MOG and CFA. (B) IL-17A and IFNγ–producing cells within CD4 T cells following MOG peptide stimulation for 3 d ex vivo. Dot plots show IFNγ and IL-17A expression in gated CD4 T cells (top panel). Bar graph represents mean ± SEM of IFNγ and IL-17A producing cells within CD4 T cells from multiple mice (bottom panel) (n = 6). (C) EAE scores of immunized WT and Mnk DKO mice monitored at indicated time points and scored as indicated in Chapter 2 (n = 5). Mean ± SEM are calculated for the indicated number of mice per group. Data shown are representative of two or more independent experiments. *p < 0.05. (Student t test)
4.2.7 Combined Mnk1/2 deficiency does not affect CD8 T cell responses to L. monocytogenes infection

The data described above have revealed that Mnk1 and Mnk2 are dispensable for T cell activation in vitro. We used the L. monocytogenes infection model to determine whether Mnk1/2 is required for T cell responses in vivo. Equal numbers of sorted naive WT (Thy1.1+) and Mnk DKO (Thy1.2+) Vα2+CD8+ OT1 cells were mixed and co-injected intravenously into WT Thy1.1+Thy1.2+ recipient mice. Recipients were subsequently infected with Lm-OVA (Fig. 21, A and B). Expansion of OVA peptide-specific OT1 T cells was monitored in the peripheral blood and spleen on days 7 and 14 after Lm-OVA infection. No significant difference was observed in the frequency of WT (Thy1.1+) and Mnk DKO (Thy1.2+) OT1 cells in the recipient mice 7 and 14 days after infection (Fig. 21, C and D). These results suggest that Mnk1/2 might not play a critical role in the expansion and early contraction phases of antigen-specific CD8 T cell responses, at least in the Lm-OVA model.

4.2.8 Mnk1/2-deficient mice mount normal primary and memory CD8 responses to LCMV infection

We next sought to better understand the effects of Mnk1/2 deficiency on polyclonal primary and memory CD8 T cell responses to viral infection. To this end, we infected WT and Mnk DKO mice with the Armstrong strain of LCMV that causes acute infection (Fig. 22A).
Figure 21: Combined Mnk1/2 deficiency does not affect antigen-specific primary CD8 T cell responses \textit{in vivo}
Figure 21. (A) Schematic representation of experimental design showing competitive adoptive transfer of WT Thy1.1+ and Mnk DKO Thy1.2+ OT1 T cells, and the Lm-OVA infection model. (B) Thy1.1 and Thy1.2 staining of mixture of sorted naive WT and Mnk DKO OT1 cells before injection. (C) Representative FACS analysis of peripheral blood cells and splenocytes detecting Vα2+ CD8 T cells (top panel), and Thy1.2/Thy1.2 congenic markers within the gated Vα2+CD8 T cell population (bottom panel) at indicated time points after infection. (D) Percentages of WT OT1 (Thy1.1+) and Mnk DKO OT1 (Thy1.2+) cells among total Vα2+CD8 cells in the peripheral blood and spleen. Mean ± SEM was calculated after correcting for the input ratio. Each dot represents one mouse. Data shown are representative of three independent experiments.
Figure 22: Mnk1/2 deficiency does not affect primary or memory anti-viral CD8 responses in vivo
Figure 22. (A) Schematic representation of experimental design showing primary infection with LCMV Armstrong, adoptive transfer of memory cells, and rechallenge. (B and C) Primary response. (B) Representative FACS plots of peripheral blood samples obtained at indicated time points and stained with anti-CD8 antibody and TetG. (C) Mean ± SEM presentation of percentages of TetG·CD8 cells in the peripheral blood at the indicated time points (n = 6 WT; n= 7 Mnk DKO). (D and E) Memory response. (D) Representative FACS plots of peripheral blood and spleen samples from recipient mice that received WT or Mnk DKO memory cells. Top panels show CD8 and TetG staining of peripheral blood and splenocytes. Bottom panels show Thy1.1 expression in the gated TetG·CD8 population. (E) Mean ± SEM presentation of percentages of Thy1.1− TetG·CD8 cells in peripheral blood and spleen samples from recipient mice (n = 5). Data shown are representative of two independent experiments.
We then examined by flow cytometry at 1, 2, and 4 weeks after infection the frequency of CD8 cells in the peripheral blood that could recognize the LCMV GP33–41 peptide presented on H-2D\(^b\) tetramers (TetG\(^+\) cells). Our results showed that the frequencies of TetG\(^+\) cells were comparable in WT and Mnk DKO mice at these time points (Fig. 22, B and C). Similar results were obtained when we determined the frequency of CD8 T cells that could recognize the LCMV NP 396–404 peptide presented on H-2D\(^b\) tetramers (data not shown). Together, these results suggest that Mnk1/2 deficiency might not alter polyclonal primary CD8 responses, at least in the LCMV model.

To understand whether Mnk1/2-deficient memory CD8 T cells generated after primary LCMV infection can respond robustly to pathogen rechallenge, we sorted out CD8\(^+\)CD44\(^+\) memory cells from WT (Thy1.1\(^−\)-Thy1.2\(^+\)) and Mnk DKO (Thy1.1\(^−\)-Thy1.2\(^+\)) mice 8 weeks after infection. These memory cells were adoptively transferred into WT Thy1.1\(^−\)-Thy1.2\(^+\) congenic recipients after normalizing the number of TetG\(^+\) cells. Recipient mice were challenged the next day with LCMV Armstrong, and the memory response was examined in the peripheral blood and spleen 7 d after infection. Results from flow cytometric analysis indicated a comparable expansion of adoptively transferred (Thy1.1\(^−\)) WT and Mnk DKO TetG\(^+\) memory cells in the peripheral blood and spleen (Fig. 22, D and E), suggesting that Mnk1/2-deficient memory CD8 cells can respond robustly to rechallenge. Taken together, these results support and extend those
from the Lm-OVA model, suggesting that Mnk1/2 function is dispensable during primary and memory CD8 responses to intracellular pathogens.

### 4.2.9 Effects of Mnk1/2 deficiency on iNKT cell development and function

The iNKT cells are a rare subset of T cells with the ability to bridge innate and adaptive immunity by rapidly producing and secreting copious amounts of cytokines. The mechanisms regulating cytokine production in iNKT cells are not well understood. We have recently demonstrated that proper iNKT cell development requires tight regulation of DAG-mediated signaling. Deficiency of RasGRP1 or enhanced activation of DAG-mediated signaling owing to DGKα and ζ deficiency can lead to defects in iNKT cell development (Shen et al., 2011a; Shen et al., 2011b). Because Mnk1 and Mnk2 are downstream effector molecules of the DAG-RasGRP1-Ras-ERK1/2 pathway, we investigated whether Mnk1 and Mnk2 have a role in regulating iNKT cell development. Like conventional T cells, individual or combined deficiency of Mnk1/2 did not affect the development of iNKT cells in the thymus, spleen, or liver (Fig. 23A). Percentages and absolute numbers of iNKT cells were similar in WT and Mnk DKO mice (Fig. 23B). Further analysis of iNKT cell developmental stages based on CD44 and NK1.1 expression did not reveal obvious differences between WT and Mnk DKO mice (Fig. 23A), suggesting normal development of iNKT cells in the absence of Mnk1/2.
Figure 23: Effect of combined Mnk1/2 deficiency on iNKT cell development and activation
Figure 23. (A) Normal iNKT cell development in the absence of Mnk1/2. Thymocytes, splenocytes, and liver mononuclear cells from age- and sex-matched Mnk DKO mice and WT controls were stained with CD1d-Tet, anti–TCRβ, anti-CD24, anti-CD44, and anti-NK1.1 followed by FACS analysis. Top panels show CD1d-Tet and anti–TCRβ staining on live cells. Bottom panels show NK1.1 and CD44 expression on gated CD1d-Tet−CD24− iNKT cells. (B) Total iNKT cell numbers in the indicated organs. (C) Mnk1 and Mnk2 are dispensable for IFNγ and IL-17 production by iNKT cells. WT and Mnk DKO thymocytes were stimulated with α-GalCer in vitro for 72 h with the addition of PMA, ionomycin, and GolgiPlug for the last 5 h. Cultured cells were then stained with CD1d-Tet and anti–TCRβ, and intracellularly stained with anti–IFNγ and anti–IL-17. Dot plots show IFNγ and IL-17 expression in live-gated CD1dTet+TCRβ+iNKT cells. (D) Assessment of iNKT cell proliferation. CFSE-labeled WT and Mnk DKO thymocytes were left unstimulated or were stimulated with α-GalCer for 72 h. Overlaid histograms show CFSE intensity in gated WT and Mnk DKO CD1dTet+TCRβ+iNKT cells. Data shown are representative of three independent experiments.
It has been reported that inhibition of Mnk1/2 by CGP57380 decreased cytokine production from iNKT cells following α-GalCer stimulation (Nagaleekar et al., 2011). We stimulated WT and Mnk DKO iNKT cells with α-GalCer in vitro for 72 h and intracellularly stained for IFNγ and IL-17 production. The percentages of IFNγ and IL-17–producing Mnk DKO iNKT cells were similar to those of WT iNKT cells (Fig. 23C). Using a CFSE dilution assay, we also examined iNKT cell proliferation following α-GalCer stimulation for 72 h. Mnk DKO iNKT cells appeared to proliferate slightly better than WT iNKT cells (Fig. 23D). These observations indicate that Mnk1 and Mnk2 are dispensable for iNKT cell development, cytokine production and proliferation.

4.2.10 Effects of Mnk1/2 inhibitor CGP57380 on activation of Mnk DKO T cells

The discrepancies between our data from Mnk1/2 double-deficient mice and those generated by chemical inhibition of Mnk1/2 with CGP57380 raise concerns about the selectivity of CGP57380 for Mnk1/2 and about the conclusions drawn from studies based on this inhibitor. To determine whether CGP57380 contains activities beyond inhibiting Mnk1/2, we examined the effects of CGP57380 on ERK1/2, Mnk1/2, and eIF4E phosphorylation following TCR engagement. As shown in Fig. 24A, CGP57380 inhibited not only eIF4E phosphorylation but also Mnk1/2 phosphorylation following TCR engagement. Moreover, it inhibited both WT and Mnk DKO T cell proliferation to similar extents (Fig. 24B), and reduced IFNγ but not IL-17 production by both WT and Mnk DKO iNKT cells (Fig. 24C).
Figure 24: Effects of Mnk1/2 inhibitor CGP57380 on T cells
Figure 24. (A) Effects of Mnk1/2 inhibitor CGP57380 on TCR signaling. WT thymocytes were rested in PBS at 37°C for 30 min, then either left unstimulated or stimulated with an anti-CD3ε Ab (500A2, 5 μg/ml) in the presence of indicated concentrations (nM) of CGP57380 at 37°C for 5 min. Cell lysates were subjected to immunoblotting analysis with the indicated antibodies. (B) CGP57380 inhibits WT and Mnk1/2DKO T cell proliferation. CFSE-labeled WT and Mnk DKO splenocytes were left unstimulated or stimulated with anti-CD3ε (2C11, 0.1 μg/ml) in the presence or absence of CGP57380 (10 nM) at 37°C for 72 h. Cells were then stained for CD4 and CD8. Histograms show CFSE intensity of live-gated CD4 and CD8 T cells. (C) Effects of CGP57380 on cytokine production by WT and Mnk DKO iNKT cells. WT and Mnk1/2 thymocytes were stimulated with α-GalCer (125 ng/ml) in the presence or absence of CGP57380 (10 nM) for 72 h. In the last 5 h of stimulation, cells were also treated with PMA plus ionomycin and GolgiPlug. Cells were then stained with anti-TCRβ antibody and CD1d-tetramer followed by intracellular staining for IFNγ and IL-17A. Contour plots show IL-17A and IFNγ expression in gated TCRβ+CD1d-tetramer+cells. Data shown represent two independent experiments.
These observations are consistent with findings that CGP57380 is able to inhibit other protein kinases such as MAPK kinase-1, casein kinase 1, and brain-specific kinase 2 (Bain et al., 2007). Thus, the effects of CGP57380 on T cells might not solely be attributed to Mnk1/2.

4.3 Discussion

Mnk1 and Mnk2 are downstream substrates for the Ras-MEK1/2-ERK1/2 and MKK3/MKK6-p38 kinase pathways. The importance of these pathways in T cells, the ability of Mnk1/2 to phosphorylate eIF4E, and the extremely dynamic nature of T cells during development and immune responses raise the possibility that Mnk1 and Mnk2 could have important roles in T cells by promoting protein translation via eIF4E regulation. Several recent studies lend credence to the idea that Mnk1/2 may be important regulators of the immune system. Inhibition of Mnk1/2 by CGP57380 has been found to reduce the translation of proinflammatory cytokines in keratinocytes (Kjellerup et al., 2008), macrophages (Rowlett et al., 2008) and dendritic cells (Mikkelsen et al., 2009). CGP57380 has also been shown to decrease cytokine production by iNKT cells and IL-17 production in CD4 T cells during Th17 differentiation (Nagaleekar et al., 2011; Noubade et al., 2011).

In this report, we have demonstrated that TCR-induced Mnk1/2 activation is promoted by Ras-ERK1/2 signaling and is negatively controlled by DGKα and ζ. In addition, both Mnk1 and Mnk2 are expressed at high levels in naive T cells, but are
downregulated in activated T cells. Using Mnk1/2 double-deficient mice, we have demonstrated that Mnk1 and Mnk2 are dispensable for the development of conventional \( \alpha \beta \) T cells, natural regulatory T cells, and \( \text{iNKT} \) cells. Moreover, Mnk1/2 double deficiency does not obviously affect activation of conventional T cells and \( \text{iNKT} \) cells, or \( \text{TH differentiation in vitro} \). The conclusion of a minimal role for Mnk1/2 in T cell activation is further strengthened by the observation that Mnk1/2 deficiency does not impair \( \text{in vivo} \) CD8 T cell responses in a bacterial model or a viral model of infection. Furthermore, our data also raise concerns over CGP57380 as a Mnk1/2–specific inhibitor because CGP57380 reduces eIF4E and Mnk1/2 phosphorylation following TCR engagement, inhibits both WT and Mnk DKO CD4 T cell proliferation, and decreases both WT and Mnk DKO \( \text{iNKT} \) cell production of IFN\( \gamma \).

Mnk1/2 deficiency does not affect \( \text{TH1} \) or \( \text{TH17} \) differentiation \( \text{in vitro} \), suggesting that there might be no obvious intrinsic defect of Mnk DKO CD4 T cells in \( \text{TH} \) differentiation. However, \( \text{TH1} \) and \( \text{TH17} \) differentiation is diminished in Mnk DKO mice in an EAE model, suggesting the possibility that Mnk1/2 deficiency affects \( \text{in vivo} \) \( \text{TH} \) lineage differentiation in a T cell–extrinsic manner. Additional experiments are required to explore these extrinsic mechanisms, including the possibility that Mnk1/2 functions in APCs to shape \( \text{TH} \) differentiation. This notion is supported by a recent report demonstrating that Mnk1 and Mnk2 regulate innate immune responses by modulating NF-\( \kappa \)B activity (Herdy et al., 2012).
Signals generated from the TCR can trigger the activation of T cells from naive or resting conditions, resulting in a significant increase in transcription, protein synthesis, and DNA synthesis (Cooper, 1969; Crabtree and Clipstone, 1994). Translation is one of the early events in activated T cells that can contribute to protein synthesis. Translational control is a key process by which cells generate crucial gene products quickly from preexisting mRNA without delay that results from mRNA transcription and RNA processing (Gebauer and Hentze, 2004; Sonenberg and Hinnebusch, 2009). Translation is a complex process and involves at least 10 translation initiation factors called eukaryotic translational initiation factors (eIFs), scaffolding or adaptor proteins, and 40S ribosomes. Binding of eIF4E to the mRNA is the foremost regulatory step in the formation of a pre-initiation complex that further leads to the formation of complete translational machinery (Gingras et al., 1999). However, how eIF4E is regulated is poorly understood.

Several studies have put forth a notion that Mnk1/2 signaling can regulate cap-dependent translation through phosphorylation of eIF4E at S209 (Pyronnet, 2000; Pyronnet et al., 1999). However, the role of eIF4E phosphorylation at S209 in translation initiation has been controversial (McKendrick et al., 2001; Scheper and Proud, 2002). Similarly, the germline deletion of Mnk1/2 in mouse models resulted in ablation in eIF4E phosphorylation without global effects on protein translation in mouse embryonic fibroblasts. In addition, Mnk DKO mice display normal growth and development. Consistently, TCR-induced eIF4E phosphorylation is abolished in Mnk DKO T cells,
suggesting that eIF4E phosphorylation at S209 is not essential for T cell development, proliferation, activation and cytokine expression, and might not globally control protein translation in T cells.

It has been demonstrated that mice that carry a non-phosphorylatable form of eIF4E (S209A) and Mnk1/2 double-deficient mice are more resistant to tumorigenesis than WT counterparts. This resistance was shown to be via translational control of a specific subset of genes related to tumorigenesis, such as vascular endothelial growth factor C, baculoviral IAP repeat-containing protein 2, and matrix metalloproteinase-3 (Furic et al., 2010; Ueda et al., 2010). Although we have demonstrated that Mnk1 and Mnk2 are dispensable for T cell development and activation in general, we cannot rule out the possibility that they might be selectively required for efficient translation of specific subsets of proteins that may affect particular aspects of T cell function.
5. The role of tumor suppressor TSC1 in regulating antigen-specific primary and memory CD8 T cell responses to bacterial infection

5.1 Introduction

Antigen-specific CD8 responses were described in section 1.1.3. A number of studies have correlated the kinetics of CD8 responses with external factors like the duration of antigen availability and the presence of co-stimulation (Badovinac et al., 2004; Porter and Harty, 2006). More recently, a role for TCR-mediated signaling (Ou-Yang et al., 2013; Teixeiro et al., 2009) and cytokine-mediated signaling (Keppler et al., 2012; Thompson et al., 2006) in shaping primary and memory CD8 responses has also come to the fore. However, the signaling mechanisms that integrate these diverse extracellular cues to modulate the magnitude and quality of CD8 immune responses remain to be fully understood.

As stated previously, mTOR is an evolutionarily conserved serine/threonine kinase that plays a critical role in integrating environmental cues such as the presence of amino acids, nutrients, growth factors and cytokines to determine cell growth and metabolic outcomes in eukaryotic cells (Sengupta et al., 2010; Zoncu et al., 2011). For a detailed discussion of TCR-mediated mTOR activation please see section 1.2.3. Recent studies have elegantly demonstrated that mTOR regulates a number of key aspects of T cell biology, including CD4 T helper cell differentiation and CD8 cell effector-memory differentiation (Araki et al., 2011; Araki et al., 2009; Chi, 2012; Delgoffe et al., 2009;
Delgoffe et al., 2011; O’Brien and Zhong, 2012; Rao et al., 2010; Waickman and Powell, 2012; Xu et al., 2012). In particular, these studies have shown that treatment with the mTORc1 inhibitor rapamycin increases the number of MPECs during the expansion phase and accelerates memory cell differentiation during the contraction phase during anti-viral CD8 responses, suggesting that mTORc1 activity may negatively regulate these processes (Araki et al., 2009). Ex vivo, the temporal kinetics of mTORc1 activity during CD8 cell activation were found to play a critical role in determining effector versus memory fate by differentially regulating the expression of the transcription factors T-bet and Eomes (Rao et al., 2010).

As mentioned in section 1.2.3, the tuberous sclerosis complex (TSC), a heterodimer of the tumor suppressor proteins TSC1 and TSC2, is an upstream negative regulator of mTORc1 activity (Inoki et al., 2002). Under resting conditions, GAP activity of the TSC complex maintains the Ras-family GTPase Rheb (Ras homolog enriched in brain) in an inactive GDP-bound form. In the presence of nutrients, growth factors or cytokines, receptor-mediated signals inhibit TSC activity, and active GTP-bound Rheb promotes mTORc1 activity by stimulating mTOR phosphorylation at Ser2448 (Huang and Manning, 2008; Inoki et al., 2003). Several recent studies have demonstrated a vital role for TSC1 in maintaining T cell quiescence, survival and mitochondrial homeostasis (O’Brien et al., 2011; Wu et al., 2011; Yang et al., 2011b; Zhang et al., 2012). Mice with a conditional deficiency of TSC1 in T cells showed a dramatic reduction of CD4 and CD8
cells in the spleen, correlating with enhanced apoptosis via the intrinsic pathway. This was accompanied by a cell-autonomous loss of T cell quiescence and hyper-responsiveness to TCR stimulation. Given that mTORc1 activity is critical in determining CD8 effector versus memory fate, we hypothesized that TSC1 would play a critical role in regulating CD8 responses. Preliminary results from a previous study suggest that TSC1f/f CD4Cre mice contained fewer antigen-specific CD8 cells and fewer IFNγ-producing CD8 cells than WT counterparts upon bacterial infection (Yang et al., 2011b). However, since TSC1f/f CD4Cre mice have fewer mature T cells, a lower frequency of naïve cells, and a higher frequency of apoptotic T cells as compared to WT counterparts, these results prove difficult to interpret.

Here we use a model of naïve TCR-transgenic (OT1) CD8 cell adoptive transfer followed by infection with Listeria monocytogenes expressing a cognate antigen (Condotta et al., 2012), to investigate a T cell-intrinsic role for TSC1 in regulating antigen-specific CD8 responses. The OT1 TCR contains Vα2 and Vβ5 variable segments and recognizes the SIINFEKL (OVA257-264) epitope of ovalbumin presented on H-2Kb. Using both individual and competitive adoptive transfers with WT counterparts, we show that TSC1 deficiency impairs antigen-specific primary CD8 responses. Fewer TSC1-deficient CD8 cells were present as compared to WT cells at the peak of the response, correlating with defective in vivo proliferation during the expansion phase. Results also showed a bigger proportion of dead cells among the TSC1 KO pool as compared to WT
counterparts. The TSC1 KO population contained an increased ratio of SLECs to MPECs at the peak of the response, correlating with enhanced contraction. Upon competitive adoptive transfer into WT recipients, fewer TSC1 KO memory OT1 cells were present at days 6 and 7 than WT OT1 memory cells, suggesting that TSC1 deficiency may also affect the quality of memory cells formed. Taken together, our findings demonstrate a previously unknown role for TSC1 in regulating the kinetics of antigen-specific primary and memory CD8 responses by repressing cell death, promoting proliferation, and regulating effector-memory differentiation.

5.2 Results

5.2.1 TSC1-deficiency impairs antigen-specific CD8 responses in vivo

To better understand the role of TSC1 in regulating primary CD8 responses, we adoptively transferred $10^4$ naïve Va2⁺ CD8 cells sorted from spleen and LN cells of WT OT1 or TSC1⁺/⁻ CD4Cre OT1 donors (CD45.1⁺CD45.2⁺) into congenically marked WT recipients (CD45.1⁻CD45.2⁻). The recipient mice were subsequently infected with Listeria monocytogenes expressing recombinant ovalbumin (Lm-Ova) (Fig. 25A). Adoptively transferred cells were tracked in the peripheral blood at 1, 2, 4 and 7 weeks post infection by flow cytometry based on their congenic markers.
Figure 25: TSC1 deficiency impairs antigen-specific CD8 responses
Figure 25. (A) Schematic representation of experimental design showing individual adoptive transfers of naïve WT OT1 or TSC1 KO OT1 cells into WT CD45.1+CD45.2+ recipients. (B) Representative FACS analysis of peripheral blood samples showing Vα2+CD8 cells among total PBMCs (top panel) and adoptively transferred (CD45.1-CD45.2+) and endogenous (CD45.1+CD45.2+) populations within the gated Vα2+CD8 population (bottom panel) at the indicated times post infection. (C) Percentages of WT and TSC1 KO cells among total PBMCs at indicated times post infection. Mean ± SEM are calculated for five mice per group. Data shown are representative of three independent experiments. *p < 0.05, **p < 0.01 (Student t test)
In our analysis, we first gated on the Vα2+ CD8 population, which contains both adoptively transferred OT1 cells and the recipient’s endogenous CD8 cells that use Vα2. Within this Vα2+ CD8 pool, adoptively transferred cells formed a distinct CD45.1-CD45.2+ population that was readily distinguishable from CD45.1+CD45.2+ endogenous cells. Results from these flow cytometric analyses showed that adoptively transferred WT cells underwent robust clonal expansion reaching peak numbers at week 1 and declining rapidly thereafter leaving behind a stable pool of memory cells (Fig. 25, B and C). However, the frequency of TSC1 KO OT1 cells was significantly lower than that of WT OT1 counterparts at all time points, suggesting that TSC1-deficiency may impair the ability of CD8 cells to mount a robust response to antigens. The reduced frequency of TSC1 KO cells at week 1 and later time points suggests that TSC1 may be required for optimal clonal expansion and memory formation/maintenance respectively.

To account for possible differences in antigen clearance and to measure the response of WT OT1 and TSC1 KO OT1 cells in the same host, we next performed competitive adoptive transfer experiments. In these experiments, a mixture containing equal numbers of WT OT1 (CD45.1+) and TSC1 KO OT1 (CD45.2+) cells was injected into each WT (CD45.1+CD45.2+) recipient (Fig. 26A). Impairment of the TSC1 KO CD8 response was more striking in the competitive model with nearly four times as many WT cells present among PBMCs at week 1 as compared to TSC1 KO cells (Fig. 26, B and C). This trend should perhaps not be surprising, given that TSC1 KO OT1 cells compete...
for antigen with an equal number of WT OT1 cells in the mixed-transfer system, as opposed to a rare population of endogenous T cells bearing a Vα2+ Vβ5+ TCR in the individual-transfer system. The TSC1 KO population also showed dramatic contraction, becoming less frequent than endogenous Vα2+ CD8 cells by week 2 (Fig. 26B). This was correlated with a marked paucity of TSC1 KO memory cells, as seen at weeks 4 and 7. Taken together, results from the individual and competitive adoptive transfer systems suggest that TSC1 may play a critical cell-intrinsic role during both the expansion and contraction phases of antigen-specific CD8 responses.

5.2.2 Loss of TSC1 diminishes CD8 cell proliferation in vivo

Since significantly fewer TSC1 KO OT1 cells were present in the peripheral blood at week 1 as compared to WT counterparts, we sought to determine if the loss of TSC1 affected the proliferative capacity of CD8 cells. To this end, we first cultured naïve WT OT1 and TSC1 KO OT1 cells *ex vivo* with APCs that were either not loaded (‘unstimulated’) or pre-loaded with the SIINFEKL peptide (‘peptide stimulated’). Upon overnight culture, WT and TSC1 KO cells showed comparable upregulation of CD69, a surface marker of T cell activation (Fig. 27A). We also examined the ability of TSC1 KO OT1 cells to proliferate in response to peptide stimulation *ex vivo*, by culturing CFSE-labeled WT OT1 and TSC1 KO OT1 cells with APCs that were not loaded or SIINFKL-loaded for a period of 72h.
Figure 26: TSC1-deficient CD8 cells are severely impaired in a competitive adoptive transfer system
Figure 26. (A) Schematic representation of experimental design showing competitive adoptive transfers of naïve WT OT1 and TSC1 KO OT1 cells into WT CD45.1+CD45.2+ recipients. (B) Representative FACS analysis of peripheral blood samples showing Vα2+CD8 cells among total PBMCs (top panel) and WT (CD45.1+CD45.2+), TSC1 KO (CD45.1-CD45.2-) and endogenous (CD45.1+CD45.2-) populations within the gated Vα2+CD8 population (bottom panel) at the indicated times post infection. (C) Percentages of WT and TSC1 KO cells among total PBMCs at indicated times post infection. Mean ± SEM are calculated for five mice per group. Data shown are representative of three independent experiments. **p < 0.01, ***p < 0.001 (Student t test)
Figure 27: Defective proliferation of TSC1-deficient CD8 cells in vivo
Figure 27. (A) Representative histograms of CD69 expression on WT OT1 and TSC1 KO OT1 cells that were cultured overnight with APCs that were not loaded or loaded with SIINFEKL peptide. (B) Representative histograms showing CFSE dilution in WT OT1 and TSC1 KO OT1 cells that were cultured for 72h with APCs that were not loaded or loaded with SIINFEKL peptide. (C) Representative density plots showing BrdU incorporation in WT OT1 and TSC1 KO OT1 cells in the peripheral blood and spleen. Mice were injected with BrdU on day 5 post infection and tissues were harvested after 16 hours for staining and flow cytometric analysis. (D) Percentage of BrdU⁺ cells among WT OT1 and TSC1 KO OT1 populations in the peripheral blood and spleen. Mean ± SEM are calculated for four mice per group. Data shown are representative of two independent experiments. *p < 0.05, **p < 0.01 (Student t test)
Analysis of CFSE dilution among OT1 cells by flow cytometry revealed that WT and TSC1 KO cells had undergone comparable rounds of cell division (Fig. 27B). Together, these results suggest that loss of TSC1 deficiency may not affect T cell activation or proliferation ex vivo. To determine if WT OT1 and TSC1 KO OT1 cells proliferated comparably in vivo, we analyzed BrdU incorporation in these populations over a 16h period during the expansion phase. Surprisingly, results from this experiment showed that a smaller pool of TSC1 KO cells had proliferated during this period as compared to WT cells in both the peripheral blood and spleen (Fig. 27, C and D). Perturbations in proliferation in vivo, but not ex vivo, suggest the possibility that TSC1 deficiency may somehow limit the ability of CD8 cells to access antigens in vivo. (Please see section 5.3 for a detailed discussion of this issue.)

5.2.3 Enhanced CD8 cell death in the absence of TSC1

Based on results from previous studies which showed an increase in T cell apoptosis in the absence of TSC1, we hypothesized that enhanced cell death may also contribute to the effete expansion of TSC1 KO OT1 cells. To examine this possibility, we stained freshly isolated splenic and peripheral blood cells on day 6 post-infection with the exclusion dye 7AAD (Fig. 28A). Flow cytometric analysis revealed that an increased proportion of TSC1 KO OT1 cells failed to exclude 7AAD as compared to WT OT1 cells (Fig. 28, A and B), indicating that enhanced cell death in the TSC1 KO population may play a role in curtailing expansion.
Figure 28: TSC1 deficiency enhances CD8 cell death *in vivo*

Figure 28. (A) Representative FACS plots showing 7AAD staining within WT OT1 and TSC1 KO OT1 populations in the peripheral blood and spleen on day 6 post-infection. (B) Percentage of 7AAD+ (non-viable) cells within WT OT1 and TSC1 KO OT1 populations in the peripheral blood and spleen on day 6 post-infection. Mean ± SEM are calculated for five mice per group. Data shown are representative of two independent experiments. **p < 0.01, ***p < 0.001 (Student t test)
5.2.4 TSC1 deficiency alters effector-memory differentiation and enhances contraction

Given that defective proliferation and enhanced cell death contribute towards impaired expansion of TSC1 KO OT1 cells, we next sought to determine if the absence of TSC1 could affect the dynamics of the CD8 response by altering effector-memory differentiation. To examine if effector-memory differentiation is affected by the loss of TSC1, we examined the frequency of SLECs and MPECs among the WT OT1 and TSC1 KO OT1 populations at the peak of the response. Flow cytometric analysis revealed that the TSC1 deficient population contained a higher ratio of SLECs (KLRG1hi IL-7Rαlo) to MPECs (KLRG1lo IL-7Rαhi) than the TSC1 sufficient population (Fig. 29, A and B), both in the peripheral blood and spleen. These results are consistent with ones from previous studies, which showed that sustained mTORc1 activity promoted T-bet expression and effector differentiation (Rao et al., 2010). We reasoned that a higher SLEC to MPEC ratio might be correlated with enhanced contraction. When we examined the frequency of cells surviving at week 2 post-infection as a percentage of the cells that were present at week 1, we found that only about 5% of TSC1 KO cells survived the contraction phase as compared to more than 10% of WT cells in the peripheral blood (Fig. 29C). A similar trend was observed in the spleen, and these findings together suggest that loss of TSC1 may promote enhanced CD8 contraction.
Figure 29: Loss of TSC1 alters CD8 cell effector-memory differentiation
Figure 29. (A) Representative FACS plots showing KLRG1 and IL-7Rα staining within WT OT1 and TSC1 KO OT1 populations in the peripheral blood and spleen at week 1 post-infection. (B) Ratio of SLECs to MPECs within WT OT1 and TSC1 KO OT1 populations in the peripheral blood and spleen at week 1 post-infection. (C) Frequency of WT OT1 or TSC1 cells surviving in the peripheral blood and spleen at week 2 post-infection, as a percentage of the cells that were present at week 1. (D) Representative FACS plots showing intracellular staining for T-bet and Eomes (with appropriate isotype controls) among WT OT1 and TSC1 OT1 cells that were cultured \textit{ex vivo} for 72h with Ova peptide-loaded APCs. (E) Representative FACS plots showing IFNγ staining of WT OT1 and TSC1 OT1 cells that were cultured \textit{ex vivo} for 72h with Ova peptide-loaded APCs and subsequently stimulated with PMA and ionomycin (in the presence of Golgi plug) for 5h. Mean ± SEM are calculated for five mice per group. Data shown are representative of four (A-C), two (D) and three (E) independent experiments. *$p < 0.05$, ***$p < 0.001$ (Student $t$ test)
To better understand the mechanisms by which TSC1 regulates effector-memory differentiation, we examined the expression of transcription factors T-bet and Eomes in WT OT1 and TSC1 OT1 cells that had been stimulated \textit{ex vivo} with Ova peptide-loaded APCs for 72h. Intracellular staining and flow cytometric analysis revealed that more TSC1 KO OT1 cells expressed T-bet and fewer expressed Eomes, as compared to WT OT1 counterparts (Fig. 29D). A bevy of elegant studies have previously revealed a complex interplay between T-bet and Eomes in CTL differentiation. While the two closely related T-box transcription factors act redundantly to induce CD8 effector functions, they have also been shown to act reciprocally to drive effector and memory cell differentiation respectively (Kallies, 2008). Based on findings from these studies, the increase in T-bet-expressing cells and decrease in Eomes-expressing cells appears to be consistent with the higher SLEC to MPEC ratio that was observed at the peak of the response (Fig. 29, A and B).

Previous studies have also delineated roles for both T-bet and Eomes in IFN$\gamma$ expression. We stimulated WT OT1 and TSC1 KO OT1 cells \textit{ex vivo} with Ova peptide-loaded APCs for 72h and subsequently queried them for IFN$\gamma$ competence by stimulation with PMA and ionomycin. By intracellular staining and flow cytometric analysis, we found that TSC1 KO OT1 cells produced less IFN$\gamma$ than WT counterparts (Fig. 29E), consistent with previous data reported by Yang et al (Yang et al., 2011b). In
sum, these observations suggest that loss of TSC1 may perturb effector-memory differentiation and enhance CD8 contraction in the Lm-Ova model.

5.2.5 Moderate impairment of CD8 memory responses in the absence of TSC1

To assess the quality of TSC1-deficient memory cells, in terms of their ability to respond to antigen re-exposure, we adoptively transferred equal numbers of WT OT1 and TSC1 KO OT1 memory cells into congenically marked naïve WT recipients (CD45.1+CD45.2+). These recipients were subsequently challenged with a ten-fold higher dose of Lm-Ova, and the memory response was monitored in the peripheral blood and spleen on days 6 and 7 post challenge (Fig. 30A). Though the ratio of adoptively transferred WT and TSC1 KO memory cells was close to 1:1 (Fig. 30B), flow cytometric analysis revealed a moderate reduction in TSC1 KO OT1 cell frequencies at days 6 and 7, as compared to WT counterparts (Fig. 30, B and C). These differences were more apparent on day 7 than day 6, but were statistically significant at both time points in the peripheral blood and spleen. Ex vivo Ova-peptide stimulation of splenocytes at days 6 and 7 post infection revealed no significant differences in the production of effector cytokines such as IFNγ and TNFα (Fig. 30D). Though TSC1 deficiency severely limits the quantity of memory formation, these results suggest that the effects on memory cell quality might be subtler. Memory cells differ from their naïve counterparts both in their migration patterns and thresholds for activating stimuli (Berard and Tough, 2002). It is possible that these differences may mitigate the effects of TSC1 deficiency in memory
cells, allowing them to expand more efficiently than TSC1-deficient naïve cells. Further studies are required to examine this possibility.

Figure 30: CD8 memory responses are moderately impaired in the absence of TSC1
Figure 30. (A) Schematic representation of experimental design showing competitive adoptive transfers of WT OT1 and TSC1 KO OT1 memory cells into WT CD45.1+CD45.2+ recipients. (B) Representative FACS analysis of peripheral blood and spleen samples showing Vα2+ CD8 cells among total PBMCs and splenocytes (top panel) and WT (CD45.1+CD45.2+), TSC1 KO (CD45.1-CD45.2+) and endogenous (CD45.1+CD45.2+) populations within the gated Vα2+ CD8 population (bottom panel) at the indicated times post challenge. (C) Percentages of WT and TSC1 KO cells among total PBMCs and splenocytes at indicated times post challenge. (D) Percentages of IFNγ+ cells and TNFα+ cells within the WT and TSC1 KO splenocyte populations at indicated times post challenge, as detected by intracellular staining and flow cytometry after 5h of Ova-peptide stimulation. Mean ± SEM are calculated for five mice per group. Data shown are representative of three independent experiments. *p < 0.05, **p < 0.01 (Student t test)
5.3 Discussion

In this study, we demonstrated that loss of TSC1 in CD8 cells diminishes antigen-specific CD8 responses, resulting in a weak expansion phase, exaggerated contraction phase and poor memory generation. We showed that weak expansion of TSC1-deficient cells was correlated with defects in survival and proliferation in vivo, while exaggerated contraction was associated with an increased ratio of SLECs to MPECs in the effector cell population. This perturbation in effector-memory differentiation was concomitant with enhanced T-bet expression and decreased Eomes expression among activated TSC1 KO cells. Upon competitive adoptive transfer with WT counterparts and antigen re-challenge, TSC1-deficient memory cells showed moderate defects in expansion but not cytokine production. Our results provide direct evidence of a CD8 cell-intrinsic role for TSC1 in regulating antigen-specific primary and memory responses, corroborating growing recognition of the TSC1-mTORc1 axis as an attractive target for modulating immune responses.

Previous studies have examined a role for mTOR and TSC1 in regulating T cell proliferation. In ex vivo experiments, mTOR-deficient CD4 cells proliferated less than WT counterparts, and that this defect was correlated with impaired upregulation of cyclin D3 expression (Delgoffe et al., 2009). Results from our ex vivo assays, in which naïve WT OT1 and TSC1 KO OT1 cells were stimulated with Ova peptide-loaded APCs, closely mirrored those from previous studies in which polyclonal TSC1-deficient CD4
and CD8 cells were stimulated with anti-CD3 antibodies. (O'Brien et al., 2011). In both cases, TSC1 deficient cells did not show defects in proliferation, and in fact appeared to proliferate slightly more than WT counterparts. Together, these results suggest that unlike a lack of mTOR activity, excessive mTOR activity may not be directly detrimental to T cell proliferation. Surprisingly, results from our in vivo BrdU incorporation experiments demonstrated a significant reduction in proliferating TSC1 KO OT1 cells during the expansion phase, suggesting that TSC1 may in fact regulate proliferation in vivo. One scenario that could reconcile these seemingly discrepant observations is that TSC1 deficiency might diminish the ability of CD8 cells to home to appropriate regions of secondary lymphoid organs and interact with APCs. This idea is supported by evidence that mTOR activity plays a role in down-regulating the expression of CD62L and CCR7 (Sinclair et al., 2008), an adhesion molecule and chemokine receptor, respectively, that are critical for lymph node and splenic white pulp homing of T cells (Ebert et al., 2005; Nolz et al., 2011; Weninger et al., 2002). Future studies are required to test the hypothesis that chronic mTORc1 activity in TSC1-deficient CD8 cells can alter their trafficking patterns in a manner that limits interaction with APCs.

A complex interplay between the closely related T-box family transcription factors T-bet (T-box, expressed in T cells) and Eomes (eomesodermin) underlies several aspects of CD8 cell function including IFNγ production and effector versus memory differentiation (Pearce et al., 2003; Szabo et al., 2002). Studies have also identified that
the transcription factor Runx3 may synergize with T-bet and Eomes during CD8 differentiation (Cruz-Guilloty et al., 2009). T-bet and Eomes show functional homology in several aspects of T cell function (Intlekofer et al., 2005), but are inversely regulated and thought to act antagonistically during CD8 differentiation. Previous work has shown that IL-12, a hallmark cytokine of cell-mediated immunity, promotes T-bet expression and represses Eomes expression in CD8 cells during Listeria monocytogenes infection (Takemoto et al., 2006). A search for underlying mechanisms revealed that IL-12 signaling sustained and enhanced mTOR activity in CD8 cells, and that inhibition of mTOR activity by rapamycin treatment could lead to a loss of T-bet expression and reverse IL-12 induced effector functions (Rao et al., 2010). With regard to IFNγ production, studies have shown that T-bet is quickly induced upon TCR stimulation and required for early cytokine production, while subsequent Runx3-mediated Eomes induction is important for sustained IFNγ expression (Cruz-Guilloty et al., 2009). However, a recent study has shown that IFNγ production from T-bet Eomes DKO CD8 cells, but not single knockout cells, was significantly lower than WT counterparts, suggesting that neither factor may be individually essential for IFNγ production (Zhu et al., 2010b). Here, we found that TSC1 deficiency was associated with an increased frequency of T-bet expression, and a reduced frequency of Eomes expression and IFNγ-production in activated CD8 cells. Further studies are required to clearly define the
molecular mechanisms by which TSC1 deficiency may modulate the complex transcriptional network that drives IFNγ expression in CD8 cells.

While TSC1 deficiency enhances mTORc1 activity, previous studies have shown that it is also associated with decreased mTORc2-Akt activity in T cells (O’Brien et al., 2011; Yang et al., 2011b). Though it stands to reason that increased mTORc1 activity might play a major role in perturbing antigen-specific CD8 responses in the absence of TSC1, further studies are required to dissect the contributions of the two mTOR-complexes to the observed phenotype. One challenge in this regard is to optimize approaches such as low-dose rapamycin treatment or conditional Raptor haplo-insufficiency to allow for the restoration of mTORc1 activity to near-normal levels in TSC1 KO cells. In conclusion, our study identifies a critical role for TSC1 in regulating both the quantitative and qualitative aspects of antigen-specific CD8 responses, and suggests that impaired proliferation, enhanced cell death and altered effector-memory differentiation contribute to impaired CD8 responses in the absence of TSC1.
6. General Discussion and Future Directions

6.1 Comparative analysis of calKKβ and DGKαζDKO mice

Analyzing the similarities and differences between calKKβ and DGKαζDKO mice is likely to prove helpful in gaining a better understanding of how chronic IKK-NF-κB signaling impairs T cell function during DGK deficiency. At the phenotypic level, both IKK and DGKαζDKO mice develop splenomegaly and liver disease. In IKK mice splenomegaly is accompanied by a disruption of red pulp-white pulp architecture likely stemming from extramedullary erythropoiesis. Whether splenomegaly in DKO mice is associated with similar histological changes remains to be investigated. Both IKK and DKO mice display mononuclear cell infiltration into liver tissues. Such infiltration is more severe in DKO mice and associated with liver injury as characterized by increased levels of liver parenchymal cell-specific transaminases (AST and ALT) in the serum. In IKK mice, the infiltrating cells were characterized predominantly as CD4 cells and CD11b+ myeloid cells, whereas DKO infiltrates are primarily composed of CD8 cells. Together, these results suggest that hyperactive IKK-NF-κB signaling might be sufficient to trigger at least some aspects of autoimmunity that are manifested in DGKαζDKO mice, but that the mechanisms by which these seemingly similar phenotypes develop may be divergent.

On the other hand, a stark point of contrast between these mice is that IKK T cells are hypo-responsive to TCR-mediated stimulation, while DKO T cells are hyper-
responsive. IKK T cells show defects in both proximal and distal signaling events and proliferate less than WT cells, whereas DKO T cells show hyper-activation of NF-κB, Ras-ERK and mTOR pathways and proliferate more than WT cells. These observations suggest the possibility that hyper-proliferation of DKO cells might be attributable to enhanced signaling via pathways other than (or in addition to) IKK-NF-κB. Since DKO cells do not suffer from signaling defects analogous to those of IKK cells, it also stands to reason that the hyper-activation of Ras-ERK or mTOR pathways could somehow prevent or alter the negative feedback mechanisms triggered by hyperactive IKK-NF-κB signaling.

Another point of comparison between IKK and DKO mice is thymocyte viability. DKO mice show a severe block in thymocyte development at the DP stage with a marked paucity of mature SP cells (Guo et al., 2008). This is associated with increased cell death among TCRβhi mature thymocytes. IKK thymocytes show an analogous, albeit much less severe, block in thymocyte development at the DP stage with a substantial reduction in absolute numbers of CD4SP and CD8SP cells (Fig. 5B). This developmental blockade was associated with increased cell death in mature thymocyte compartments, though the results did not reach statistical significance (Fig. 6, A and B). The milder impairment in thymocyte development and survival observed in IKK mice suggests that the severe developmental blockade observed in DGKαζDKO mice is likely attributable to the hyper-activation of multiple signaling pathways including IKK-NF-κB. This idea
is consistent with previous observations that the Ras-ERK pathway is highly activated in thymocytes undergoing negative selection (Mariathasan et al., 2000). When it comes to peripheral T cells, IKK cells show increased cell death in the periphery than WT cells (Fig. 5F), whereas no such increase is seen in DKO cells (our unpublished observations).

Firstly, this disparity between thymocyte and mature T cell survival suggests that the viability of these closely related populations may in fact be differentially regulated. Secondly, given the pro-survival effects of both Ras-ERK and mTOR pathways in a number of cellular contexts including oncogenesis, these results also indicate that such pro-survival signals may be sufficient to overcome the increased propensity for apoptosis associated with IKK-NF-κB hyper-activity in T cells.

In summary, a comparison of the similarities and differences between IKK and DGKaζDKO mice suggests that parallels seen at the phenotypic level may belie disparities in underlying mechanisms.

### 6.2 Feedback inhibition of TCR-proximal signaling events by constitutive IKKβ activity

Our results suggest that signaling mechanisms as proximal to the TCR as ZAP70-phosphorylation are defective in the presence of constitutive IKKβ activity. While increased Blimp1 levels in IKK T cells played a causal role in attenuating TCR-mediated activation, Blimp1 deficiency did not restore activation to normal levels suggesting that other mechanisms may be involved. Further studies are required to delineate some of these mechanisms, but data available in the literature present two exciting possibilities.
First, negative regulation of TLR-mediated NF-κB activation involves the transcriptional upregulation of dominant negative adaptor proteins (Ruland, 2011). Studies have shown that stimulation of murine embryonic fibroblast cells with LPS can induce de novo generation of a shorter isoform of the adapter MyD88 via alternative splicing (Burns et al., 2003; Janssens et al., 2003). Dimers of full-length MyD88 bring IRAK1 and IRAK4 together to facilitate IRAK4-mediated phosphorylation of IRAK1 and subsequent NF-κB activation during normal TLR-mediated signaling. NF-κB activity drives the expression of the shorter MyD88 isoform, which lacks the domain required for IRAK4-binding, thereby preventing further NF-κB activation. Another example of a dominant negative adaptor in TLR-mediated NF-κB signaling is IRAK-M, a kinase-inactive member of the IRAK family (Kobayashi et al., 2002). Based on these findings, it is tempting to speculate that dominant-negative isoforms of TCR signaling-related adaptor proteins may exist and play a role in inhibiting TCR-proximal signals upon NF-κB activation in T cells.

Secondly, other studies have identified a critical role for PKCθ in regulating the stability of immunological synapses (Zanin-Zhorov et al., 2011). The immunological synapse consists of a central supra-molecular activation cluster (cSMAC) that mainly contains TCR microclusters, surrounded by a peripheral supra-molecular activation cluster (pSMAC) that contains adhesion molecules such as LFA1 and ICAM1. Formation of the synapse is thought to play a key role in stabilizing T cell-APC interactions and facilitating complete T cell activation by sustaining signal transduction (Dustin, 2008).
Studies have shown that PKCθ is selectively recruited to the cSMAC-pSMAC junction (Yokosuka et al., 2008) and plays a critical role in the formation of “symmetry breaks” in SMACs that allow for T cells to migrate locally before synapse re-formation (Sims et al., 2007). Since ZAP70 phosphorylation in IKK T cells appears comparable to WT cells at early time points (2 min) but is dramatically lower at later time points (15 min) (Fig. 8C), it is plausible that constitutive IKKβ activity may be associated with defects in synaptic stability. Given the critical role of PKCθ in maintaining synaptic stability, further studies should explore if sustained NF-κB signaling could set in motion negative feedback mechanisms that modulate PKCθ function to perturb the stability of the synapse.

6.3 Future directions in examining Mnk1/2-function

T cell activation is accompanied by a dramatic increase in cell size and requires the rapid synthesis of large amounts of structural proteins and other specialized proteins such as cytokines. As stated previously, cap-dependent translation initiation is the primary mode of ribosomal recruitment to mRNA in eukaryotic cells, and about 95% of cellular mRNAs are translated in a cap-dependent manner. In this context, the hypothesis that Mnk1/2 proteins (which phosphorylate eIF4E, the rate-limiting initiation factor in cap-dependent translation) may play a critical role in normal T cell activation and function seems reasonable. However, results from our studies suggest that despite the complete loss of eIF4E phosphorylation at Ser209, Mnk1/2-deficient mice show normal T cell development, activation, proliferation, cytokine-production, CD4 T H
differentiation, and primary and memory CD8 responses. Results to the contrary from previous studies that used a chemical inhibitor of Mnk1/2 activity were undermined by findings that the inhibitor possesses off-target activity.

Despite the absence of obvious changes in T cell development and function, it remains possible that a subset of genes may be differentially expressed in T cells in the absence of Mnk1/2 activity, specifically affecting one or more aspects of T cell function that were not examined in our current study. Studies with a model of tumorigenesis have shown that Ser209 is the only phosphorylation site on eIF4E. Results from polysome profiling experiments showed that phosphorylation at this site selectively increased the translation efficiency of a set of 35 mRNAs that code for pro-tumorigenic factors (Furic et al., 2010). Among these mRNAs were those encoding the closely related chemokines CCL2 and CCL7. While CCL2 is thought to recruit monocytes, memory T cells and dendritic cells to sites of inflammation, CCL7 specifically attracts macrophages (Zlotnik and Yoshie, 2000). Based on findings that CD8 cell-mediated macrophage recruitment plays an important role in adipose tissue inflammation in obesity (Nishimura et al., 2009), it would be interesting to study the development of obesity-related inflammation and metabolic disease in Mnk DKO mice. However, the generation of conditional Mnk1/2 knockouts would be required to tease out the role of T cells in this process.
This line of study is further supported by results from polysome profiling, which showed that eIF4E phosphorylation was required for efficient translation of caspase 4. Caspase 4 is a pro-inflammatory caspase that has recently been shown to be required for the activation of caspase 1 and the NLRP3 inflammasome in macrophages (Sollberger et al., 2012). Since the NLRP3 inflammasome has been shown to play a critical role in obesity-induced macrophage-T cell activation in adipose tissues (Vandanmagsar et al., 2011), the reduced translation efficiency of CCL2, CCL7 and caspase 4 in Mnk1/2 DKO mice collectively argues for an immune phenotype that may render these mice less susceptible to obesity-induced inflammation and metabolic disease.

Results from our study show that while ex vivo Th1 and Th17 differentiation of CD4 cells is unimpaired by Mnk1/2 deficiency, a smaller pool of MOG-reactive Th1 and Th17 cells is generated in vivo in Mnk1/2 DKO mice as compared to WT counterparts in an EAE model. These results suggest that T cell-extrinsic factors may play a predominant role in dampening in vivo Th1 and Th17 differentiation in Mnk DKO mice. Data from previous studies suggest a role for MAPK pathways in regulating IL-12 production by APCs, lending credence to notion that Mnk1/2 proteins (that are located downstream of ERK and p38) may also play a role in this process. For instance, results from one study showed that macrophages and dendritic cells that lacked MKK3 (a MAPK kinase that phosphorylates p38) displayed defects in IL-12 production (Lu et al., 1999). Though the effect of MKK3 deficiency can be partly explained by reduced
transcription of IL-12, it remains possible that p38-mediated activation of Mnk1/2 may play an important role in optimal IL-12 translation in APCs. Future studies should investigate the role of Mnk1/2 in TLR-induced production of IL-12, IL-6 and TGFβ by APCs.

6.4 Future directions in examining TSC1-mediated regulation of CD8 responses

While results from our study suggest that TSC1 deficiency alters CD8 effector cell differentiation to enhance the ratio of short-lived effectors to memory precursors (presumably via hyper-active mTORc1 signaling), the mechanisms underlying these perturbations remain to be investigated. One promising possibility in this regard, is that signaling via the TSC1-mTORc1 pathway might affect the expression of transcriptional regulators Id2 and Id3, which control the formation of distinct CD8 cell subsets. Previous studies using reporter mice have shown that precursors of long-lived memory cells can be identified prior to the peak of the CD8 response by the expression of high levels of Id3 and intermediate levels of Id2 (Yang et al., 2011a). These Id3hi cells showed a gene expression profile similar to that of long-lived memory cells before the expression of surface memory markers, and ‘preferentially’ differentiated into KLRG1lo IL-7Rαhi MPECs upon adoptive transfer into infection-matched hosts. Loss of Id3 expression in T cells was associated with defects in long-lived memory formation, whereas Id2 deficiency resulted in higher Id3 expression and loss of KLRGhi cells. Interestingly, treatment with cytokines such as IL-12 was able to influence the expression of these
transcriptional regulators, with lower Id3 expression and higher Id2 expression being observed in the presence of IL-12. Since IL-12 signaling has previously been shown to influence CD8 cell effector-memory differentiation by regulating the temporal kinetics of mTORc1 signaling (Rao et al., 2010), these data suggest the hypothesis that sustained mTORc1 activity in TSC1-deficient CD8 cells could promote increased SLEC to MPEC frequencies by altering expression levels of Id2 and Id3. Findings that (a) Blimp1 plays a key role in repressing Id3 expression and CD8 memory formation (Ji et al., 2011), (b) C/EBPβ can promote the expression of Blimp1 in plasma cells (Pal et al., 2009), and (c) signaling via the mTOR pathway can alter the expression of C/EBPα and C/EBPβ isoforms (Calkhoven et al., 2000), collectively suggest the possibility that mTOR activity could regulate the expression of Id2 and Id3 via a pathway that involves C/EBP isoforms and Blimp1.

Dynamic changes in metabolic activity accompany the transition of a CD8 cell from its quiescent naïve state to an active effector state and back to a quiescent memory state (Prlic and Bevan, 2009; van der Windt and Pearce, 2012). Previous work has shown that defects in mitochondrial fatty acid metabolism can have deleterious effects on CD8 memory formation (Pearce et al., 2009), and that memory formation involves switching from a bio-energetically unstable effector state (with low mitochondrial mass to total cell mass ratio) to a bio-energetically stable memory state (with high mitochondrial mass to total cell ratio) (van der Windt et al., 2012). Given that a central role for the TSC1-mTOR
pathway in mediating metabolic changes and mitochondrial homeostasis has come to the fore, it would be interesting to examine whether perturbations in metabolic pathways underlie the impairment in CD8 responses caused by TSC1 deficiency.

6.5 Concluding remarks

Here, we analyzed the effects of dysregulated signaling via DAG-mediated pathways on T cell function. Constitutive activation of the IKK-NF-κB pathway was found to be detrimental to T cell function and survival. TSC1 deficiency (which causes mTORc1 hyper-activation) impaired antigen-specific CD8 responses by curtailing expansion, promoting contraction, and perturbing effector-memory differentiation. Mnk1/2 proteins were found to be dispensable for T cell development and several aspects of T cell function (including proliferation, CD4 TH differentiation and antigen-specific CD8 responses), but were important for EAE pathogenesis. Together, the findings from these studies provide deeper insight into the regulation of T cell function by DAG-mediated pathways, and may have implications for the design of immune-modulation strategies during vaccination and immunotherapy.
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Biography

Sruti Krishna was born on June 8th 1985, in Chennai, India. Though she grew up in a family of accountants and businessmen, Sruti was strangely drawn towards biology from a young age. As a high school graduate, she was one of four students selected through a country-wide competition to represent India at the International Biology Olympiad in 2002. Sruti won a bronze medal at the Olympiad in Riga, Latvia, and subsequently began her college education at Birla Institute of Technology and Science in Pilani, India. She graduated with an integrated Masters degree in Biology and a Bachelor’s degree in Computer Science in 2007, and was consistently placed among the top 10 students in her class of 900 during this period. Having developed a keen interest in the workings of the immune system by this time, Sruti was eager to pursue doctoral research in United States. She was accepted into Duke University’s Department of Immunology in the fall of 2007 and received the James B Duke fellowship, a prestigious award reserved for the brightest students entering graduate school. Sruti conducted her doctoral research in the laboratory of Dr. Xiao-Ping Zhong, publishing four first author papers including ‘Chronic activation of the kinase IKKβ impairs T cell function and survival’, ‘Mnk1 and 2 are dispensable for T cell development and activation but important for the pathogenesis of experimental autoimmune encephalomyelitis’, ‘Role of diacylglycerol kinases in T cell development and function’, and ‘Regulation of lipid signaling by diacylglycerol kinases during T cell development and function’. 