FORKHEAD TRANSCRIPTION FACTORS FOXP1 AND FOXP4 REGULATE T CELL DEVELOPMENT AND FUNCTION

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ABSTRACT

FORKHEAD TRANSCRIPTION FACTORS FOXP1 AND FOXP4 REGULATE T CELL DEVELOPMENT AND FUNCTION

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Transcription factors regulate T cell fates at every stage of development and differentiation. Members of the FoxP family of Forkhead transcription factors are essential for normal T lineage development; Foxp3 is required for regulatory T cell generation and function, and Foxp1 is necessary for the development of naïve T cells. FoxP family member Foxp4 is highly homologous to Foxp1 and has been shown to dimerize with other FoxP proteins.

In this thesis, we report the first studies of Foxp4 in T lymphocytes. Using a CD4Cre-mediated conditional knockout approach we evaluated the roles for Foxp4 regulation in the T lineage. T cell development and homeostasis are normal in the absence of Foxp4. Despite effective control of infection with *Toxoplasma gondii* or acute Lymphocytic choriomeningitis virus *in vivo*, cytokine production during antigen-specific rechallenge is reduced in the absence of Foxp4. We conclude that Foxp4 is dispensable for T cell development, but necessary for normal memory T cell recall responses to antigen in acutely or chronically infected mice.

Next we determined whether FoxP family members compensate for one another in Foxp1- or Foxp4-knockout models. We utilized a similar CD4Cre approach to delete

both Foxp1 and Foxp4 in T cells. Foxp1/4-deficient T cells exhibit abnormal thymic development and T cell receptor signaling. Loss of Foxp1/4 results in significantly reduced T cell numbers, and altered T cell effector function, reminiscent of $Foxp1^{cKO}$ T cells.

Lastly, we examined the functions of Foxp1/4 in Foxp3+ regulatory T cells (Tregs). Tregs are critical for prevention of autoimmunity and controlling immune responses during infection. While conditional deletion of either Foxp1 or Foxp4 in T cells has little effect on Tregs, combined deletion results in abnormal Treg generation. Foxp1/4-deficient Tregs exhibited significant defects in both development and homeostasis. Under competitive conditions, double-deficient Tregs are at a significant developmental disadvantage relative to wild-type competitors. Furthermore, Foxp1/4-deficient Tregs exhibit impaired cytokine-induced STAT5 phosphorylation and reduced expression of Foxp3, suggesting Foxp1/4 is required for normal Treg generation.

Together, these findings demonstrate that the FoxP family regulates multiple facets of T cell development and function, and actively contributes to the maintenance of immunological tolerance.

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CHAPTER I

Introduction

A brief introduction to the adaptive immune system

The purpose of the immune system is to protect the host from infection with pathogens, including bacteria, viruses, fungi, and parasites. There are two branches of the immune system: innate and adaptive. The innate immune system exists in different forms in all multicellular organisms, whereas the adaptive immune system evolved in vertebrates in the animal kingdom. While innate immune mechanisms provide initial protection to the host, adaptive mechanisms mount specific responses to control and clear foreign substances and pathogens.

The primary cellular components of the adaptive branch of the immune system are a type of white blood cell called lymphocytes, which are further divided into T cells and B cells. Compared to cells of the innate system, T and B lymphocytes are uniquely suited to initiate specialized soluble and cellular responses and to conduct a systemic response upon exposure to a pathogen. For instance, activated lymphocytes undergo clonal expansion in which rapid cellular division produces an exponential increase in identical daughter cells poised for further activation and effector differentiation.

Because of the potent functions of lymphocytes, activation of these cells is tightly regulated from the early stages of development. During lymphocyte maturation, somatic rearrangement of antigen receptor genes causes each cell to express a unique antigen receptor complex on the cell surface. In the T lineage, this antigen receptor is called the T cell receptor (TCR). A successfully rearranged TCR complex licenses a single specificity to each cell. A large percentage of developing lymphocytes are selectively culled, to ensure that each antigen receptor rearrangement signals appropriately and that clones with self-reactive antigen receptors are eliminated. Similar processes occur in B cells to restrict the survival of potentially autoreactive lymphocytes.

During development, lymphocytes are further committed to distinct subsets that best suit their antigen receptor specificity. Within the T lineage, cells are divided into two main T cell subsets. CD4+ TCR+ lymphocytes (CD4 T cells) respond to immunologic challenges originating outside host cells, such as bacterial infections and allergenic antigens. CD8+ TCR+ cells (CD8 T cells) are cytotoxic lymphocytes that clear intracelullar pathogens, like viruses, by killing infected host cells. Signals specific for each kind of immunologic insult activate the correct cell type and induce effector cell differentiation, proliferation, and expression of gene programs encoding lymphocyte functions.

Mature lymphocytes circulate through blood and lymphatic vessels to patrol host tissues for pathogens. To ensure inflammatory lymphocyte reactions occur only when warranted, layers of transcriptional regulation prevent non-specific initiation of effector programs. Naïve, or antigen-inexperienced, lymphocytes remain functionally silent until they encounter cognate antigen. Activation of T and B cells requires antigen receptor

ligation, which triggers a cascade of intracellular signaling and initiation of an adaptive immune response.

Successful clearance of infection requires expansion of lymphocytes expressing antigen receptor complexes specific for epitopes unique to the pathogen. Of the millions of lymphocytes circulating in the body, only a fraction will be specific to any one pathogen. Upon activation, these pathogen-specific T cells and B cells expand exponentially to produce significant numbers of identical daughter cells, equally reactive to pathogenic epitopes. B cell clones become activated and produce soluble antibodies against the pathogen, which aid in detection and killing of extracellular pathogens. T cells orchestrate responses by secreting cytokines that recruit other immune cells to infected tissues. CD8 T cells can also participate by mounting cytotoxic responses against cells harboring intracellular pathogens. This process of clonal expansion and activation affords the host access to an army of effector T or B cells to control the spread of bacterial, fungal, viral or parasitic pathogens at the time of infection, while maintaining fewer lymphocytes at steady-state.

Following pathogen clearance, there is no need for the large numbers of daughter effector cells; further, prolonged maintenance of these cells can harm the host. Effector cells undergo apoptosis during the contraction phase, which occurs after an infection is controlled. However, a population of pathogen-specific lymphocytes is retained and persists in the host to guard against repeated infection. These cells allow the host to mount more rapid and larger immune responses upon re-exposure to a specific pathogen. A cardinal feature of adaptive immunity is the provision of this immunological memory, which benefits the longevity of the host by preventing chronic illness.

The protective benefits of adaptive immunity described above are offset by the potential damage caused by dysregulated immune reactions. At every point in the life cycle of lymphocytes, both environmental and intracellular signals direct cell fate decisions to maintain a diverse repertoire of quiescent antigen-specific T and B cells in a healthy host. Unprovoked expansion and activation of T or B cell clones commonly results in non-specific immune responses and damage to host tissues. Therefore, the selective activation of pathogen-specific cells is tightly regulated. Lymphocyte specificity, controlled activation, and restrictions on self-reactive lymphocyte development are examples of regulatory mechanisms in the adaptive immune system. These precautions provide protection for vertebrate organisms against foreign pathogens, while minimizing the risk of uncontrolled cytotoxic and inflammatory responses.

Forkhead transcription factors govern cell fate decisions

In every nucleated cell in the body, cell fate decisions are regulated by both extrinsic and intrinsic mechanisms. Environmental cues such as soluble growth factors support cell metabolism, migration, and survival. Intrinsic processes include cell cycle checkpoints and manipulation of genomic DNA to express proteins necessary for proliferation and differentiation. Successful execution of gene expression requires both temporal and contextual specificity. In eukaryotic cells, regulatory mechanisms have evolved to correctly interpret extrinsic and intrinsic signals to prevent unproductive gene expression. A fundamental source of regulation is a class of proteins called transcription factors that translate extracellular signals and control the expression or silencing of gene programs.

One family of transcription factors is the forkhead family. Members of this family are expressed in all eukaryotic cells except plant cells (Jonsson and Peng, 2005). All forkhead box (Fox) proteins possess a highly conserved sequence of 80-100 amino acids, originally identified in Drosophila in 1990 (Kaufmann and Knochel, 1996). X-ray crystallography and nuclear magnetic resonance (NMR) studies of the forkhead domain determined this sequence forms three alpha helices and three beta sheets flanked with two loops or wings, giving the three-dimensional structure a butterfly-like appearance (Hannenhalli and Kaestner, 2009). This winged-helix structure facilitates binding of Fox proteins to the major groove of DNA sequences containing consensus binding sites (Myatt and Lam, 2007). Fox proteins recognize sites with the core sequence 5' (A/G)(C/T)(A/C)AA(C/T)A 3' (Carlsson and Mahlapuu, 2002; Myatt and Lam, 2007).

More than 100 genes in the human genome encode forkhead proteins that are organized into 27 subfamilies based on phylogenetic hierarchy and sequence homology (Hannenhalli and Kaestner, 2009; Uhlenhaut and Treier, 2011). In humans, expression of forkhead proteins has been implicated in the generation of most tissues in the body. Forkhead family members are required to orchestrate organogenesis throughout embryonic development, and mutations in forkhead proteins can result in embryonic lethality. Defective Fox regulation in children and adults is associated with pathological disorders and increased incidence of cancer (Myatt and Lam, 2007).

Forkhead proteins are required for normal hematopoiesis and the maintenance of both innate and adaptive immunity. In both myeloid and lymphocyte blood cell lineages, expression of forkhead proteins is essential for normal development and function (Coffer and Burgering, 2004; Myatt and Lam, 2007). Fox transcription factors directly regulate thymic organogenesis (Foxn1), lymphocyte proliferation and survival (FoxO family

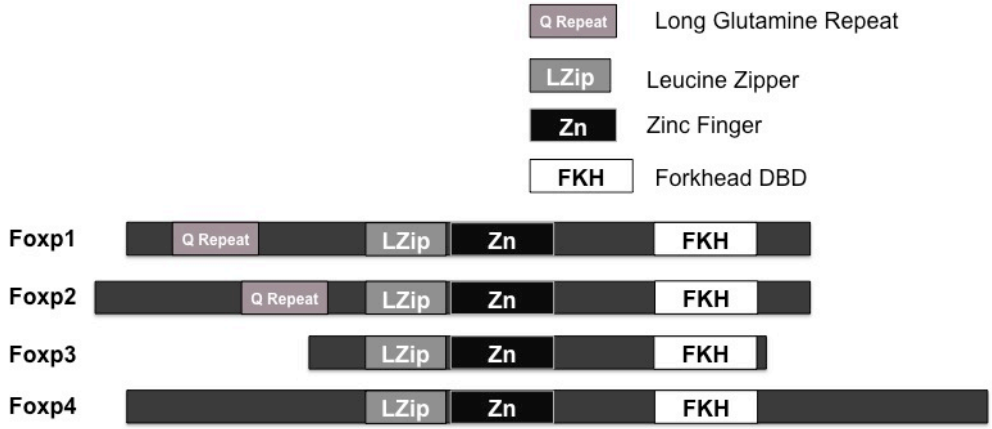
proteins), and differentiation of multiple blood cell lineages (Foxj1) (Coffer and Burgering, 2004). Therefore, forkhead proteins play a crucial role in maintaining adaptive immunity by dictating cell fate decisions in the hematopoietic lineage.

The FoxP subfamily of transcription factors

The FoxP subfamily is comprised of four proteins, Foxp1-Foxp4, which generally act as transcriptional repressors (Lopes et al., 2006; Teufel et al., 2003). FoxP family proteins share common characteristics including a conserved leucine zipper domain, a zinc finger motif, a long glutamine repeat, and the forkhead box DNA-binding domain (Teufel et al., 2003; Wang et al., 2003). The sequences of *FoxP* member genes *foxp1*, *foxp2* and *foxp4* are more closely related, while *foxp3* is least like its family members (Lu et al., 2002; Uhlenhaut and Treier, 2011). While FoxP proteins recognize the nucleotide sequence unique to forkhead DNA binding, sequence variation among the family members is thought to license each protein to recognize independent targets and assume distinct regulatory roles. For example, Foxp3 binds the nucleotide sequence 5' (G/a)TAAACA 3', while Foxp1 binds the sequence 5' AA(C/t)A(C/t)AAATA 3' (Koh et al., 2009; Wang et al., 2003). Each contains the core sequence recognized by forkhead transcription factors, but also illustrates variable specificity in gene targets of the FoxP family. Furthermore, the flanking sequences around the forkhead domain in FoxP proteins are all unique, which may affect how these proteins bind DNA targets and other proteins.

The FoxP proteins are unique from all other forkhead subfamilies because of the way these proteins dimerize to bind DNA. The leucine zipper domain is required for protein-protein dimerization and for regulation of gene targets (Chokas et al., 2010).

FoxP family members can also heterodimerize with one another via the leucine zipper motif. Mutation of the highly conserved leucine residues within the leucine zipper domain disrupts protein-protein interactions and inhibits transcriptional regulation by FoxP dimers (Li et al., 2004a; Liu et al., 2012; Zhou et al., 2009). Heterodimerization provides for regulation of specific combinations of gene sequences, depending on which proteins participate in the FoxP complexes. Ectopic overexpression studies have demonstrated that Foxp1, Foxp2, and Foxp4 can homo- and heterodimerize, and that Foxp3 can form heterodimers with Foxp1. FoxP family heterodimerization may offer another level of DNA site recognition, elegantly controlled by the expression or availability of different FoxP partners.



Schematic 1. **The *FoxP* family**. Representation of the features and relative length of the four members of the FoxP family of transcription factors.

More complexity of FoxP protein regulation is provided by interactions with other transcription factors and enzymes that regulate DNA architecture through chromatin

remodeling. Conformational changes in FoxP proteins as they bind partners may also expose different sites involved in protein-protein interactions. Competition for FoxP protein binding sites by potential partners and the stability of multi-protein complexes may affect transcription factor function. Therefore, the conserved FoxP family characteristics support complex protein interactions, and highly specific regulation of gene programs.

FoxP family transcriptional regulation in mammalian development

Each member of the FoxP family regulates diverse tissue-specific cell fate decisions throughout development (Carlsson and Mahlapuu, 2002; Kaufmann and Knochel, 1996; Shu et al., 2001). Although Foxp1, Foxp2, and Foxp4 are detected in adult tissues, expression of these transcription factors is highest during embryonic development of neural, intestinal, pulmonary, and cardiovascular tissues. Foxp3 expression is detected during development in the embryonic neuronal tissues, but is otherwise limited to immune cells, which will be discussed later.

The function and regulation of FoxP proteins have been characterized through germline deletion, overexpression, and gene mutation. Initial studies demonstrated that germline deletion of Foxp1, Foxp2, or Foxp4 blocks development, resulting in embryonic lethality (French et al., 2007; Li et al., 2004a; Wang et al., 2004). However, more recent studies describe the effects of lineage-specific FoxP family deletion, which bypass blocks in embryonic development. This growing body of literature illustrates the versatility of FoxP transcription factor functions, including regulation of cellular proliferation, differentiation, localization, and survival.

Studies of cardiopulmonary development offer excellent examples of the ways FoxP proteins regulate cell fate decisions. Foxp1 expression is highest in developing lung tissue and is required for the normal development of the distal pulmonary epithelium. Foxp4 is co-expressed with Foxp1—together, these proteins repress abnormal cell differentiation of pulmonary epithelium throughout development. Deletion of either Foxp1 or Foxp4 does not alter differentiation of epithelial cells, but Foxp1/Foxp4 double deficiency has profound effects on secretory cell development in the pulmonary airway that cause neonatal morbidity. Co-regulation of pulmonary development by Foxp1 and Foxp4 highlights the ability of these homologous Fox proteins to compensate for one another to preserve cell fate potential. In contrast, Foxp2 is also required for normal lung development, but regulates the development of proximal-distal epithelium independently of Foxp1 and Foxp4. The expression patterns and regulatory functions of Foxp1/Foxp2/Foxp4 neatly demonstrate the roles FoxP members can play independently and in combination to direct cell fate decisions.

In addition to directing tissue differentiation, FoxP family proteins regulate cell survival and proliferation. The FoxP family is essential for normal cardiac development, both in the formation of vasculature and cardiomyocyte development. Foxp1 and Foxp2 are implicated in cardio vasculogenesis. Germline deletion of Foxp1 causes defects in heart valve and ventricle formation, resulting in death by day 16.5 of embryogenesis (E16.5) (Wang et al., 2004). This defect is further characterized by dysregulated myocardium maturation, which is dependent on Foxp1 regulation. Foxp1-/- cardiomyocytes exhibit hypertrophy, increased proliferation, and failure to differentiate normally (Bai and Kerppola, 2011). These findings suggest Foxp1 is required for repression of abnormal cell division and growth in cardiac tissues as well as in the lung.

Like Foxp1 and Foxp2, Foxp4 is expressed in and required for development of cardiopulmonary tissues. Foxp4 expression in the developing embryo programs localization of cardiac tissues and proper heart development. Foxp4-deficient mice exhibit embryonic lethal cardia bifida (Li et al., 2004b). Foxp4-deficient precardiac tissues fail to migrate toward the midline of the fetus, resulting in two symmetrical hearts, bilateral to the midline (Li et al., 2004b). Defects in the developing foregut of embryonic Foxp4-/- mice also suggest Foxp4 not only directs localization, but also directs the survival of critical tissues during embryogenesis.

Defects observed in cardiopulmonary development demonstrate transcriptional regulation by Foxp1 and Foxp2 represses cellular proliferation. Dysregulation of cellular proliferation is a hallmark of cancer, and FoxP family members have been implicated in tumor repression. Oncogenic cell growth occurs with loss of Foxp1 and Foxp2 transcriptional regulation in multiple tissues (Myatt and Lam, 2007). FoxP family member Foxp3 also protects against tumor development (Heinze et al., 2011; Li et al., 2011; Wang et al., 2010). Mutations that perturb Foxp3 regulation lead to cancer development in the breast, brain and intestine (Hannenhalli and Kaestner, 2009; Myatt and Lam, 2007; Triulzi et al., 2013).

Together, these studies demonstrate how FoxP family proteins regulate multiple kinds of cell fate decisions across a variety of tissues. FoxP-mediated regulation of target genes affects development, survival, and differentiation. FoxP proteins direct these processes individually and through cooperation with other transcription factors.

FoxP transcription factors in the adaptive immune system

All members of the FoxP family are expressed in blood cells, except Foxp2. Previous studies in lymphocytes demonstrate how Foxp1 and Foxp3 regulate cell fate decisions and impact adaptive immunity. In B cells, Foxp1 initiates B lymphopoiesis, and controls antigen receptor gene recombination later in development (Hu et al., 2006). Expression of the enzyme RAG (recombination activating gene) is required for normal B cell receptor rearrangement and B cell selection (Hu et al., 2006). Deletion of Foxp1 impairs RAG expression and blocks B cell development. Humoral immunity may also rely on expression of Foxp4. Preliminary data from our work suggest Foxp4 deficiency causes abnormal B cell development. In contrast, loss of FoxP family proteins does not block T cell development. FoxP members do not regulate somatic rearrangement of TCR genes, but instead regulate cell fate decisions during differentiation and commitment to T cell subsets.

Of the FoxP proteins, the function of Foxp3 transcriptional regulation is best defined. Expression of Foxp3 in T lymphocytes is critical for the maintenance of self-tolerance (Brunkow et al., 2001; Hori et al., 2003; Ramsdell and Ziegler, 2003). Foxp3 was first identified in mouse models of autoimmunity, and later associated with IPEX (Immunodysregulation polyendocrinopathy enteropathy X-linked) syndrome (Bennett et al., 2001; Gambineri et al., 2003). Patients with IPEX inherit gene mutations in Foxp3 that cause dysregulation of immune cells, manifesting in symptoms of severe autoimmunity (Bennett et al., 2001; Gambineri et al., 2003).

The mechanism of Foxp3-dependent tolerance was traced to a subset of CD4+ T cells, called regulatory T cells, or Tregs (Hori et al., 2003; Rudensky, 2011; Sakaguchi et al., 2006). This population of CD4 T cells is required to suppress self-reactive immune

responses, and to return balance to immune cell homeostasis following infection (Khattri et al., 2001). Foxp3 is regarded as a master regulator of Treg commitment among the scores of transcription factors expressed in T cells, since it is necessary to initiate Treg-associated gene programs. In both animal models and humans, Tregs normally develop during thymic selection, but conversion of conventional CD4 T cells during immune responses also occurs upon Foxp3 induction (Chen et al., 2003; Fu et al., 2004; Zheng et al., 2004). In addition to Foxp3 expression, Tregs are identified by cell surface expression of the high affinity IL-2 receptor alpha chain (CD25), GITR (a member of the tumor necrosis factor receptor family) (Cuzzocrea et al., 2005; Gavin and Rudensky, 2003). Several groups have determined Foxp3 regulates transcription of these cell surface proteins and other gene programs required for tolerance, although the precise mechanisms of Treg suppression are not well understood.

Initial studies in Foxp1-deficient T cells determined Foxp3+ Treg differentiation does not require Foxp1 expression (Feng et al., 2010). Instead, Foxp1 is necessary for normal development of conventional T cells (Feng et al., 2010; Feng et al., 2011). Deletion of Foxp1 in the T lineage demonstrated that Foxp1 normally represses activation-induced gene programs in both CD4 and CD8 T cells. Foxp1-deficient T cells acquired an activated phenotype, including upregulation of the cell surface marker CD44 (Feng et al., 2010). Furthermore, T cells lacking Foxp1 expression exhibited effector functions, including increased proliferation and cytokine production. Experiments in which Foxp1 was temporally deleted after normal T cell development demonstrated that loss of Foxp1 immediately resulted in abnormal activation (Feng et al., 2010). Therefore, sustained Foxp1 expression is required to repress effector T cell fate and to maintain the repertoire of naïve T lymphocytes.

As observed in other cell types, Foxp1 also regulates survival and proliferation of T lymphocytes. The survival of both B and T lymphocytes is impaired without Foxp1 expression despite increased expression of the interleukin-7 receptor (IL-7R) (Feng et al., 2011). Foxp1 acts as a transcriptional repressor of the *il7r* gene, which encodes the alpha chain of the Interleukin-7 receptor (IL-7R) (Feng et al., 2010; Hu et al., 2006). Foxp1 and forkhead factor Foxo1 compete for a forkhead binding site in the *il7r* promoter. In the absence of Foxp1, T cells express increased levels of IL-7R, and CD8 T cells proliferate abnormally to IL-7 stimulation (Feng et al., 2011). However, despite increased sensitivity to anti-apoptotic IL-7 signals, survival is impaired. Loss of Foxp1 causes T lymphopenia, which becomes more severe with age. Therefore, Foxp1 regulation maintains the quality and quantity of the T lymphocyte repertoire.

Preliminary work has demonstrated Foxp4 expression in lymphoid tissues (Teufel et al., 2003). However, the function of Foxp4 in lymphocytes had not been described. Like all FoxP family members, Foxp4 forms homodimers, and can heterodimerize with Foxp1 and Foxp2 in non-lymphoid cells (Li et al., 2004a). Examples of Foxp4 regulation in other cell types indicate this transcription factor also directs cell differentiation and survival. Yet it was unclear what processes Foxp4 regulates in adaptive immune cells, and whether Foxp4 expression is required to the same degree as its FoxP family members for normal T cell development.

FoxP protein Interactions in T cells

FoxP transcription factors regulate gene expression by participating in multi-protein complexes. Multiple domains, including the conserved FoxP forkhead domain, mediate interactions between FoxP proteins and a variety of protein partners, leading to

functional alterations in gene transcription. Foxp3 binding partners include other transcription factors: Foxp1, NFAT, NFκB, Runx family members, retinoic acid-related orphan receptor (ROR). FoxP proteins also interact with chromatin remodeling proteins such as histone deacetylases (HDACs), which are proteins capable of altering DNA accessibility (Bettelli et al., 2005; Du et al., 2008; Li et al., 2006; Li et al., 2007; Ono et al., 2007; Wu et al., 2006). Direct interaction between Foxp4 and HDACs has also been reported in transfected fibroblasts, but not yet in lymphocytes (Chokas et al., 2010). The ability of FoxP proteins to work with a variety of partners ostensibly increases the variety of potential gene targets.

Interactions with different binding partners rely on conserved motifs within the FoxP protein sequence. Models of Foxp3 dimerization indicate this protein changes conformation as it binds another Foxp3 protein, other partners, or target genes. For example, homodimerization of Foxp3 exposes a number of hydrophobic amino acid residues encoded in sequences flanking the leucine zipper domain (Zhou et al., 2009). Normally, these residues would provide better stability for dimerization if aligned along the axis of protein-protein interaction. Instead these residues are interspersed around and throughout the leucine zipper domain. Such unusual placement suggests these sequences provide a scaffold for other proteins to bind to Foxp3 dimers. Furthermore, Foxp3 binds different target sequences along the major or minor groove of DNA, which also changes the availability of motifs (Myatt and Lam, 2007; Song et al., 2012; Zhou et al., 2009). FoxP platforms may be fundamental for the recruitment of transcriptional machinery or for binding of chromatin remodeling proteins. Sequence variability around conserved motifs in each FoxP member may shape protein-protein interactions, or generate unique conformational landscapes.

Structure of Thesis

This thesis begins by specifically addressing how Foxp4 regulates T cell development and function. Preliminary evidence of Foxp4 expression in lymphoid tissues suggested this FoxP protein plays a role in adaptive immunity. Requirements for related proteins Foxp1 and Foxp3 for normal immune regulation, as well as the high degree of homology with the Foxp1 gene sequence, suggested Foxp4 could participate in similar regulation of lymphocyte development. These findings raised several hypotheses that provoked investigation of Foxp4 regulation in lymphocytes.

Studies described in Chapter II of this thesis assessed the effects of Foxp4 deletion on the development and function of the T lineage. Unlike its FoxP relatives, deletion of Foxp4 does not affect T cell development. Foxp4 does not appear to cooperatively regulate Treg and conventional T cell gene programs with Foxp1 or Foxp3. Instead, our data demonstrate Foxp4 regulates memory T cell recall responses and may be important for maintaining long-term immunity.

Although our work suggested Foxp1 and Foxp4 have independent functions in T cells, we had not formally ruled out the possibility that Foxp1 also compensates for loss of Foxp4, and can assume the roles normally filled by Foxp4. In Chapter III, we expanded our studies to examine how loss of both Foxp1 and Foxp4 affected T cell development. Combined deletion of FoxP proteins provides the first characterization of Foxp1- and Foxp4- deficient T cells. Double-deficient T cells were compared to T cells in which Foxp1 or Foxp4 were deleted. Without Foxp1/4, many Foxp1-associated defects were exaggerated in both CD4 and CD8 T cells.

Deletion of both Foxp1 and Foxp4 altered the expression of the CD4 co-receptor on both CD4 and CD8 T cells. In Chapter IV, we examine the defect in CD4 expression in depth, and demonstrate cell-intrinsic dysregulation of this critical co-receptor at the transcriptional level. Furthermore, the expression patterns of CD4 were significantly dysregulated in double-deficient T cells, but not in Foxp1- or Foxp4-deficient conventional T cells or in the Treg subset of CD4 T cells that express Foxp3. These data suggest FoxP members can compensate for one another in select gene programs, and expression of at least one FoxP protein is sufficient to regulate levels of CD4.

Chapter V addresses how Foxp1, Foxp3, and Foxp4 regulate peripheral tolerance. Deletion of Foxp1 and Foxp4 revealed a critical role for FoxP family members in Foxp3+ Treg development. Defects appeared in double-deficient Treg differentiation, but not in Foxp1 or Foxp4 knockout T cells, suggesting expression of Foxp3 with at least one FoxP family member was sufficient for normal generation of regulatory T cells. Signaling pathways downstream of cytokine and TCR stimulation are dysregulated in Foxp1/4-deficient cells, resulting in abnormal Treg development, survival, and function.

Together, this work expands the model of FoxP family transcriptional regulation in T lymphocytes to include Foxp4-specific functions. Unlike Foxp1 and Foxp3, Foxp4 regulation is limited to memory T cell responses. With few exceptions, our studies indicate Foxp4 otherwise serves as a redundant regulator of Treg development under steady-state conditions. Combined deletion of Foxp1 and Foxp4 causes a unique set of developmental and survival defects, which are exacerbated under competitive conditions. Studies of double knockout T cells demonstrate some cases in which Foxp1 and Foxp4 may compensate for one another to support T cell survival and generation of regulatory T cells. This thesis concludes with discussion of these results, and

implications for Foxp4 regulation of adaptive immune cell effector and memory responses.

CHAPTER II.

Foxp4 regulation is dispensable for T cell development, but essential for memory recall responses.

Introduction

In the immune system, members of the FoxP family of transcription factors regulate gene programs important for normal development and function. FoxP family member Foxp4 regulates developmental programs in heart and lung tissues, and shares a high degree of homology with Foxp1 (Li et al., 2004b). Preliminary work has demonstrated expression of Foxp4 in lymphoid tissues (Teufel et al., 2003). However, the function and role of Foxp4 in T cells has not been described.

FoxP family members regulate gene expression by forming homo- and heterodimers (Li et al., 2007). The FoxP protein Foxp3 also interacts with other transcription factors through participation in multi-protein complexes. Multiple domains, including the conserved DNA-binding forkhead domain, mediate interactions between Foxp3 and a variety of partners (Bettelli et al., 2005; Li et al., 2006; Ono et al., 2007; Wu et al., 2006). Studies in transfected fibroblasts indicate Foxp4 forms homo- and heterodimers. In non-lymphoid cells, Foxp4 has been shown to heterodimerize with Foxp1 and Foxp2 (Li et al., 2004a). Foxp4 protein-protein interactions with members of other transcription factor families and chromatin remodeling proteins have also been

demonstrated. Direct interaction of Foxp4 with histone deacetylases (HDAC) has also been reported in transfected fibroblasts, but not in lymphocytes (Chokas et al., 2010).

This is the first study of Foxp4 expression and function in T lymphocytes. Foxp4 is expressed in immature thymocytes and in mature T lymphocyte subsets. In contrast to previously reported studies of FoxP family members, we find Foxp4-deficient thymocytes undergo normal development to become naïve, quiescent peripheral T cells. Foxp4 expression has no effect on development of Foxp3+ T regulatory cells, induction of Foxp3 in naïve CD4 T cells *in vitro,* or Treg function. T cell-specific deletion of Foxp4 does not immediately suggest functional disruption of gene programs required for adaptive immunity.

To further investigate the role of Foxp4 in T cell responses, mice lacking Foxp4 expression in the T lineage were challenged *in vivo* and assessed for normal adaptive immune responses. In this study, we utilized two model pathogens: *Toxoplasma gondii* and lymphocytic choriomeningitis virus (LCMV).

Toxoplasma gondii is an obligate intracellular protozoan parasite that can infect both humans and mice through contaminated food or water (Dubey, 2008; Dupont et al., 2012). In immunocompetent hosts, innate and adaptive responses control infection and pathogen replication, but fail to clear the parasites from the body completely. (Dubey, 2008; Johnson, 1992; Weiss and Dubey, 2009) Immune pathology declines during transition to chronic toxoplasmosis when immune responses contain *T. gondii* to tissue cysts in muscle and neural tissues. However, the parasite remains in the host in the form of dormant bradyzoites (Dupont et al., 2012; Johnson, 1992).

Previous studies have highlighted the importance of regulating T cells responses during *T. gondii* infection, as depletion of T cells in infected mice results in uncontrolled

disease in the brain and increased immune pathology (Araujo, 1992). Regulation by T helper Th1 (Th1) and Tregs are important to orchestrate immune cell responses (Hall et al., 2012; Stumhofer et al., 2006). Loss of either pro-inflammatory or suppressive signals causes mice to succumb to infection, indicating that a balance of lymphocyte function is required to maintain effective immune control over parasite replication.

LCMV is a rodent-borne arenavirus, originally isolated after an outbreak in St. Louis, Missouri more than 80 years ago . Models of classic antiviral immune responses and immunological memory have emerged from extensive studies of LCMV infection. Additionally, an impressive array of reagents has been developed to examine both acute and chronic LCMV-specific responses, enabling researchers to observe and study lymphocyte activation, expansion, contraction and memory T cell development. In this study, we utilized the Armstrong strain of LCMV, which is cleared 7-8 days post infection; a population LCMV-specific memory cells capable of mounting recall responses develop and reach stasis approximately 30 days after infection.

Infection of Foxp4-deficient mice demonstrated Foxp4 was not required to control infection with either *T. gondii* or LCMV. However, the capacity to produce effector cytokines was reduced in peripheral T cells generated by either pathogen. Rechallenge with cognate antigen revealed reduced memory recall responses in the absence of Foxp4 regulation. Therefore, Foxp4 appears to be unnecessary for T cell development, but necessary for the maintenance of long-term host immunity.

Results

Foxp4 is expressed in T lymphocytes

foxp4 transcript expression was detected by northern blot in multiple tissues, including the spleen (Teufel et al., 2003). We extended these studies to assess Foxp4 expression in developing thymi. In collaboration with Drs. Ed Morrisey and Shanru Li, protein was detected by immunohistochemistry in thymic tissues of fetal E18.5 day-old mice. Immunostaining of transverse sections with Foxp4 polysera revealed Foxp4 protein expression in the thymus, particularly in cortical regions (Figure 2.1A, right panel). Foxp4 expression in the thymus was compared to other FoxP family members. Staining with Foxp1-specific polysera of embryonic thymi appeared a darker brown color, denoting Foxp1 protein expression (Figure 2.1A, left panel). In contrast, Foxp2 staining of the thymi exhibited reduced expression, which correlates with previous reports that Foxp2 is not expressed in the hematopoietic lineage (Figure 2.1A center panel) (Shu et al., 2001). These data are consistent with published findings, and demonstrate Foxp4 protein expression in the thymus.

Next, we examined Foxp4 expression in developing T lymphocyte populations using highly purified T lymphocyte subsets isolated from wild-type thymi (Figure 2.1B). Steady-state Foxp4 mRNA levels were assessed using a TaqMan-based real-time polymerase chain reaction (PCR) PCR assay. Thymocytes were sorted from 8-week-old C57Bl/6 wild-type mice using flow cytometry, based on expression of CD4 and CD8. Lineage markers were used to exclude CD11c, CD11b, and F4/80 expressing non-T cells. $CD4^{-}CD8^{-}$ double negative (DN) thymocytes were further separated based on CD25 and c-Kit expression. In all thymocytes populations, Foxp4 mRNA was expressed at detectable levels, with the highest expression found in the $CD4^{-}CD8^{-}CD25^{+}$c-Kit^{-}

(DN3) subset. Following β-selection, we observed that Foxp4 expression declined but remained constitutively expressed in both CD4 and CD8 single positive T cell subsets.

Previously, studies have demonstrated Foxp4 expression in peripheral lymphoid tissues. However, these studies did not determine the specific cell types expressing Foxp4. Real-time PCR was used to determine relative Foxp4 levels in various sorted T cell populations. Foxp4 mRNA was expressed in both peripheral mature CD4 and CD8 T cells (Figure 2.1C). Based on CD44 and CD62L expression, CD4 and CD8 T cells can be further divided into $CD44^{lo}CD62L^{hi}$ naïve, $CD44^{hi}CD62L^{lo}$ effector memory (EM), and $CD44^{hi}CD62L^{hi}$ central memory (CM) populations. While Foxp4 was expressed in sorted sub-populations, no statistically significant differences in Foxp4 levels were detected in naive, EM, or CM T cells of both the CD4 and CD8 T lineage. Therefore, Foxp4 expression is dynamically regulated throughout thymic development but decreased in mature cells. In peripheral T cells, Foxp4 is constitutively expressed.

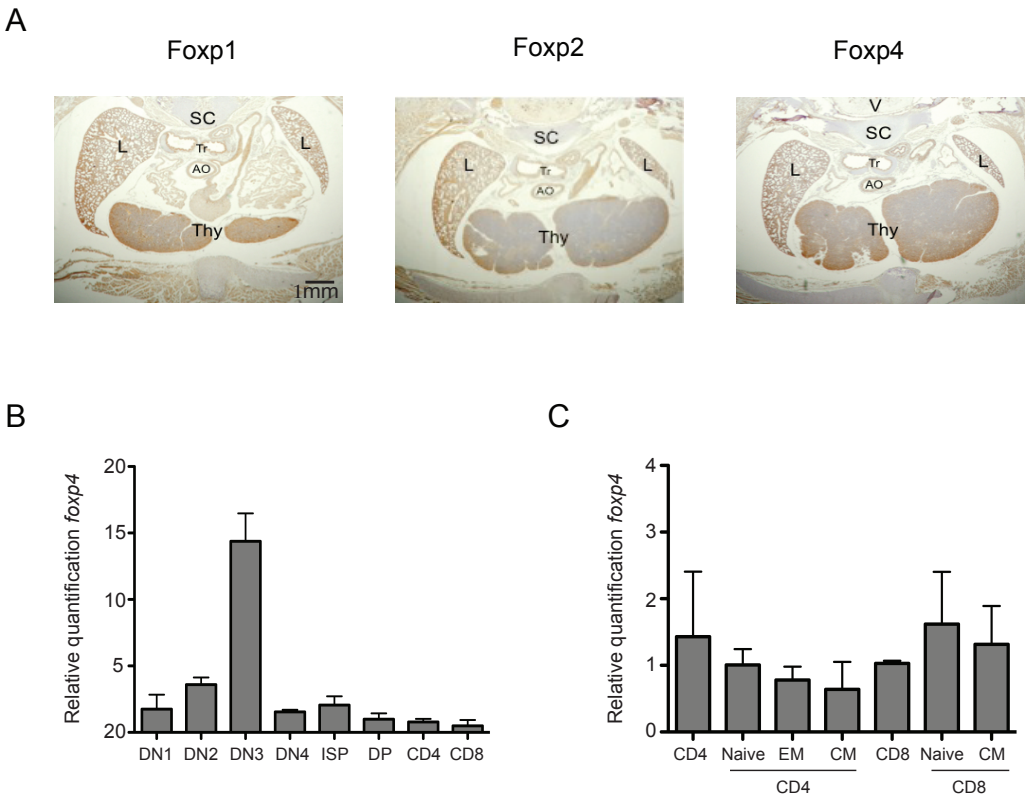


Figure 2.1. Foxp4 is expressed in lymphoid tissues and T cell subsets. A) Expression of Foxp1, Foxp2 and Foxp4 in wild-type embryonic thymi by immunostaining with FoxP member antisera. 10µm cryosections of E18.5 mice were mounted and fixed with formaldehyde and H&E stained. Frames are magnified 40X. Representative of two independent experiments. L=lung, SC=spinal cord, AO=aortic arch, Tr=trachea, Thy=thymus. B) Thymocytes from C57BL/6 mice were stained with a lineage cocktail, CD4, CD8, CD44 and CD25, and HSA. Stained cells were collected using flow cytometric cell sorting. Steady state mRNA levels of Foxp4 and ß-actin were determined by TaqMan PCR. Foxp4 levels were normalized to ß-actin. Double positive thymocytes were given a relative value of 1. All isolated populations were lineage negative. DN1: CD4-CD8-CD25-CD44+; DN2: CD4-CD8-CD25+CD44+; DN3:CD4-CD8-CD25+CD44-; DN4: CD4-CD8-CD25-CD44-; ISP: CD4-CD8+HSA+CD5-; DP: CD4+CD8+; CD4: CD4+CD8-; CD8: CD4-CD8+HSA-CD5+. C) C57BL/6 splenocytes were stained with CD4, CD8, CD44, CD62L and CD25 and collected by flow cytometry. Relative levels of Foxp4 were determined as in (A). Total CD8+ were given a normalized value of 1. Naive: $CD44^{lo}CD62L^{hi}$, EM: $CD44^{hi}CD62L^{lo}$; CM: $CD44^{hi}CD62L^{hi}$. Error bars represent standard deviation among triplicate wells from TacMan assay. Data is representative of two independent experiments.

Foxp4 can be efficiently deleted in thymocytes

To investigate the role of Foxp4 in T lymphocyte development and function, we sought to generate Foxp4-deficient thymocytes. Germline deletion of Foxp4 results in embryonic lethality at day E12.5 due to cardiac defects (Li et al., 2004b), necessitating a conditional deletion approach. To study the effect of Foxp4 deletion specifically in the T lineage, we used mice with LoxP/LoxP sites flanking exons 12-13 of *foxp4* that encode the forkhead domain (Figure 2.1A). $Foxp4^{FLOX}$ mice were then crossed with CD4Cre mice in which Cre recombinase expression is under transcriptional control of the *cd4* promoter (Lee et al., 2001). Under *cd4* promoter regulation, Cre is first expressed at the transition from the double negative to double positive stage in the thymus. Gene deletion is observed in DP, mature SP thymocytes, and peripheral T cells. To increase likelihood of generating Foxp4 null cells, we used mice carrying one germline null allele and one floxed allele ($Foxp4^{Flox/null}$ $CD4Cre^{+}$, henceforth referred to as cKO). Throughout this study, cKO animals were compared to conditional heterozygous ($Foxp4^{Flox/+}CD4Cre^{+}$, henceforth referred to as cHET) littermates that expressed one wild-type allele and one floxed allele. Thus, cHET T cells express one allele of Foxp4, while cKO mice lack both genomic Foxp4 alleles following Cre-mediated deletion.

To confirm efficient deletion of Foxp4, we first isolated total thymocytes (>95% DP and SP cells) and evaluated deletion in genomic DNA using a PCR-based assay. Deletion of Foxp4 was verified by detection of a wild-type Foxp4 PCR product at 412 kb, a $Foxp4^{FLOX}$ product including the inserted flanking LoxP sites at 508 kb, or the deleted $Foxp4^{\Delta}$ product at 475kb in total thymocytes and purified peripheral CD4 T cells from cKO mice (Figure 2.2A and data not shown). In RNA isolated from Foxp4 cKO purified

splenic CD4 T cells the level of Foxp4 message was undetectable, as determined by real-time PCR (Figure 2.2B). Taken together, CD4Cre-mediated deletion results in Foxp4 deficient thymocytes and peripheral T cells, and allows for further investigation of the role of Foxp4 in the T lineage.

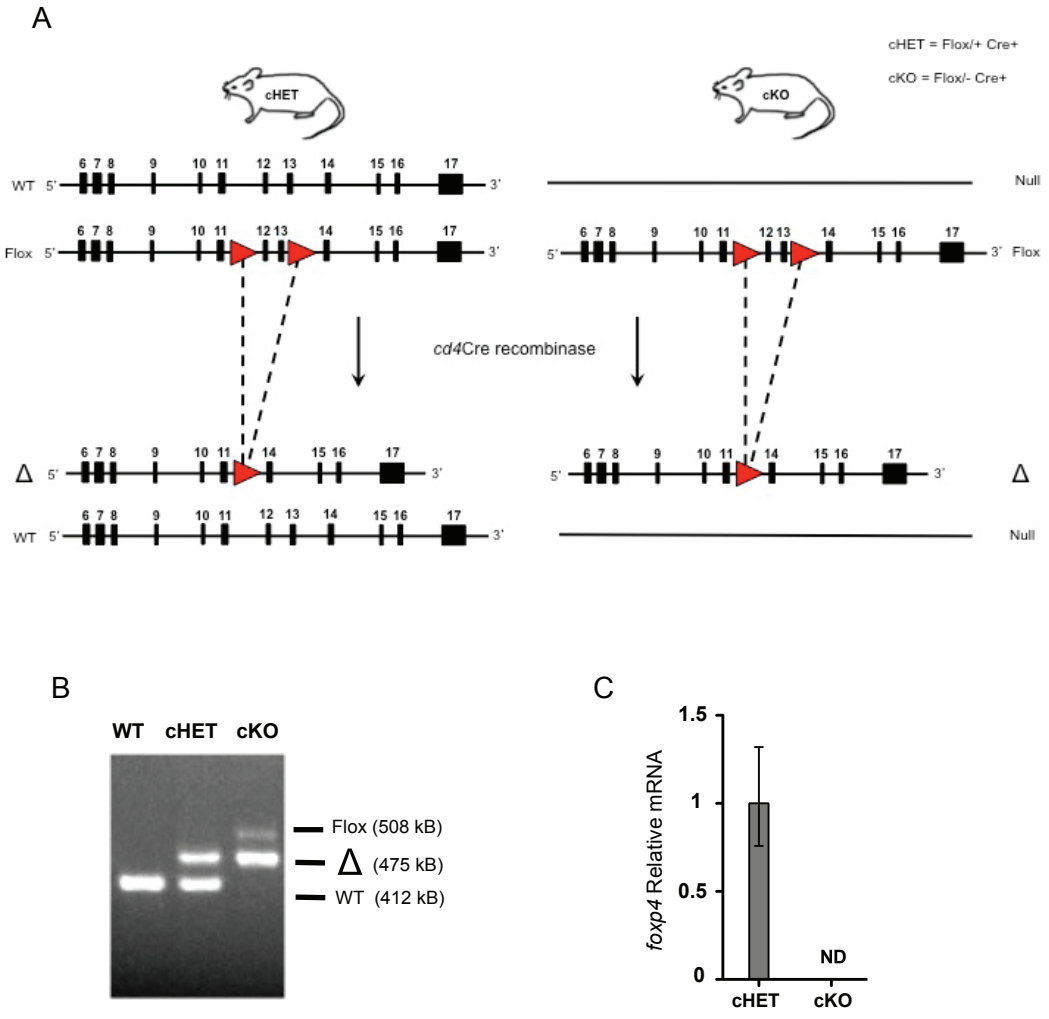


Figure 2.2. Generation of Foxp4 cKO mice. A) Schematic of conditional deletion of Foxp4 in cHET and cKO mice. LoxP sites (red triangles) were cloned into genomic DNA flanking exons 12 and 13 sequences, to generate the Foxp4^{FLOX} mutant allele. cHET mice express one Foxp4^{FLOX} allele and one wild-type Foxp4 allele. cKO mice were bred to express one Foxp4^{FLOX} allele and a Foxp4 mutant allele. Cre recombinase under the

cd4 promoter results in excised DNA between LoxP sites. B) DNA isolated from cHET and cKO thymocytes was amplified using primers to detect wild-type, floxed, and deleted allelic sequences. Band sizes and identities are indicated. Representative of ten experiments. C) RNA isolated from WT (C57BL/6) and cKO thymocytes was assessed for Foxp4 by real time PCR. ND=not detectable. Representative of three experiments.

Foxp4 is not required for T cell development and peripheral homeostasis

To determine if thymocyte development was normal in the absence of Foxp4, the phenotype and cellularity of thymic populations from cHET and cKO animals were compared. From initial observation, the size and cellularity of Foxp4 cKO thymi was comparable to cHET and Cre control thymi (Figure 2.3A, and data not shown). Throughout these studies, there were no changes observed between cHET and Cre control mice, suggesting loss of one allele of Foxp4 had no impact on T cell development. The relative frequency of DP, and SP populations, and absolute numbers were similar between both cohorts of mice (Figure 2.3B, C). ISP, DP and SP populations were stained for phenotypic expression of markers of development (Figure 2.3D). Loss of Foxp4 resulted in comparable expression of CD5 or the TCRβ chain between cHET and cKO mice. CD44 levels on Foxp4 cKO cells were also evaluated, as Foxp1 conditionally deficient CD4SP and CD8SP T cells acquire a $CD44^{hi}$ phenotype. In Foxp4 cKO mice, however, CD4SP and CD8SP cells maintain low levels of CD44 expression, providing the first evidence that Foxp1 and Foxp4 have non-overlapping roles in thymocyte development. Furthermore, these data suggest that deletion of Foxp4 during transition from DN to DP has no effect on the development of single positive thymocytes.

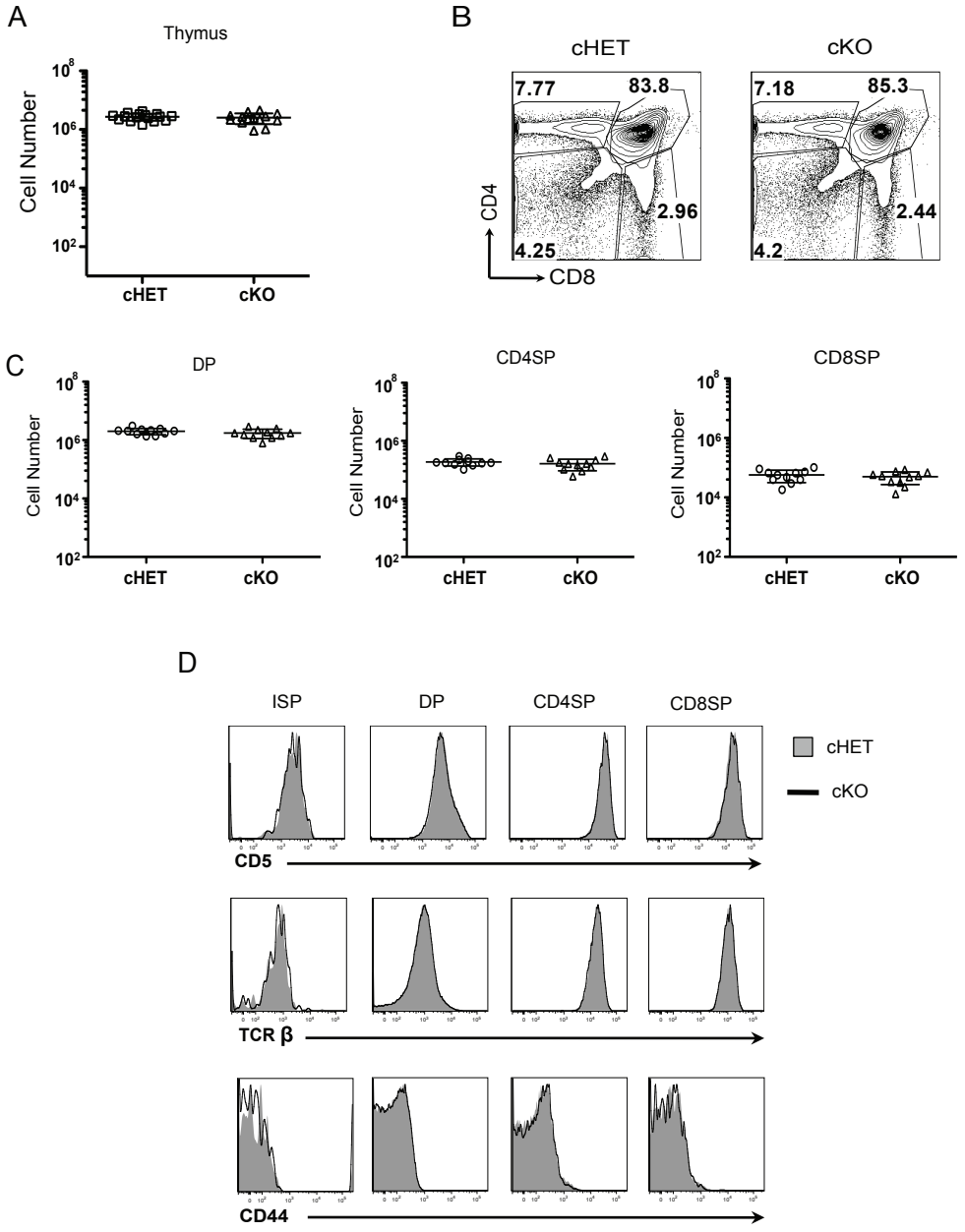


Figure 2.3. Deletion of Foxp4 at the DP stage does not alter thymocyte development. A) Total cellularity was assessed in both cHET and cKO thymi from four-week-old littermates. Each point represents a single mouse. Mean and standard deviation are indicated. B) Thymocytes were stained for CD4 and CD8 and analyzed by polychromatic flow. Contour plots shown are previously gated on live, singlet-gated cells, negative for myeloid lineage markers (CD11b, CD11C, CD19, B220). Gated frequencies are indicated. Representative of 12 cHET and 12 cKO mice. C) Absolute numbers of thymocyte populations were determined. Each point represents an individual mouse. D) Thymocytes were stained with CD4, CD8, CD5 and HSA. Cells are gated on

populations as indicated at the top of each column and assessed for CD5, TCRβ, and CD44 expression. Solid histograms are from cHET and bold lines are from cKO mice. Representative of 12 cHET and 12 cKO mice.

Because thymocytes appear to develop normally, we were able to assess the effect of Foxp4 deficiency in peripheral T cell populations. Splenic cellularity in cKO mice was similar to that seen in cHET mice (Figure 2.4A, left panel). Similarly, the frequency and absolute number of cKO CD4 and CD8 T cells was comparable to controls (Figure 2.4A, center and right panels, Figure 2.4B). As observed in developing Foxp4 cKO thymocytes, the majority of Foxp4 cKO splenic T cells remain phenotypically naïve, expressing low levels of CD44 and high levels of CD62L (Figure 2.4B). Similar frequencies of $CD44^{hi}$ endogenous memory T cells were present in Foxp4 cHET and cKO mice. This is in contrast to Foxp1 cKO T cells, which are uniformly $CD44^{hi}$. We assessed other markers of activation for any indication of aberrant T cell development, but we found comparable expression of CD69 and the TCRβ chain on splenic CD4 and CD8 T cells (Figure 2.4C, D). Similarly, Foxp4 deletion does not affect levels of the IL-2 or IL-7 receptor on either CD4 or CD8 T cells (Figure 2.4E), suggesting Foxp4 does not regulate mechanisms of T cell commitment or homeostasis. Foxp4 cKO mice were observed for more than 12 months, and did not develop any sign of abnormal immune activation or overt pathology (data not shown). Thus, Foxp4 deficiency does not result in abnormal activation or lead to overt autoimmune pathology, allowing for interpretation of function studies.

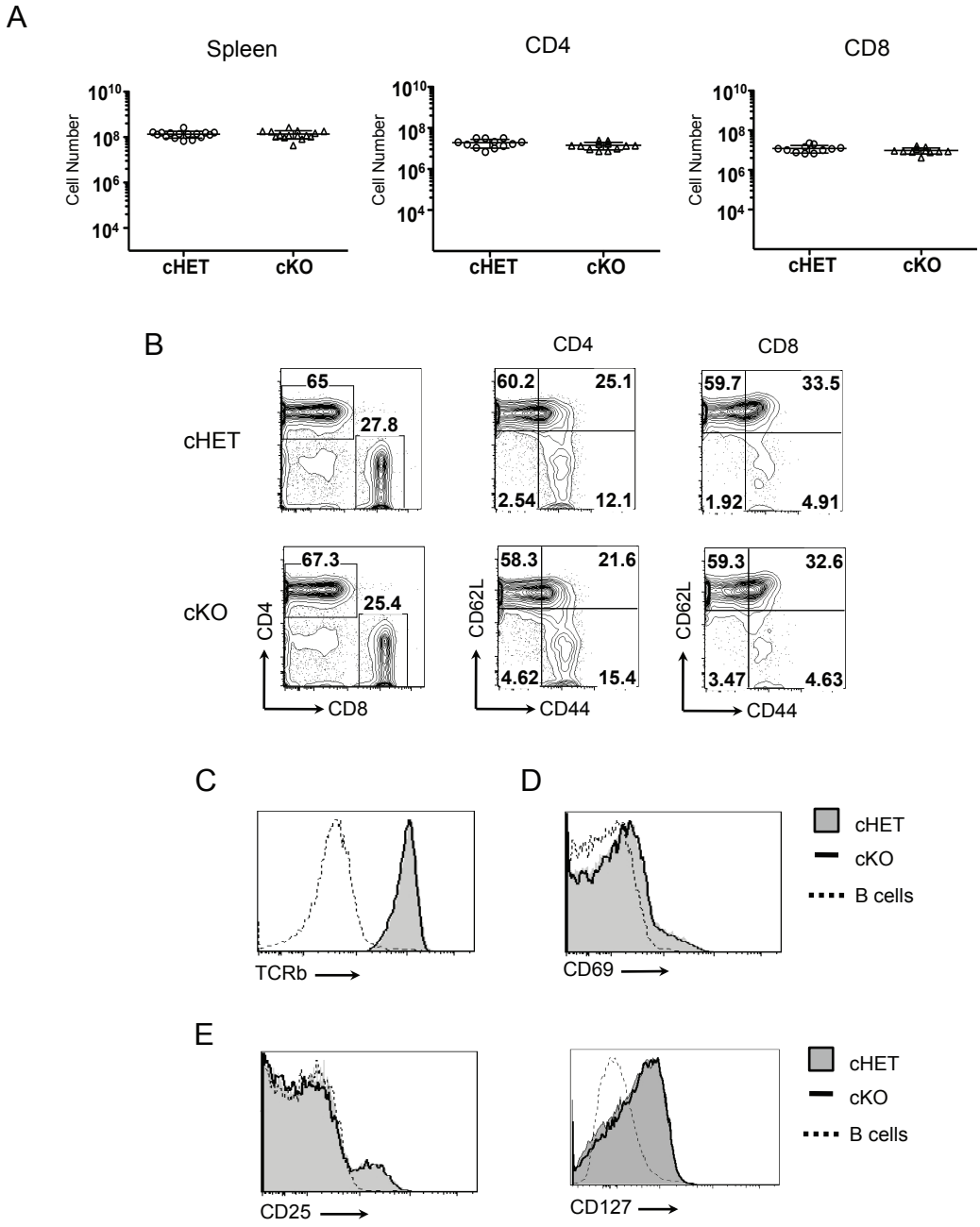


Figure 2.4. Foxp4 is not required for the generation of peripheral CD4 and CD8 T cells. A) Absolute numbers from spleens from four-week-old cHET and cKO mice, including total spleen cellularity (top panel), numbers of $CD4^+$ T cells (middle panel) and CD8+ T cells (bottom panel). B-E) Splenocytes cells from 6-8 week-old cHET and cKO mice were stained for expression of CD4, CD8 and CD44 and CD62L, or CD25, CD69, TCRβ, and IL-7 receptor (CD127) for immunophenotyping by polychromatic flow cytometry on LSRII. Histograms are gated on live, singlet $CD4^+$ T cells. Representative of 4 independent experiments.

Normal Regulatory T cell generation in Foxp4 cKO CD4 T cells.

Interaction of FoxP family members in homo- and heterodimers suggested Foxp3+ regulatory T cells (Tregs) could be affected by Foxp4 deletion. Foxp4-deficient mice were assessed for normal $Foxp3^{+}$ regulatory T cell development (Treg) (Figure 2.5). The frequency of CD4 T cells expressing the high affinity interleukin-2 receptor alpha chain (CD25) and Foxp3 was similar between cHET and cKO mice (Figure 2.5A, and data not shown). There was no difference in the total cell number of this population, or in expression of Foxp3 protein, as indicated by the mean fluorescence intensity of Foxp3 staining (Figure 2.5A-B).

Induced regulatory T cells (iTreg) develop when naïve CD4 cells are activated in the presence of exogenous IL-2 and transforming growth factor β (TGFβ) *in vitro*. To assess whether Foxp4 is necessary for the induction of Foxp3 expression in mature CD4 T cells, sorted naïve $CD44^{low}$ CD4 cHET or cKO T cells were cultured *in vitro* with TGFβ and IL-2. After four days under polarizing conditions with or without TGFβ, cells were assessed for Foxp3 and CD25 co-expression (Figure 2.5C). In the presence of TGFβ and IL-2, similar frequencies of cHET and cKO CD4 cells acquire a regulatory T cell phenotype, and Foxp3 induction is normal. Culture of Foxp4 cKO CD4 cells with IL-2 but not TGFβ did not result in Foxp3 expression (Figure 2.5C, left panels). Thus, Foxp4 is not necessary for the development of nTreg or generation of iTreg cells.

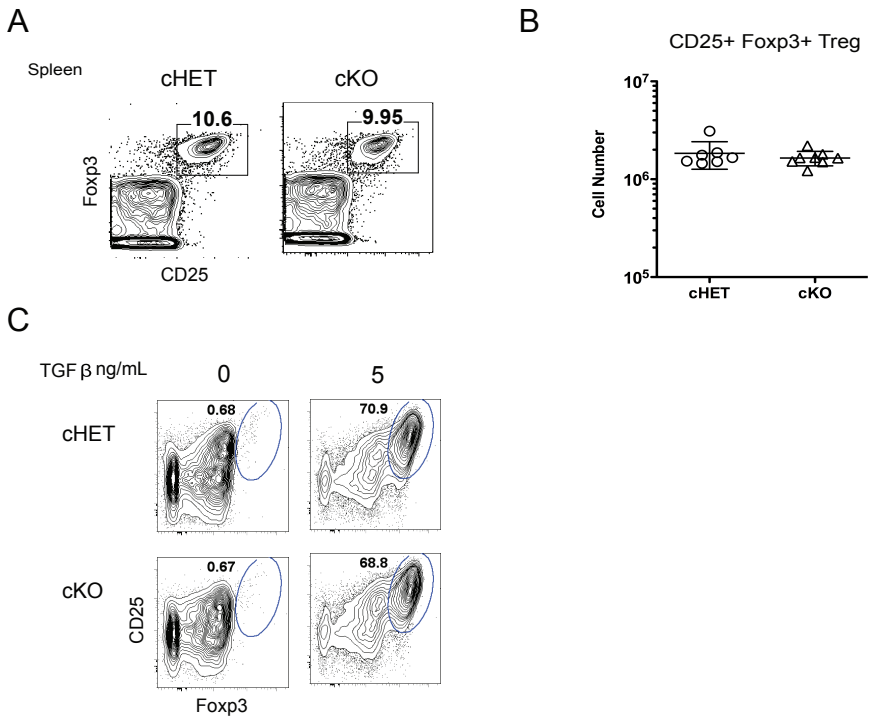


Figure 2.5. Foxp3+ Regulatory T cell development does not require Foxp4 expression. A) Splenic Foxp3$^+$ nTreg cells were identified by flow cytometry based on expression of CD4, CD25 and intracellular staining for Foxp3 protein. Gated frequencies are indicated. Representative of 7 cHET and 7 cKO mice. B) Frequencies of Treg were calculated using the splenic cellularity and relative frequency. Each point represents an individual mouse. Mean and standard deviation are indicated. C) Naïve CD4 T cells from cHET or cKO mice were polarized to induce iTreg differentiation for four days *in vitro* in the presence of anti-CD3 and IL-2 with (left) or without (right) TGFβ. Wells were harvested and cells were assessed for expression of CD25 and Foxp3. Plots are gated on live, CD4$^+$ cells. Representative of 4 experiments.

Foxp4 is not necessary for T cell proliferation or activation.

We next assessed how Foxp4 levels are regulated in response to T cell receptor stimulation and activation. CD4 T cells from wild-type C57BL/6 mice were stimulated with anti-CD3 and anti-CD28, and harvested 24 or 48 hours later. *foxp4* transcript levels were measured using real-time PCR. *foxp4* message is increased three-fold 24 hours

after TCR stimulation and returns to pre-activation levels by 48 hours (Figure 2.6A). The change in Foxp4 expression in response to TCR and CD28 costimulation suggests that Foxp4 regulation may be involved in transcriptional programs of activation.

To determine whether Foxp4 upregulation is necessary for normal T cell activation, Foxp4 cKO T cells were stimulated *in vitro*. T cell stimulation induces activation of several fundamental signaling pathways, resulting in upregulation of cell surface proteins associated with T cell activation and proliferation. T cell proliferation was assessed by stimulating CFSE-labeled T cells *in vitro* with anti-CD3 and anti-CD28. Foxp4 cKO CD4 and CD8 T cells dilute CFSE comparably to cHET T cells (Figure 2.6B and data not shown). Proliferation of cKO cells was equivalent to cHET cells regardless of the concentration of anti-CD3 used. Activated cKO T cells were stained for expression of cell surface effector markers typically induced during effector responses. Overnight stimulation of CD4 and CD8 T cells resulted in equivalent induction of CD69 and decreased levels of IL-7R (Figure 2.6C), suggesting Foxp4 does not regulate Foxp1 targets, IL-7R. Similarly, splenocytes stimulated for four days exhibited increased CD44 expression and concomitant downregulation of CD62L expression similar to control cHET cells (Figure 2.6D). Together, these data suggest that the loss of Foxp4 does not alter the proliferative response or the profile of cell surface receptors that accompanies T cell activation.

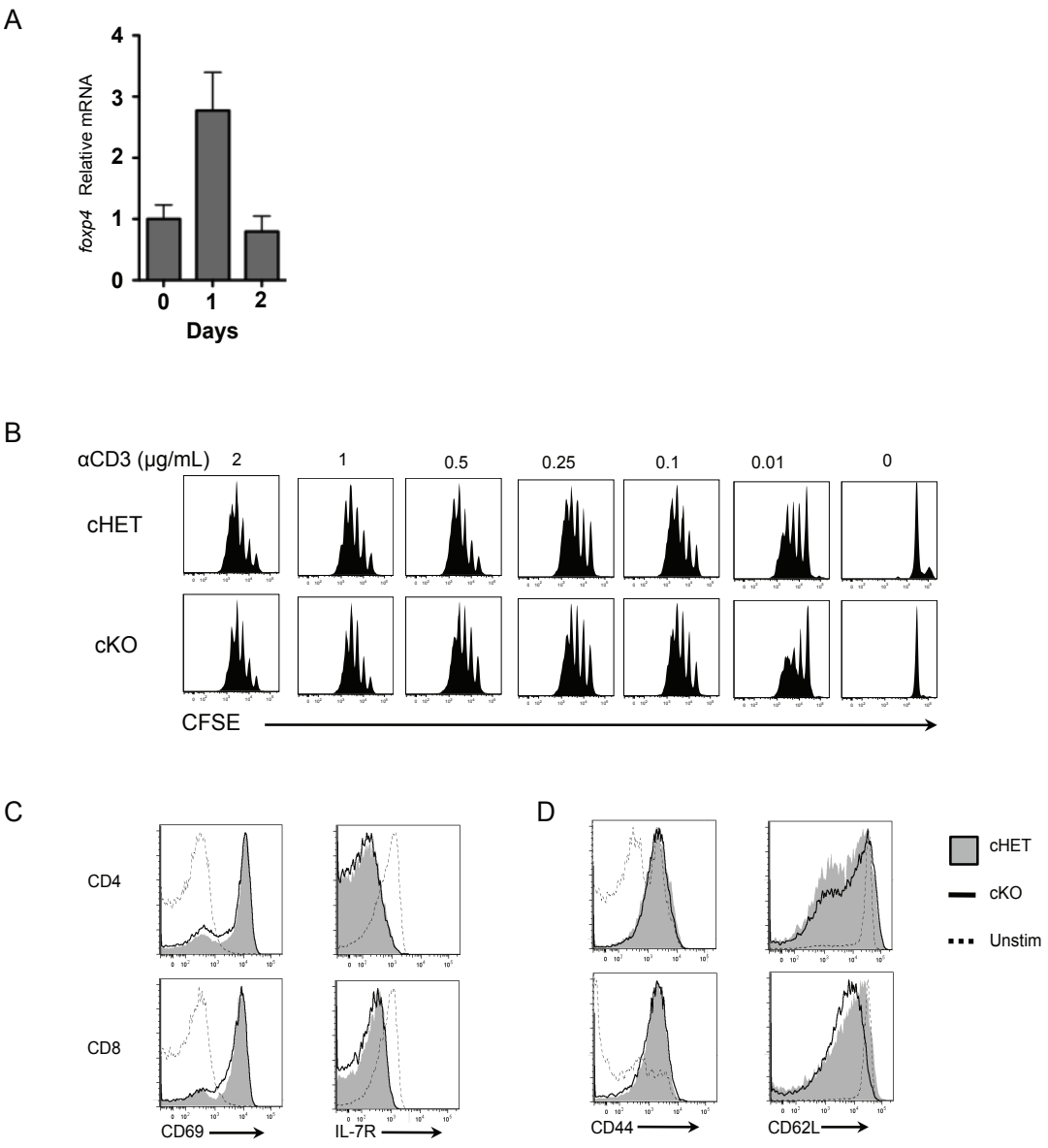


Figure 2.6. T cell activation induces normal proliferation and effector T cell differentiation in the absence of Foxp4. A) C57BL/6 $CD4^+$ T cells were isolated and plated in wells coated with 1μg/mL anti-CD3 and 5μg/mL soluble anti-CD28. Wells were harvested on the indicated days. RNA was isolated and cDNA was generated as described in Materials and Methods. Foxp4 relative to β-actin transcript levels were normalized to day 0. Representative of 2 experiments. B) $CD4^+$ T cells were labeled with CFSE, and cultured 4 days with indicated concentrations of aCD3 and soluble anti-CD28. Dilution of CFSE was assessed on day 4. Representative of 4 cHET and cKO mice. C) CD4 T cells from cHET and cKO mice were stimulated overnight in wells coated with anti-CD3 plus soluble anti-CD28. Harvested cells were stained for CD69 and IL-7R. Histograms are gated on CD4 or CD8 $TCR\beta^+$ cells. Representative of 2 experiments. D) cHET and cKO $CD4^+$ T cells were stained for expression of CD44 and CD62L after 4 days in culture with anti-CD3 and anti-CD28. Representative of 4 cHET and 5 cKO mice.

Foxp4 does not regulate T helper cell commitment

Effector CD4 T cell differentiation induces commitment to a T helper subset and specific regulation of cytokine production. Preliminary experiments demonstrated that CD4 and CD8 cKO T cells were capable of producing interferon gamma (IFNγ), tumor necrosis factor (TNFα), and IL-2 during a short restimulation immediately *ex vivo* (data not shown). To determine how Foxp4 might regulate effector T cell development, we exploited the T helper cell model of *in vitro* polarization. Naïve CD4 T cells can commit to several different lineages, depending on environmental signals during activation. To assess whether Foxp4 deficiency affects T helper cell commitment, CD4 T cells were enriched from spleens of cHET and cKO mice by MACS column and stimulated *in vitro* in cytokine cocktails. Committed T helper cells produce lineage-specific cytokines upon restimulation. To demonstrate T helper lineage commitment, cultured cells were restimulated with phorbol myristate acetate and ionomycin. Cytokine export was inhibited with addition of monensin. After 4 hours, cells were stained intracellularly for production of IL-2, IFNγ, and interleukin-17 (IL-17), indicating Th0, Th1, and Th17 differentiation, respectively.

cHET and cKO T cells were able to commit to specific T helper lineages and produce prototypical cytokines in response to restimulation (Figure 2.7A). Both cHET and cKO CD4 T cells cultured with IL-12 under Th1 lineage conditions produced IFNγ, but not the Th17 lineage-specific cytokine IL-17. Conversely, T cells cultured with TGFβ and interleukin-6 (IL-6) to induce Th17 commitment produced IL-17, but not IFNγ. T cell activation in the presence of IL-2 resulted in expected production of IL-2 and IFNγ. Specific T helper lineage cytokine production demonstrates intact regulation of T helper gene programs in Foxp4-deficient CD4 T cells.

To investigate Foxp4 expression in T helper lineages, expression of Foxp4 was determined in wild-type polarized CD4 T cells. Wild-type cells were cultured *in vitro* to induce Th0, Th1, or Th17 polarization. After four days, wells were harvested to assess *foxp4* transcript by real-time PCR. Foxp4 expression is increased in Th17, relative to starting levels in naïve T cells, and to other T helper subsets (Figure 2.7C). Together, these data suggest Foxp4 expression is induced after T cell activation and remains elevated under Th17 conditions,

Foxp4 cKO T cells exhibited a trend toward increased IL-17 production *in vitro*. As Foxp4 is thought to be a transcriptional repressor (Li et al., 2004a), this led us to investigate additional anomalous cytokine expression by polarized cKO T cells. Interestingly, a higher frequency of Th17 skewed Foxp4 cKO cells produced IL-2, relative to identically stimulated cHET cells (Figure 2.7A, bottom left panel, and 2.7B). To quantitate the relative percentages of IL-17 or IL-2 producing Th17 cells over a number of experiments, the percentages of IL-2+ or IL-17+ cKO cells were normalized to matched cHET controls (Figure 2.7B). Foxp4 cKO Th17 cells averaged increased frequencies of IL-2 or IL-17 producers, but not IFNγ. Maintenance of IFNγ repression indicates normal commitment to Th17 differentiation in cKO T cells. Instead, increased IL-2 and IL-17 production suggested Foxp4 may be necessary for repressing excessive cytokine responses in CD4 Th17 cells.

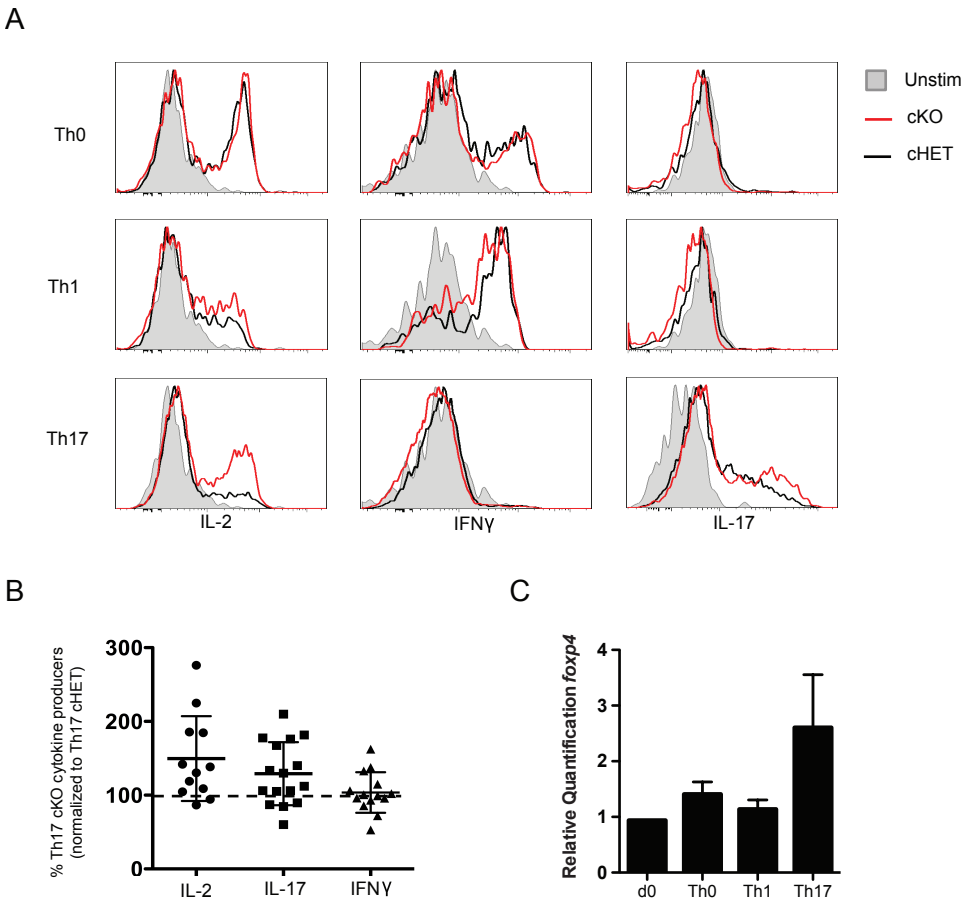


Figure 2.7. Foxp4 is not required for T helper cell differentiation. A) CD4 T cells were enriched from cHET or cKO spleens and cultured in wells coated 1µg/mL anti-CD3 and 5µg/mL soluble anti-CD28. Antibody cocktails were prepared as described in Materials and Methods and added to induce T helper commitment. After 4 days wells were restimulated for 4 hours with PMA and Ionomycin. Cytokine export was inhibited by Brefeldin A. Wells were harvested and cells were stained for expression of CD4, TCRβ, CD44 and intracellularly for cytokine production of prototypic T helper cytokines IFNγ, IL-2, and IL-17. Representative plots of eight experiments. B) CD4+ CD44hi T cells harvested from Th17 polarized wells and stained for cytokine production. Compiled frequencies of live CD4+ CD44hi T cells producing the indicated cytokines were normalized to frequencies of control cells in each independent experiment. Compiled from eight experiments. C) C57BL/6 $CD4^+$ T cells were isolated and plated in wells coated with 1µg/mL anti-CD3 and 5µg/mL soluble anti-CD28. Exogenous IL-2 was added to cultures to induce Th0 polarization. Recombinant IL-12 was added to Th1 wells, and TGFβ and IL-6 induced Th17 polarization. Wells were harvested after four days. RNA was isolated and cDNA was generated as described in Materials and Methods. Foxp4 relative to β-actin transcript levels were normalized to day 0. Representative of 2 experiments.

Foxp4-deficient T cells control chronic infection

We determined Foxp4 expression was not necessary for T cell development, and *in vitro* T helper commitment was not greatly affected. It was unclear how T cell-specific Foxp4 deficiency might alter the physiologic immune response to pathogenic infection. T helper polarization of Foxp4 cKO T cells *in vitro* prompted us to investigate how loss of Foxp4 regulation in activated cells affected the course of host immunity. *Toxoplasma gondii* infection elicits a systemic Th1 type immune response but establishes a chronic infection approximately 30-40 days after infection.

To assess the response to chronic toxoplasmosis, Me49 *T. gondii* cysts were injected intraperitoneally to cHET and cKO cohorts, and $Foxp4^{FLOX}$ Cre-negative littermates. Immune dysregulation during onset of toxoplasmosis resulted in weight loss and increased morbidity in other systems (Stumhofer et al., 2006). No differences were observed between Foxp4 cHET and Cre control mice, suggesting Foxp4 haplo-insufficiency had no effect on pathogen control. Body weight of experimental cohorts was monitored throughout the course of infection. Weights were then normalized as a percentage relative to starting weight, and averaged across cohorts (Figure 2.8A). No differences were observed in the weight or morbidity between groups of Cre^{NEG}, cHET and cKO mice, and no overt immune pathology developed (data not shown).

We sought to determine if the infection was controlled normally in Foxp4 cKO mice. To assess parasite clearance, brain tissues were harvested for immunohistopathology, and for determination of parasite burden by quantitative PCR. Foxp4 cKO tissues and littermate controls were assessed in a blinded fashion for cyst counts, with no appreciable difference across the cohorts (data not shown). Parasite burden in the brain and liver was measured by real-time PCR and normalized to a

standard curve to calculate parasite DNA remaining forty days post-infection (Figure 2.8B, C). Parasite load by DNA amplification indicated similar degrees of chronic toxoplasmosis in typical sites of parasite maintenance in cHET and cKO animals. No additional effects on tissue histology or animal survival were observed. These data suggest that T cell-specific loss of Foxp4 does not alter the overall ability to control *T. gondii* infection.

To further characterize the immune response again *T. gondii*, T cells from spleen, lymph node and CNS were analyzed. Overall splenic cellularity, CD4 and CD8 T cell numbers were comparable between cKO and control mice (Figure 2.8D, E). Additionally, brain mononuclear cells (BMNCs) were isolated from perfused brain tissues. Total numbers were comparable, which suggests Foxp4 cKO T cells were not trafficking into the CNS abnormally (Figure 2.8F). Frequencies and relative numbers of CD4 and CD8 T cells were also comparable in cHET and cKO cohorts (Figure 2.8G, and data not shown).

Activation and proliferation of Foxp4 cKO BMNCs and splenocytes appeared normal (data not shown). We assessed proliferation by Ki-67 staining of lymphocytes. Similar numbers of cHET and cKO T cells stained positively for Ki-67, suggesting cKO T cell numbers were maintained by normal turnover in response to *T. gondii* in both CNS and peripheral lymphoid tissues (data not shown). Furthermore, CD4 and CD8 T cells from spleens and lymph nodes of cHET and cKO mice showed comparable expression of activation markers CD44 and CD62L (data not shown), which demonstrates that Foxp4 is not necessary for the maintenance of peripheral T cells during chronic toxoplasmosis.

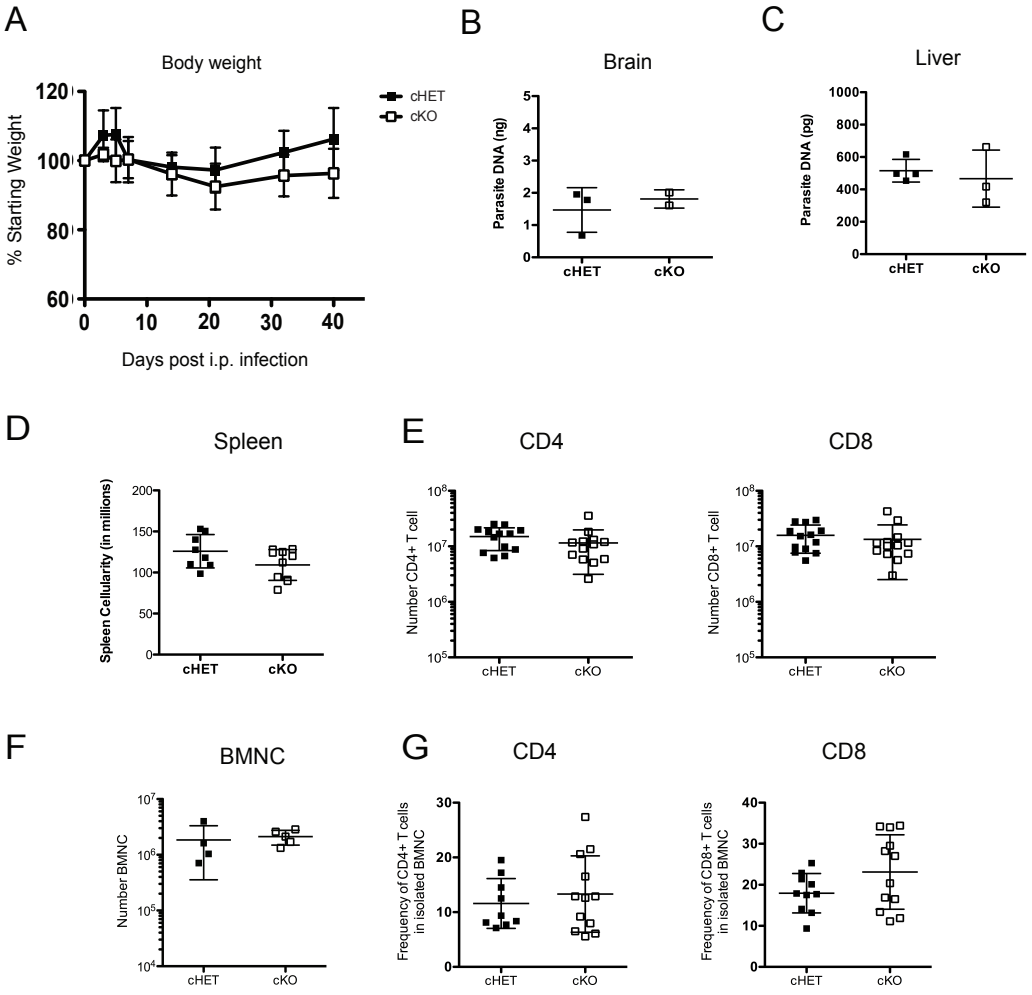


Figure 2.8. *Toxoplasma gondii* infection does not lead to wasting or lymphopenia in Foxp4 cKO mice. Eight-week-old Foxp4$^{FLOX/FLOX}$ Cre^{NEG}, cHET and cKO mice were infected with *T. gondii* by intraperitoneal injection of 20 Me49 cysts. A) Weights of mice were assessed longitudinally and plotted relative to the percentage of each respective mouse's starting weight at d0. Mean and s.d. from 9 Cre^{NEG}, 12 cHET, and 15 cKO mice. B-C) Parasite DNA was quantitated using SYBR probes specific for *T. gondii*. B) Brain tissue was perfused and harvested at day 40 post infection. C) Whole liver was homogenized for detection of parasite DNA. Levels of *T. gondii* DNA were normalized to a standard curve. Each point represents an individual mouse. Mean and standard deviation are shown. Representative of 3 individual experiments. D) Spleens were harvested at 40 days post infection and total spleen cellularity was determined. E) Total CD4 and CD8 T cell numbers were determined by staining splenocytes from mice in infected cohorts for analysis by multichromatic flow cytometry. F) Brain mononuclear cells were isolated via Percoll gradient and enumerated. G) Frequencies of TCR+ CD4 and CD8 lymphocytes in the brain were identified by multichromatic flow cytometry. D-G) Each point represents an individual mouse. Mean and standard deviation are shown. Representative of 3 experiments.

We next assessed the differentiation and functionality of Foxp4 cKO effector T cells that had been generated in response to *T. gondii* infection. During chronic infection with *T. gondii*, Th1 CD4 T cells are an important source of IFNγ, and are required for control of the parasite (Araujo, 1992; Gazzinelli et al., 1992). Cytokine production in cHET and cKO T cells was first assessed by enzyme-linked immunoassay (ELISA). Whole splenocytes were cultured for 24 hours in the presence of anti-CD3 or soluble tachyzoite antigen (STAg), or left unstimulated in media (Figure 2.6*A*). Culture supernatants were assessed for levels of IFNγ, an effector cytokine critical in controlling *T. gondii* infection and preventing cyst reactivation (Denkers, 1999; Yap and Sher, 1999). STAg stimulation, which restimulates those cells specific for *T. gondii,* resulted in statistically lower levels of soluble IFNγ detectable in cKO versus Cre^{NEG} control. A trend toward lower IFNγ was seen when comparing Cre^{NEG} to cHET and cHET to cKO cells, but the difference did not reach statistical significance. Conversely, supernatants from splenocyte cultures stimulated with anti-CD3 contain equivalent levels of IFNγ. These results suggest that Foxp4 may regulate the induction of IFNγ in an antigen-specific manner. IL-17 production in response to ant-CD3 and STAg stimulation was also measured, to determine if IFNγ responses were uniquely regulated. However, we found IL-17 production was also reduced in Foxp4 cKO cultures, demonstrating that Foxp4 deletion appears to impact the overall responsiveness of antigen specific cells to rechallenge.

The difference seen in cytokine production could be caused by several defects: a reduced number of cytokine-producing T cells, a decrease in the per cell production, or increased consumption of the cytokine. To determine if cKO T cells had altered cytokine generation on a per cell basis, unfractionated splenocytes were stimulated. Intracellular

cytokine staining (ICS) was used to measure single-cell production of IFNγ, IL-2, IL-17, and tumor necrosis factor alpha (TNFα). Cells were stimulated with PMA and ionomycin for four hours to determine whether cKO T cells were capable of producing cytokines. Analysis by flow cytometry indicated no difference in the frequency or absolute number of splenic CD4 or CD8 T cells capable of producing the cytokines assayed (Figure 2.9B, and data not shown). Furthermore, the MFI of these cytokines also suggests these cells are capable of producing similar amounts of cytokine, but does not address how antigen specific TCR stimulation elicits cytokine production.

The brain is a site of constant antigen presentation during toxoplasmosis. We isolated mononuclear cells from the brain (BMNCs) and assessed cytokine production by ELISA and ICS (Figure 2.9C, D). Similar to splenic T cells, there was no difference in the levels of IFNγ in response to anti-CD3 (Figure 2.9C). In wells stimulated with STAg, IFNγ levels are statistically decreased in cKO BMNCs, relative to BMNC responses from cHET and Cre^{NEG} mice. Foxp4 cKO BMNCs were further assessed by ICS in a four-hour recall response. Cells isolated from the brain tissues are capable of producing IFNγ, IL-2, IL-17, and TNFα at similar frequencies to controls, despite the lower quantity detected by ELISA (Figure 2.9D, and data not shown).

Predicted T helper responses to restimulation suggest Foxp4 is not necessary for differentiation or commitment to T helper lineages during infection. Therefore, the decreased cytokine response by chronically infected Foxp4 cKO cells is not caused by reduced IFNγ production on a per cell basis, deficient Th1 CD4 effector function, or a striking lack of lymphocytes. Rather, Foxp4-deficient cells demonstrated decreased IFNγ responses to STAg stimulation by ELISA regardless of tissue, suggesting a systemic defect in antigen-specific immunity.

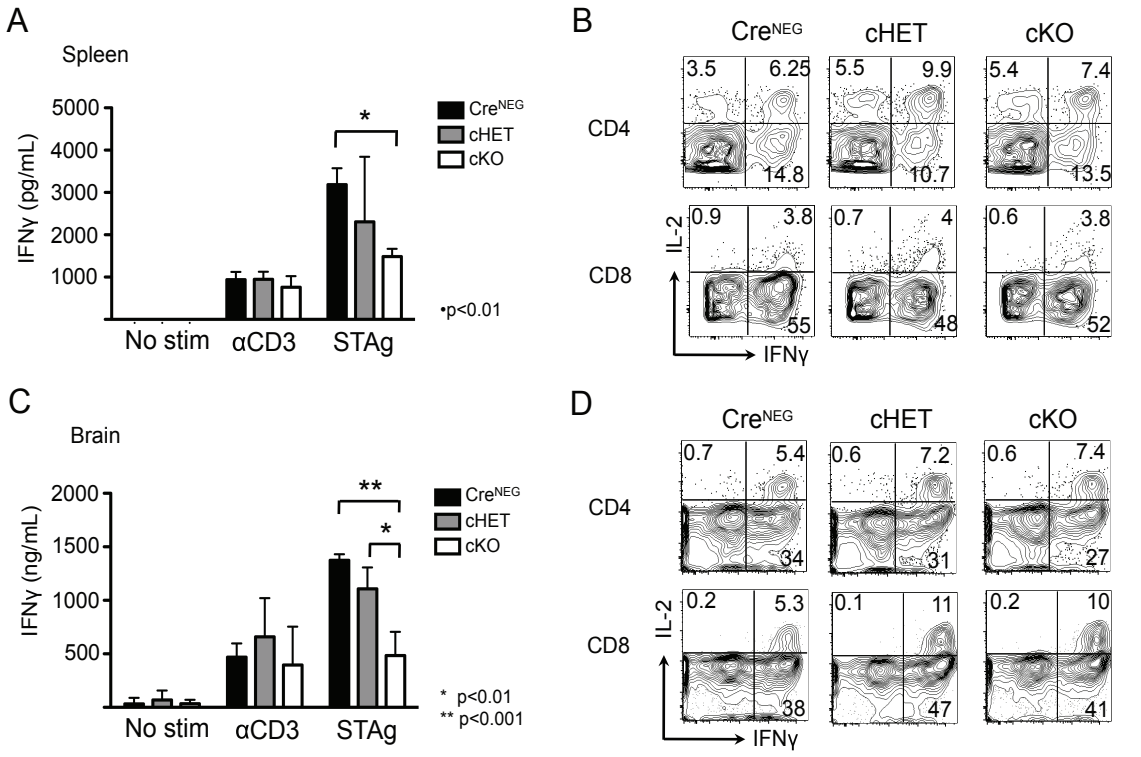


Figure 2.9. Foxp4 deletion alters recall responses to *Toxoplasma gondii* in the spleen and brain. A and C) Splenocytes and BMNC from mice infected with *T. gondii* 40 days earlier were cultured with anti-CD3, soluble tachyzoite antigen (STAg), or left unstimulated for twenty-four hours. Supernatants were analyzed for levels of IFNγ by ELISA, and normalized to a standard curve. Representative of 3 independent experiments. B and D) Splenocytes and BMNC were stimulated for four hours with PMA and Ionomycin in the presence of Brefeldin A, and assessed for intracellular IL-2 and IFNγ. Contour plots are gated on $CD4^+$ or $CD8^+CD3^+$ cells. Relative percentage is shown within each gate. Splenocytes are representative of 9 Cre^{NEG}, 12 cHET and 15 cKO. BMNC are representative of 5 CreNEG, 7 cHET, and 7 cKO mice. E) CD8-depleted splenocytes from cHET and cKO mice were cultured in Th0 (left) or Th1 (right) polarizing conditions. Cells were restimulated in the presence of monensin. Histograms are gated on $CD4^+$ T cells. cHET (filled histogram); cKO (black solid line); unstimulated (dashed line). Representative of 6 experiments.

Foxp4 is not required for acute viral clearance.

Host immunity and pathogen control and clearance differs greatly depending on the nature of the pathogen and the characteristics of the infection. Chronic infection with *T. gondii* results in sustained inflammatory conditions and presence of antigen, which may result in exhaustion and/or dysfunctional T cell responses. It was unclear whether Foxp4 was necessary during the chronic disease setting of *T. gondii* and whether constant antigen caused exhaustion and poor cytokine production by Foxp4 cKO T cells. Therefore, we investigated whether Foxp4 cKO memory cells would mount normal recall responses following an acute infection. We utilized the LCMV model of viral infection.

Cre^{NEG}, cHET and cKO mice were infected with the Armstrong strain of LCMV at day 0. To differentiate $CD44^{hi}$ endogenous memory T cells from LCMV-specific CD4 memory cells, cells were stained with MHC Class II tetramer:peptide conjugated to phycoerythrin. Additional staining for cell surface levels of CD44 determined antigen-experienced LCMV-specific T cells. For detection of LCMV-specific CD8 T cells we employed MHC Class I tetramer:peptide conjugated to allophycocyanin. CD4 splenocytes bearing the TCR specific for the LCMV epitope gp61 were identified by positive staining with $I\text{-}A^b$:gp61 tetramer, and CD8 T cells specific for LCMV epitope gp33 were identified by staining with $H2\text{-}D^b$:gp33 tetramer. Mice were bled weekly, to assess expansion and contraction of LCMV-specific CD4 and CD8 T cells. Gp61-specific or gp33-specific T cells were normalized per million lymphocytes. Foxp4 expression was not necessary in effector CD4 or CD8 T cell expansion or contraction after viral clearance (Figure 2.10A, B).

At day 30 post-infection, cohorts were sacrificed and assessed for maintenance and function of memory CD4 and CD8 T cells. Comparable frequencies of gp61-specific CD4 T cells and gp33-specific CD8 T cells were found in Cre^{NEG}, cHET and cKO mice (Figure 2.10C). Total numbers of I-A^b:gp61 tetramer+ cells or H2-Db:gp33+ cells were calculated from spleen cellularity and demonstrated similar numbers across all cohorts (Figure 2.10D, and data not shown).

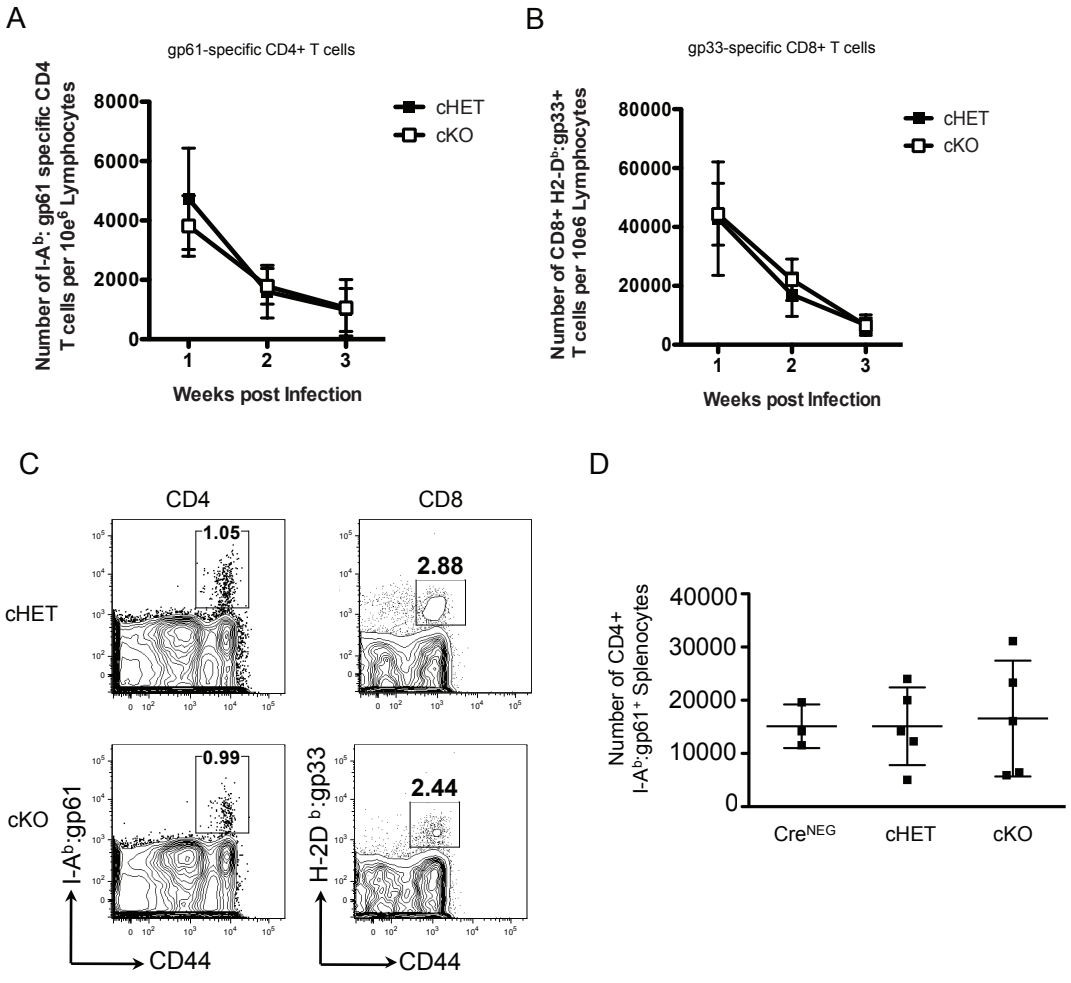


Figure 2.10. Antigen specific Foxp4 cKO T cells persist after antigen clearance. $Foxp4^{FLOX/FLOX}$ Cre^{NEG}, cHET and cKO mice were infected with the Armstrong strain of LCMV by intraperitoneal injection at day 0, allowed to clear infection, and to generate memory T cells. A-B) Peripheral blood was taken weekly from infected mice and

peripheral lymphocytes isolated using a Ficoll gradient. Frequencies of A) I-A^b:gp61+ $CD44^{hi}$ antigen specific CD4 T cells and B) H2-D^b:gp33+ $CD44^{hi}$ LCMV specific CD8 T cells were determined by tetramer staining and flow cytometric analysis. Representative of 3 Cre^{NEG}, 5 cHET and 5 cKO mice across two independent infections. C-D) At day 30 post-infection mice were sacrificed and spleens were harvested. C) Frequencies of I-A^b:gp61+ $CD44^{hi}$ CD4 T cells (left panels) and H2-D^b:gp33 $CD44^{hi}$ CD8 T cells (right panels) at day 30. Representative of 3 Cre^{NEG}, 5 cHET and 5 cKO mice across two independent infections. D) Numbers of I-A^b:gp61+ $CD44^{hi}$ CD4 T cells were calculated from whole spleen numbers. Each point represents one mouse. Mean and standard deviation are indicated.

To assess whether Foxp4 was necessary to mount recall responses following acute viral infection, single-cell cytokine production was assessed following a short recall response. Splenocytes were challenged *ex vivo* with gp61 peptide or left unstimulated, in the presence of Brefeldin A, to test CD4 cytokine responses (Figure 2.11A, and data not shown). Wells were harvested after 4 hours and cells were stained intracellularly for IFNγ, IL-2 and TNFα. Representative plots show comparable percentages of Cre^{NEG} and cHET splenocytes produce IFNγ and TNFα in response to gp61 peptide stimulation (Figure 2.11A and data not shown). However, Foxp4 cKO spleens contained fewer responders to gp61 restimulation. Cytokine production of all three cytokines is reduced in cKO mice, relative to cHET and Cre^{NEG} mice (Figure 2.11C). Frequencies of polyfunctional gp61-specific memory cells also reveal a distinct reduction in cKO splenocytes that produce IFNγ, TNFα, and IL-2, suggesting Foxp4 expression is important in CD4 memory recall responses following acute infections (Figure 2.11D). Polyclonal restimulation with PMA and ionomycin revealed an decreased percentage of CD4 T cells are capable of producing cytokine (Figure 2.11B), suggesting the total cKO CD4 population may exhibit decreased functional cytokine responses.

In contrast, when whole splenocytes were rechallenged with gp33 peptide, LCMV-specific responses by CD8 T cells appeared to be relatively similar between

cHET and cKO mice (Figure 2.11E and data not shown). Furthermore, total CD8 cells were stimulated with PMA and ionomycin and produced cytokine normally, suggesting polyclonal CD8 recall responses were intact. Therefore, Foxp4 may be important for CD4 memory recall responses, but not CD8, in the setting of acute infection.

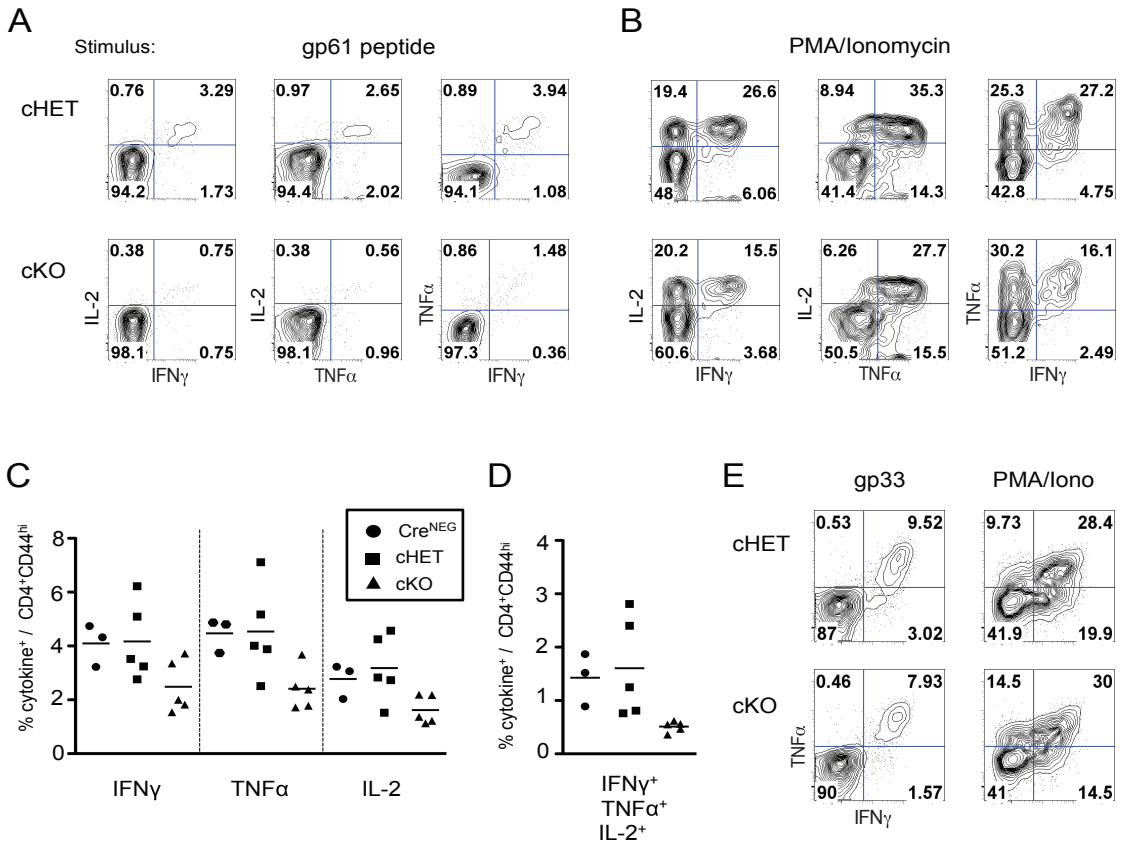


Figure 2.11. Foxp4 deficient CD4 T cells exhibit reduced memory recall responses following LCMV infection. Foxp4$^{FLOX/FLOX}$ Cre^{NEG}, cHET and cKO mice were infected with the Armstrong strain of LCMV by intraperitoneal injection at day 0, allowed to clear infection, and to generate memory T cells. At day 30 post-infection mice were sacrificed and spleens were harvested. A) Splenocytes harvested at day 30 were restimulated with gp61 peptide for four hours *in vitro*, in the presence of Brefeldin A. Cells were assessed for cell surface expression of CD4, CD8, CD44, and stained intracellularly for IFNγ, TNFα, and IL-2. Representative plots of 3 Cre^{NEG}, 5 cHET and cKO across two independent infections. B) Splenocytes from infected cohorts were stimulated with PMA and Ionomycin in the presence of Brefeldin A, and similarly stained for cytokine production as in B. C) Compiled frequencies of cells producing IFNγ, TNFα, or IL-2 (left),

or polyfunctional cells producing all three cytokines (right). D) Frequencies of polyfunctional CD4 T cells from gp61 peptide stimulation. E) Splenocytes stimulated with gp33 peptide (left panels) or PMA and Ionomycin (right panels) in the presence of Brefeldin A were assessed for IFNγ, TNFα, and IL-2. Events are previously gated on live, CD8+ CD44hi T cells. Representative plots of 3 Cre^{NEG}, 5 cHET and cKO across two independent infections.

Discussion

In this chapter, we detail exclusive requirements for Foxp4 regulation in the T lineage. We show that Foxp4 is expressed in T cells and plays a role in effector T cell cytokine responses *in vitro* and *in vivo*. We also present evidence of unique Foxp4 regulation in memory recall responses. Due to the high degree of homology between Foxp1 and Foxp4, and known heterodimerization in other cell types, Foxp4 cKO T cells might have developed an activated phenotype. Surprisingly, naïve Foxp4 deficient T cells develop normally, indicating Foxp4 is not necessary to maintain T cell quiescence. More recent studies have shown Foxp1 represses *il7ra* transcription and regulates homeostasis in T cells (Feng et al., 2011). Whether repression of IL-7R transcription is due to Foxp1:Foxp1 homodimers or Foxp1:Foxp4 heterodimers was not directly addressed. However, deletion of Foxp4 has no effect on either IL-7R levels or on peripheral homeostasis based on steady-state lymphocyte numbers and *in vivo* bromodeoxyuridine labeling (data not shown), suggesting that repression of IL-7R transcription and maintenance of a naïve phenotype is likely Foxp4-independent.

Similarly, Foxp3 expression in regulatory CD4 cells is not affected by Foxp4 deletion. Normal Treg development in Foxp4 cKO mice suggests Foxp4-specific regulation is not necessary for the generation or maintenance of natural Tregs *in vivo*. These data also indicate Foxp3:Foxp4 protein interaction is not important for Treg development. In addition, we did not find alterations in the frequency or number of Tregs

in acutely or chronically infected mice (data not shown), suggesting that Foxp4 deletion does not have an impact on the survival or maintenance of Tregs under homeostatic or infectious conditions.

Because Foxp4 expression is not necessary for normal generation of peripheral T cells, we were able to evaluate how Foxp4 deletion affected cell fate commitment of effector CD4 T cells. We expected Foxp4 expression to be important for T cell activation, and possibly differentiation, given the three-fold increase in expression following stimulation. However, Foxp4 cKO effector cells did not appear defective when evaluated by *in vitro* assays. Foxp4-deficient CD4 T cells are able to differentiate into Th1 and Th17 lineages, and to convert into Foxp3^{+} Tregs *in vitro.* Furthermore, proliferation and upregulation of known markers of activation appears normal in activated CD4 and CD8 populations. Thus, despite increased expression of Foxp4 after TCR stimulation, we have found no substantial effect of Foxp4 deletion on T cell activation or differentiation.

To more thoroughly evaluate effector function, we examined Foxp4-deficient T cell responses following pathogenic infection. During chronic infection with *T. gondii*, Foxp4 cKO mice controlled the infection similarly to controls as evidenced by similar numbers of cysts and quantitatively equal parasite burden. Total cellularity and phenotype of T cells in infected cKO mice were comparable to cHET animals. Trafficking to inflamed tissues appeared normal, although we cannot exclude the possibility that Foxp4 cKO T cells migrate abnormally to the CNS where chronic antigen stimulation induces cell death. However, if this were the case, we would expect to find signs of increased immunopathology and lymphocyte infiltration in brain tissue, which was not characteristic of cKO immunohistopathology. Lack of overt pathology or increased morbidity of Foxp4 cKO animals suggests T cell responses were largely intact.

Given the robust responses of CD4 and CD8 T cells stimulated *in vitro*, we expected Foxp4 deletion may cause increased cytokine production. Previous reports have demonstrated that Tregs and suppressive signals, including IL-10 and IL-27, are important for host survival during infection with *T. gondii*. Extraneous Th1 responses lead to increased host fatality, and aberrant Th17 responses, in IL-27-deficient animals, lead to increased inflammation in the CNS. However, Foxp4 cKO antigen specific lymphocytes exhibited reduced cytokine responses upon rechallenge. While stimulation with anti-CD3 generated similar quantities of cytokine detected, stimulation with STAg resulted in lower IFNγ and IL-17 responses. On an individual cell basis, Foxp4 cKO CD4 and CD8 T cells were capable of producing cytokine following short-term pharmacological stimulation. However, lower quantities of cytokine from 24-hour stimulation with STAg suggest Foxp4 deficient cells are less responsive to *T. gondii* antigen. Moreover, we found a dose-dependent effect of Foxp4 expression in STAg responses. Foxp4 cHET mice exhibited an intermediate defect in cytokine production, suggesting wild-type Foxp4 expression is required for normal memory recall responses.

We reasoned chronic infection could lead to fewer *T. gondii* specific effector and memory T cells, but limitations of antigen specific reagents in *T. gondii* responses prevented further investigation. The LCMV model of infection provides identification of antigen specific T cells by tetramer binding of LCMV-specific T cell clones. We found normal numbers of LCMV-specific CD4 and CD8 T cells in Foxp4 cKO mice after acute infection. However, lower frequencies of Foxp4-deficient memory CD4 T cells also produced cytokines during antigen specific recall responses. These data suggest Foxp4 may not be important for maintaining the quantity of memory T cells, but rather the quality.

Foxp4 cKO studies described defects that only appear in activated T cells. Previous evidence supports Foxp1-dependent regulation is important for naïve T cell maintenance, cell survival, and IL-7R expression. Gene array data demonstrates effector T cells express lower levels of Foxp1 (Heng and Painter, 2008; Rudra et al., 2012), presumably to release Foxp1-mediated repression of effector programs. Given the high degree of homology between Foxp1 and Foxp4, perhaps Foxp1 is able to compensate for Foxp4 deletion, until Foxp1 is downregulated in effector T cells. Reduced expression of both FoxP members then results in dysregulation of normal T cell function.

Binding of FoxP proteins to forkhead family members and to other transcription factors affords Foxp4 the opportunity to participate in a broader range of gene regulation. Some clues about Foxp4 regulation come from *in vitro* polarization studies, which indicate Th17 differentiation may be subtly dysregulated without Foxp4 expression. It is particularly interesting that comparison of polarized cells with and without CD28 signals indicated costimulation was required to induce abnormal cytokine responses (data not shown), and that the resulting cKO Th17 cells produce more IL-2. These data suggest Foxp4 may interact with NFκB or NFAT, primary regulators of IL-2 gene transcription. FoxP family members have been shown to directly bind both of these transcription factors. Therefore, Foxp4 may be important in repressing NFκB or NFAT gene targets. Foxp4 regulation appears to be central to Th17 programs, which suggests RORγT may be another factor that co-regulates with Foxp4. Alternatively, Foxp4 gene targets may be most accessible in the chromatin conformation of RORγT regulated loci. Taken together, these subtle defects invite future work to describe the Foxp4 binding partners and regulatory mechanisms.

While decreased memory responses indicate Foxp4 is important for maintaining T cell function, it does not exclude a role for antigen presenting cells (APCs) or natural killer cells. Control of chronic toxoplasmosis requires several immune cell types, and CD4 T cells orchestrate much of the cooperation of innate and adaptive components during immune responses. Expression of CD4Cre generates T lineage-specific deletion of Foxp4, but does not rule out a model in which the ability of CD4 T cells to influence the maturation and activation of accessory cell types is dependent on Foxp4 expression.

These studies are the first to characterize expression of Foxp4 in T cells and report diminished effector T cell responses in infected Foxp4 cKO mice. Development of naïve T cells and regulatory T cells is normal in Foxp4 cKO mice, indicating Foxp4 may not regulate the same gene programs as FoxP family members, Foxp1 or Foxp3. Instead, Foxp4 expression is increased in activated T cells, and *in vivo* studies suggest a role for Foxp4 in the regulation of cytokine responses. These findings differentiate Foxp4 from its family members, and offer another prospective area of immune regulation by FoxP proteins. While Foxp1 and Foxp3 are both necessary to maintain a dormant immune environment, Foxp4 appears to have a supportive role in maintaining protective immune responses.

CHAPTER III.

Foxp1 and Foxp4 direct T cell fates and functions.

Introduction

Forkhead transcription factors of the FoxP family regulate cell fate decisions in immune cells. Foxp3 regulates the balance between suppressive and inflammatory Th17 T helper lineages and provides maintenance of peripheral tolerance, while Foxp1 directs T cell subset development and differentiation (Feng et al., 2010; Ziegler, 2006; Ziegler and Buckner, 2009). Previous studies have demonstrated that Foxp1 maintains naïve $CD44^{low}$ T cell development by suppressing effector cell differentiation. Proliferation and survival is disrupted in Foxp1-deficient T cells due to altered responses to cytokine signaling (Feng et al., 2011).

In contrast, development of T cell subsets does not appear to rely on the related FoxP family member, Foxp4; however, CD4 helper T cell differentiation may be altered without Foxp4-dependent regulation. Transcriptional regulation by Foxp4 supports long-term immunity by maintaining memory T cell recall responses. Foxp4-deficient CD4 and CD8 memory T cells exhibit reduced effector function after rechallenge, specifically in cytokine responses (Wiehagen et al., 2012).

Characteristics of Foxp1 and Foxp4 protein sequences enable these FoxP proteins to interact with each other and potentially co-regulate genes (Li et al., 2004a). Additionally, homology between Foxp1 and Foxp4 suggest these transcription factors can possibly compensate for one another by recognizing similar consensus binding sites or partnering with identical transcription factors. Additional changes in gene regulation may occur in single cKO mice, but expression of Foxp1 in Foxp4^{cKO} may compensate for loss of Foxp4. Therefore, it was unclear how combined deletion of Foxp1 and Foxp4 in the T lineage would affect adaptive immunity, and whether double-deficient T cells would reveal additional genes under FoxP regulation.

Mice were generated in which T cells were deficient for both Foxp1 and Foxp4. Characterization of Foxp1/4-deficient, or cDKO, T cells is described in this chapter, including an extensive description of thymic development and T cell differentiation, survival, and cytokine production. cDKO T cell survival and development were further examined in competitive bone marrow chimeras to assess how cell-intrinsic Foxp1/4-dependent regulation affects gene programs in the T lineage.

Preliminary data suggest expression of either Foxp1 or Foxp4 is required for maintenance of adaptive immunity and control of infection. These data indicate Foxp1 has exclusive gene targets important for naïve T cell maintenance, and that Foxp4 can partially compensate for loss of Foxp1. Additionally, expression of one allele of Foxp1 is sufficient for normal lymphocyte development and function. Together, these findings inform models of Foxp1 and Foxp4 regulation in T cells.

Results

Generation of Foxp1/Foxp4 conditional double knockout mice.

To generate mice in which the T lineage is selectively deleted for Foxp1 and Foxp4, we crossed Foxp1$^{FLOX/FLOX}$, previously described (Feng et al., 2010; Li et al., 2012), and Foxp4$^{FLOX/FLOX}$ mice to generate Foxp1^{FLOX}Foxp4^{FLOX} mice, which express all four mutant alleles (Figure 3.1A). Foxp1^{FLOX}Foxp4^{FLOX} offspring were crossed with transgenic mice expressing Cre on the *cd4* promoter, as discussed in Chapter 2, to produce CD4Cre+ Foxp1^{FLOX}Foxp4^{FLOX} (cDKO) mice. Throughout these studies, deletion of Foxp1 and Foxp4 in cDKO mice was compared to deletion of Foxp1 or Foxp4 in single cKO mice (Foxp1^{cKO} and Foxp4^{cKO}, respectively). Wild-type mice expressing CD4Cre (Cre^{POS}) served as Cre controls.

	Foxp1	Foxp4	Number of alleles
Cre^{POS}			4
Foxp1^{cHET}			3
Foxp4^{cHET}			3
Foxp1^{cKO}			2
Foxp4^{cKO}			2
cDHET			2
1H4K			1
1K4H			1
cDKO			0

Table 3.1. Generation of cDKO mice and littermate controls. Solid boxes represent expression of Foxp1 (blue) or Foxp4 (green) wild-type alleles. Hatched boxes represent deletion after Cre-mediated recombination. Each row represents Foxp1/4 expression patterns in one genotype, specified in the left column. The remaining FoxP genes expressed after deletion are included in the right column.

Our breeding strategy also generated littermates in which combinations of floxed or wild-type Foxp1 and Foxp4 genes were expressed. We examined littermates in which one allele of each FoxP member was deleted ($Foxp1^{FLOX/+}Foxp4^{FLOX/+}CD4Cre^{+}$; conditional double heterozygote, or cDHET). Offspring also included mice with one allele of Foxp1 and two alleles of Foxp4 ($Foxp1^{FLOX/+}Foxp4^{FLOX/FLOX}$, or 1H4K), and mice expressing both alleles of Foxp1 and one allele of Foxp4 ($Foxp1^{FLOX/FLOX}Foxp4^{FLOX/+}$, or 1K4H). Inclusion of 1H4K and 1K4H mice, in which three of the four genes are deleted, enabled evaluation of allelic series of Foxp1 and Foxp4 expression, and definition of the contribution of both genes in dose-dependent settings. Unless otherwise stated, the studies described in this chapter included four- to eight- week-old cDKO mice with littermate controls.

Deletion of FoxP family members was verified by quantitative real-time PCR. CD4+ TCR+ mature single positive thymocytes were sorted from cDKO or wild-type thymi. Transcript levels of Foxp1 and Foxp4 were determined in wild-type and cDKO T cells and normalized to internal β-actin levels. In cDKO thymocytes, relative expression Foxp1 and Foxp4 was at least ten-fold less than wild-type thymocytes, and in some experiments, below the limits of detection (Figure 3.1B-C, and data not shown). Successful deletion of Foxp1 and Foxp4 was satisfactory, and allowed further work to survey the effects of FoxP deletion in the T lineage.

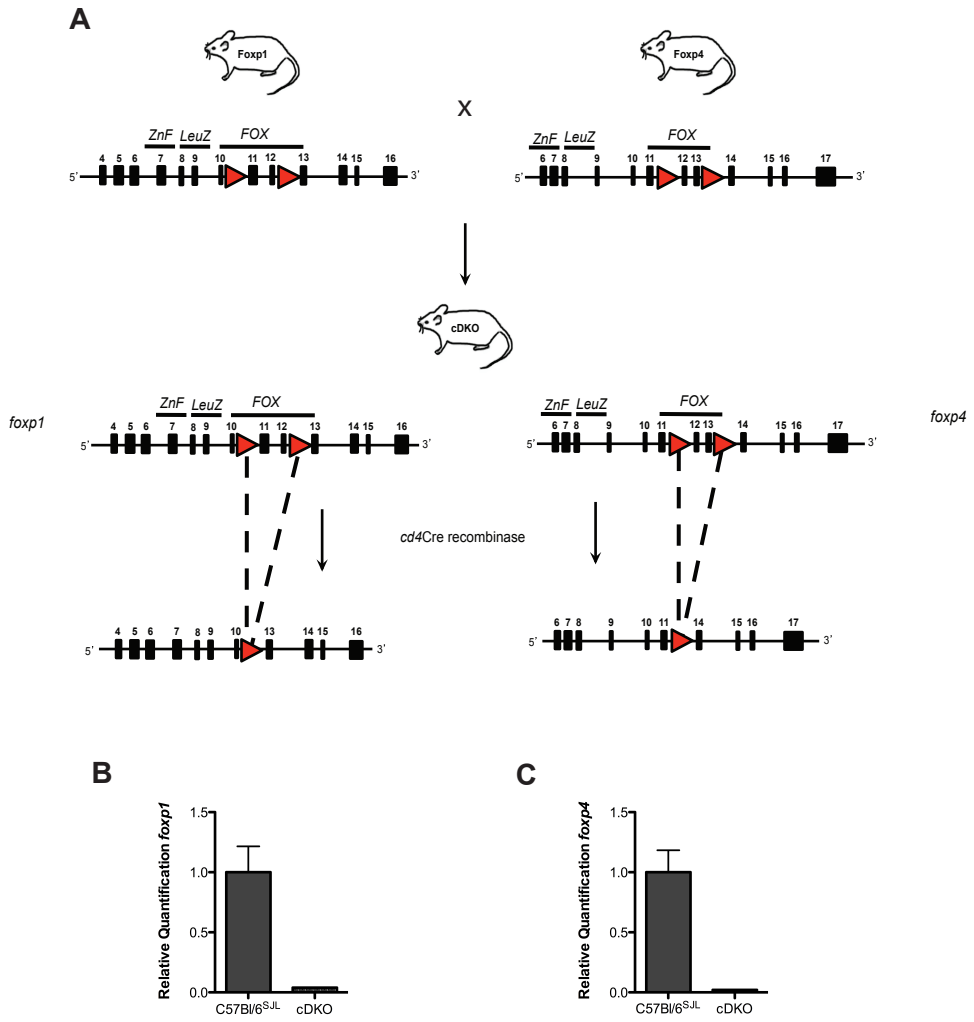


Figure 3.1. Generation of cDKO mice. A) Schematic of breeding strategy to produce offspring expressing engineered Foxp1^{FLOX} and Foxp4^{FLOX} alleles, and CD4-Cre. Foxp1^{FLOX} and Foxp4^{FLOX} alleles are similarly designed to eliminate DNA encoding the forkhead DNA-binding domain (FOX). Exons are denoted by black rectangles. Exons 11 and 12 in the Foxp1 gene encode the FOX domain and are flanked with LoxP sites (red triangles). The Foxp4 FOX domain is eliminated by excising exons 12 and 13. Cre expression begins at the DP stage of thymocyte development. B-C) Relative expression of Foxp1 (B) and Foxp4 (C) mRNA transcript detected in highly-purified CD4SP thymocytes by real-time PCR. Expression is relative to wild-type C57Bl/6 CD4SP cells.

Foxp1 and Foxp4 in thymic development.

To assess the effect of Foxp1/4 deletion on T cell development, we initially characterized thymocyte development, where Cre expression is induced. DP and SP thymocytes were detected in all cohorts but total thymic cellularity was reduced in cDKO mice compared to Foxp4 and Cre^{POS} mice (Figure 3.2A). On average, cDKO thymi were one-third smaller than Cre^{POS} thymi (Figure 3.2B). Less significant differences were observed between cDKO and other littermate cohorts, although both $Foxp1^{cKO}$ and cDKO trended toward reduced numbers in comparison to cDHET and $Foxp4^{cKO}$. Importantly, cDHET thymi were comparable to Foxp4 and Cre^{POS} numbers, indicating haploinsufficiency of Foxp1 and Foxp4 did not affect thymic development.

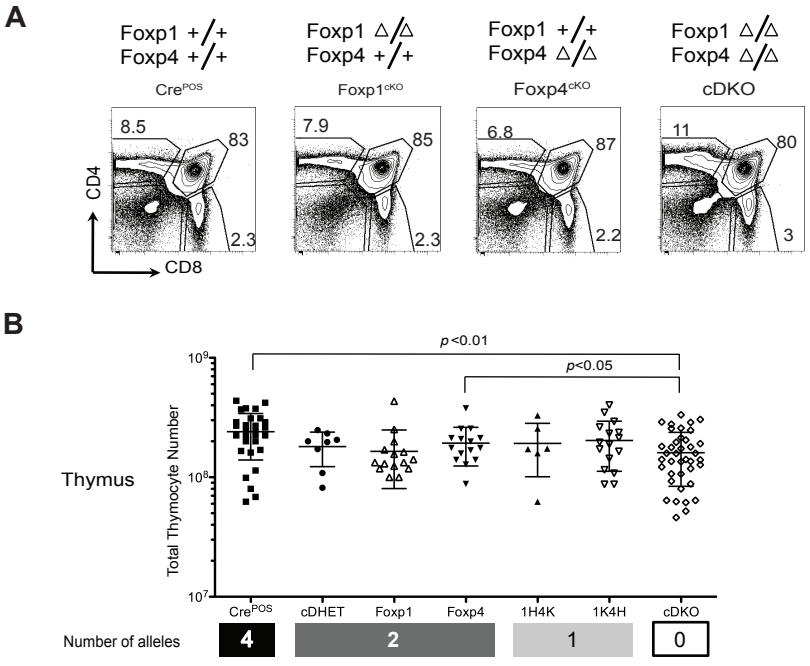


Figure 3.2. Reduced thymic cellularity in $Foxp1^{cKO}$ and cDKO mice. A) Representative plots of developing DP, CD4SP and CD8SP thymocytes in Cre^{POS}, $Foxp1^{cKO}$, $Foxp4^{cKO}$ or cDKO mice. B) Abolute cell numbers of total thymi compiled from 16 experiments, comparing multiple mouse cohorts. Cre^{POS} thymic cellularity was

compared to littermates expressing four, two, or one wild-type copy of either Foxp1 or Foxp4. Each point represents an individual mouse. Statistical analyses were determined by one-way ANOVA with Dunnett post-test. *p* values are denoted above. All other comparisons were not statistically significant.

In wild-type thymi, 75%-85% of the thymus consists of CD4+CD8+ DP cells. In cDKO mice, the reduced frequency and number of DP accounted for the decreased thymic cellularity (Figure 3.3A-B). Foxp1 deletion resulted in similarly reduced numbers of DP in Foxp1^{cKO} and 1K4H thymi, suggesting that Foxp1 deletion in these cohorts affected DP development. Expression of at least one allele of Foxp1 was sufficient for normal DP numbers, as the number of DP cells in thymi from cDHET, Foxp4^{cKO}, and 1H4K mice were comparable to thymi from Cre^{POS} mice (data not shown).

Further characterization of DP thymocytes indicated developmental differences between Cre^{POS}, Foxp1^{cKO}, and cDKO mice, suggesting altered TCR signaling. DP thymocytes can be divided into immature and mature populations based on the expression of TCRβ, CD69, and CD5. Relatively normal frequencies of immature and mature DP thymocytes were detected in Foxp1^{cKO} and cDKO thymi (data not shown). However, significantly increased expression of CD5 was consistently detected in DP thymocytes from cDKO mice compared to other cohorts (Figure 3.3C-D).

CD5 is a cell surface protein that associates with TCR complexes containing TCRβ, CD3, and CD28 (Raman, 2002). Expression of CD5 typically correlates with TCR signaling strength, which has important implications for the negative selection of developing thymocytes (Kishimoto and Sprent, 2000). CD5 inhibits TCR signaling, and earlier reports suggested CD5 regulates TCR tuning during thymocytes maturation. Increased levels of CD5 expression denoted altered thymic selection. (Azzam et al., 2001). To determine whether thymic selection was dysregulated in cDKO mice, the Vβ

usage of CD4SP and CD8SP cDKO thymocytes was compared in cDKO, single cKO, and Cre control mice (Figure 3.3E). Thymic selection was not grossly affected in cDKO mice, as the usage of Vβ families in both CD4SP and CD8SP populations was comparable to that observed in Cre^{POS} mice. The development of polyclonal SP cells allowed us to assess T cell development in Foxp1 and Foxp4 mutants more thoroughly.

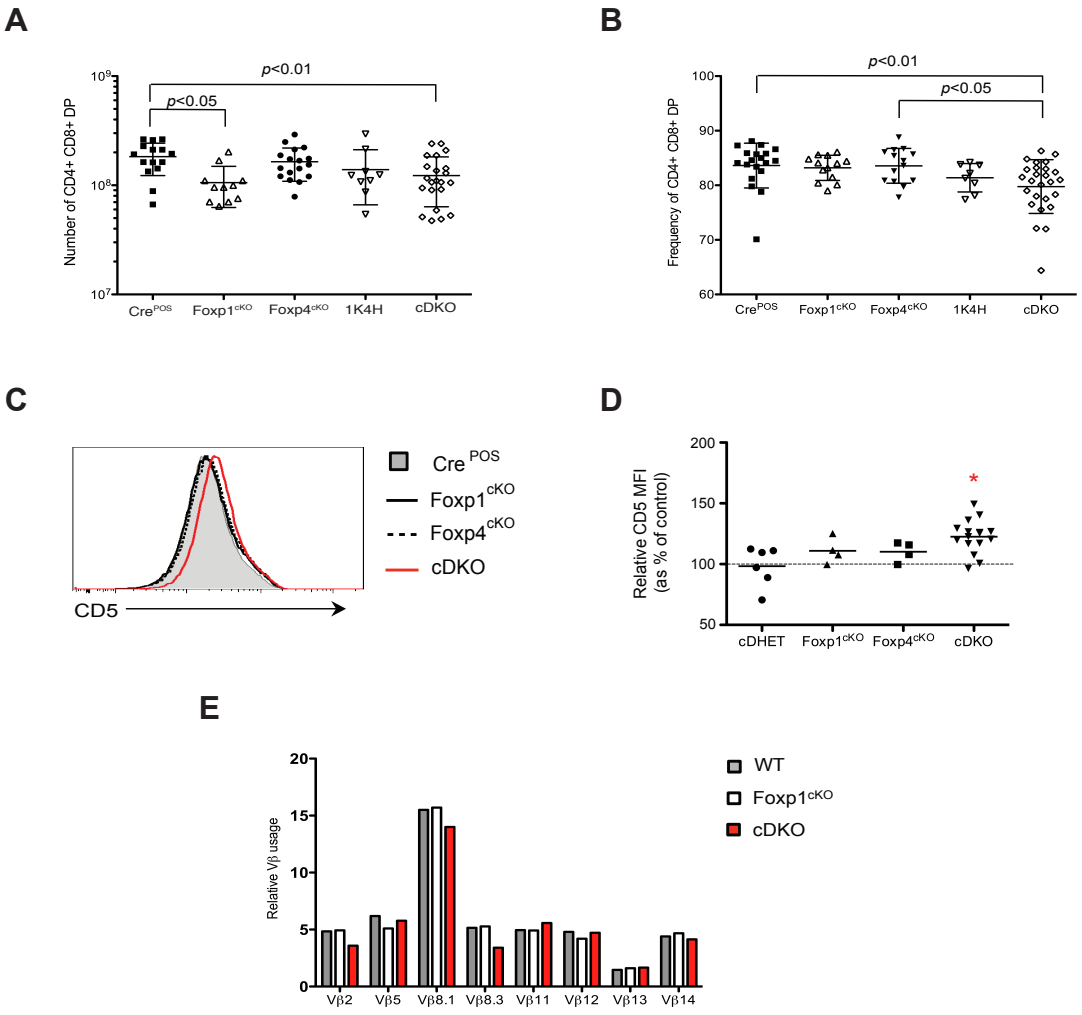


Figure 3.3. Foxp1/4 deletion alters CD5 expression and DP cellularity but not SP polyclonality. A-B) Absolute number (A) and frequency (B) of DP thymocytes compiled from 16 experiments. Compared to Cre^{POS} littermate controls, number and frequency of

cDKO DP are significantly reduced. Each point represents an individual mouse. C) Representative staining of cell surface expression of CD5 on DP from Cre^{POS} (gray histogram), $Foxp1^{cKO}$ (black line) $Foxp4^{cKO}$ (dotted black line), or cDKO (red line). D) Relative fold change in mean fluorescence intensity of CD5 staining compiled from 5 experiments. Black dotted line represents average of Cre^{POS} littermate controls to which all cohorts were relative. A-D) Statistical analyses by one-way ANOVA with Dunnett post-test. *$p<0.05$; Significant p values are shown, all other comparisons were not statistically significant. E) TCR Vβ usage across eight Vβ families by CD4SP thymocytes in Cre control (gray), $Foxp1^{cKO}$ (white) or cDKO (red) thymocytes. Frequencies calculated from percentage of cells expressing individual Vβ+ chains as a fraction of the total CD4SP population.

Our findings show that thymic development generally remained intact in the absence of Foxp1 and Foxp4 expression. CD4SP and CD8SP thymocytes develop in cDKO thymi, demonstrating that Foxp1/4 deletion in DP thymocytes did not cause a block in SP thymocyte generation (Figure 3.1A). Mature cDKO TCRβ+ CD4+ SP and CD8+ SP thymocytes were present at typical ratios of CD4SP to CD8SP thymocytes observed in wild-type and Cre^{POS} mice (data not shown). Similarly, CD4SP and CD8SP thymocytes were detected in thymi from Cre^{POS}, cDHET, 1H4K, and 1K4H mice (data not shown), indicating that Foxp1 or Foxp4 expression is not required for CD4 and CD8 SP thymocyte generation.

Numbers of CD4SP or CD8SP thymocytes were comparable regardless of the expression of various combinations of Foxp1 and Foxp4 (Figure 3.4A-B). Therefore, our data demonstrate that Foxp1 and Foxp4 were not required individually or together for generation of TCRβ+ CD4 or CD8 SP. However, an increased percentage of total cDKO thymocytes were CD4SP (Figure 3.4C). On average, cDKO CD4SP comprised 11% of total thymocytes, compared to approximately 7% of total thymocytes in control thymi. In contrast, frequencies of CD8SP and DN were not significantly different in cDKO thymi (data not shown). While this increase in relative CD4SP frequency correlated to

decreased DP frequency, relative frequencies of CD8SP were unchanged, indicating that Foxp1 and Foxp4 expression may function differently in CD4 and CD8 SP lineages.

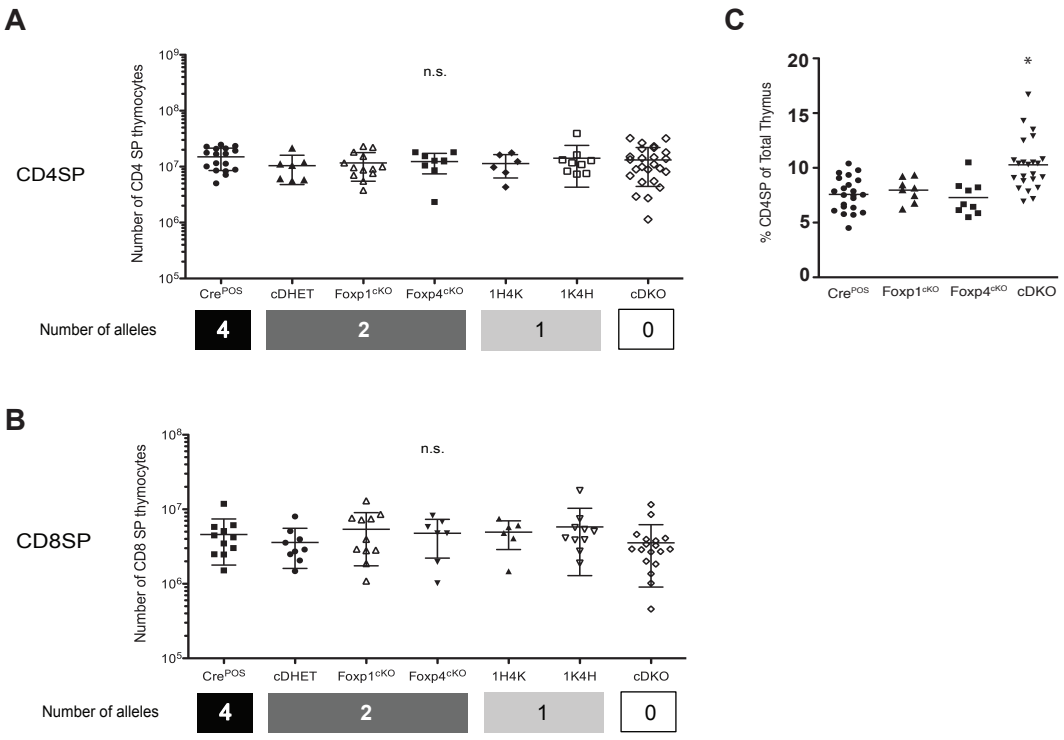


Figure 3.4. Normal numbers of SP thymocytes develop in cDKO mice. A-B) Absolute number of A) CD4SP and B) CD8SP thymocytes across Cre^{POS}, $Foxp1^{cKO}$ or $Foxp4^{cKO}$, cDKO, and littermate control cohorts compiled from 16 experiments. C) Compiled frequency of CD4+CD8- SP thymocytes of total thymi. Cells expressing CD11b, CD11c, Mac-1 Gr-1, or B220 were gated out prior to CD4SP frequency determination. Each point represents an individual mouse. Statistical analysis by one-way ANOVA with Dunnett post-test.

FoxP members maintain peripheral CD4 and CD8 T cell number

We next assessed how deletion of Foxp1 and Foxp4 affected peripheral T cells. No differences in spleen cellularity were observed across any of the littermate cohorts, and no overt signs of pathology were detected (data not shown). However, frequencies of TCRβ and B220+ cells were altered in cDKO mice. Significantly fewer TCRβ+ lymphocytes were found in the spleens of cDKO mice, compared to age-matched Cre^{POS}, single cKO, and littermate controls (data not shown). In cDKO mice, the frequency of TCRβ+ splenocytes averaged 5%-10%, while the frequency of TCRβ+ averaged 30%-40% in littermate controls. Total T cell numbers were reduced in other peripheral tissues as well. Peripheral blood, bone marrow, and pooled lymph nodes (LN) contained reduced frequencies and numbers of TCRβ+ cells in cDKO mice, suggesting T lymphopenia extended to other peripheral lymphoid tissues (data not shown).

To determine which populations within the T compartment are affected by FoxP protein deletion, the frequency and numbers of CD4 and CD8 T cells in peripheral lymphoid tissues were compared across all cohorts (Figure 3.5). Foxp1 deletion alone resulted in reduced CD8 T cells in peripheral tissues (Figure 3.5B, D). In both lymph nodes and spleen tissues, the relative percentage and total number of CD8 T cells were significantly reduced compared to Cre^{POS} controls. In contrast, CD4 T cells in $Foxp1^{cKO}$ spleen and LN were comparable to control mice. Together, these data suggest Foxp1 is not necessary to maintain CD4 T cells. However, expression of at least one allele of Foxp1 is necessary for CD8 T cell maintenance.

Combined deletion of both Foxp1 and Foxp4 resulted in more severe T lymphopenia in both LN and spleen compared to $Foxp1^{cKO}$ mice. Although SP thymocyte

numbers in the cDKO thymus were comparable to SP thymocyte numbers in control thymi, the total number and frequency of CD4 and CD8 T cells in cDKO peripheral tissues were significantly lower compared to littermate controls. (Figure 3.5A, C, and data not shown). 1K4H mice exhibited an intermediate defect with CD4 and CD8 T cell numbers falling between the average cellularity in Foxp1^{cKO} and cDKO mice. This gene dosage effect of Foxp4 deletion suggests that T cells require FoxP members for normal peripheral homeostasis. Additionally, in the absence of Foxp1, Foxp4 expression may play a role in maintaining the T lymphocyte compartment.

In cDKO mice, T cell numbers were more diminished in LN than in the spleen (Figure 3.5A-B). Numbers of cDKO CD4 T cells in pooled LN were a 100-fold lower than Cre^{POS} mice. On average, we observed less than one million CD8+ T cells in the pooled LN of cDKO mice—almost ten times lower than the average number of CD8 T cells in the pooled LN from Cre control mice. In the spleen, however, cell numbers are less than one fold lower than littermate controls. Thus, there are more striking differences between cDKO and littermate controls in lymph nodes than in the spleen, indicating that anatomic location may also play a role in cDKO T cell maintenance.

In comparison, littermate controls, including cDHET, Foxp4^{cKO}, and 1H4K mice had similar numbers and frequencies of peripheral CD4 and CD8 T cells in the LN and spleen. Expression of one allele of Foxp1 was sufficient to provide normal T cell development. Together, these data demonstrate that the deletion of Foxp1 in the T lineage results in overall decreased T cell numbers in peripheral lymphoid tissues, affecting CD8 T cell cellularity. Combined deletion of Foxp1 and Foxp4 significantly exacerbates T lymphopenia and affects both CD4 and CD8 T cell numbers.

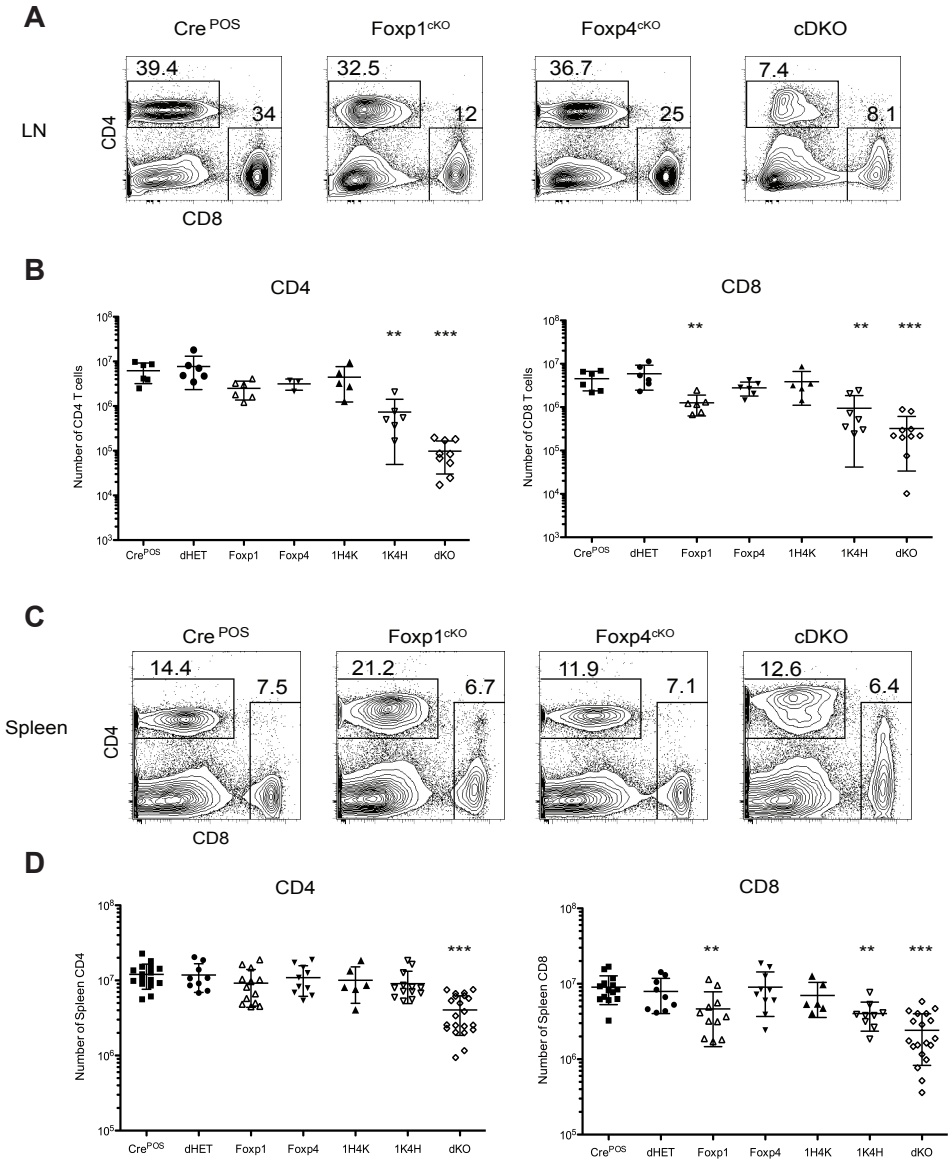


Figure 3.5. Reduced cDKO CD4 and CD8 T cells contribute to significant T lymphopenia. A, C) Representative plots of CD4 and CD8 T cells from pooled lymph nodes (A) or spleen (C) from Cre^{POS}, $Foxp1^{cKO}$, $Foxp4^{cKO}$, cDKO mice. Frequencies correspond to CD4+ or CD8+ T cells. B, D) Absolute numbers of CD4 (left) or CD8 (right) T cells. Each point represents an individual mouse. B) LN numbers compiled from 6 experiments. **p<0.005 ***p<0.0001. D) Spleen numbers compiled from 16 experiments. **p<0.05 ***p<0.001.

CD44 expression is regulated by Foxp1 but not Foxp4.

In Chapter 2, we demonstrated Foxp4 deletion does not affect the phenotype of developing SP thymocytes. In contrast, previous studies have shown that $Foxp1^{cKO}$ thymocytes develop abnormally and acquire CD44 expression upon SP maturation (Feng et al., 2010). Therefore, we sought to determine whether deletion of both Foxp1 and Foxp4 altered expression of CD44 or other cell surface markers.

Compared to Cre^{POS} or Foxp4-deficient T cells, cDKO T cells express altered levels of cell surface markers. In the thymus, CD44 expression by cDKO CD4SP and CD8SP thymocytes was similar to $Foxp1^{cKO}$ SP thymocytes, indicating Foxp1 expression is necessary to repress CD44 upregulation (Figure 3.6A). Comparison of CD44 profiles across all littermates clearly showed that expression of at least one allele of Foxp1 is sufficient for repressing CD44 expression. These results indicate that Foxp4 expression has no effect on aberrant expression of CD44 on SP thymocytes in $Foxp1^{cKO}$ or cDKO mice. Thus, regulation of CD44 expression in thymocytes represents a non-overlapping role of Foxp1 and Foxp4 in thymic development.

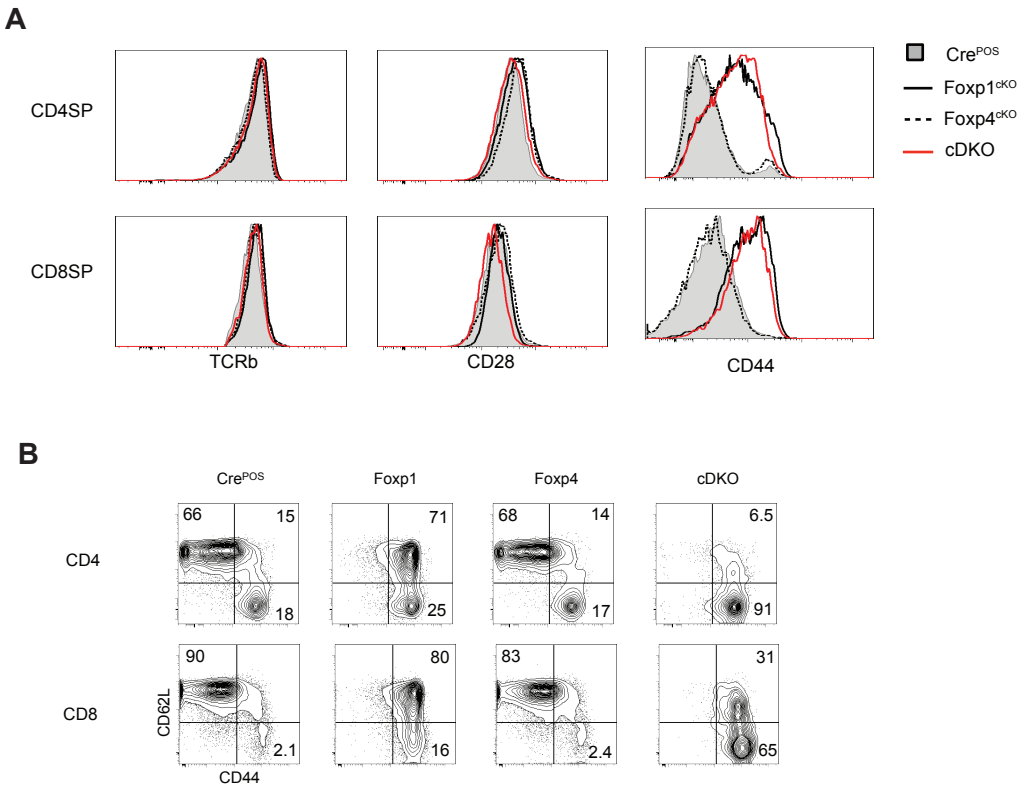


Figure 3.6. cDKO SP thymocytes and splenic T cells express T cell signaling proteins and markers of activation. A) CD4SP (top panels) and CD8SP (bottom) thymocytes from Cre^{POS} (solid histograms), Foxp1^{cKO} (black line), Foxp4^{cKO} (black dotted line), cDKO (red line) express comparable levels of TCRβ, CD28 (left panels). Foxp1^{cKO} and cDKO SP thymocytes express abnormal levels of CD44. B) Representative plots of Cre^{POS}, cDKO, and single cKO CD4 (top) and CD8 (bottom) T cell expression of markers of activation: CD44 and CD62L.

cDKO CD4 and CD8 T cells acquire an effector memory phenotype.

As observed in the thymus, Foxp1^{cKO} and cDKO T cells are CD44^{hi} in both spleen and LN. In addition to CD44, expression of CD62L is altered in both CD4 and CD8 cDKO T cells. In Cre^{POS} and Foxp4^{cKO} mice, 75%-90% of CD4 and CD8 T cells are CD62L^{hi} naïve lymphocytes. In general, we observed that 70%-85% of Foxp1^{cKO} CD4

and CD8 T cells are 70%-85% CD62L^{hi}, resembling a central memory (CM) phenotype. In contrast, approximately 10% of cDKO CD4 T cells are CD62L^{hi} CM, and 90%-95% CD4 T cells are CD62L^{low} (Figure 3.6B, top panels). CD62L expression in cDKO CD8 T cells is slightly different than CD4 T cells. CD8 T cells isolated from the spleen and LN of cDKO mice are 50%-70% CD62L^{low} (Figure 3.6B bottom panels, and data not shown). Taken together with the CD44^{hi} phenotype, the vast majority of CD4 and CD8 cDKO T cells exhibit an effector memory CD44^{hi}CD62L^{low} phenotype.

Loss of Foxp1/4 results in systemic T lymphopenia.

We reasoned it was possible the T lymphopenia we perceived in lymphoid tissues was actually due to changes in T cell migration. CD62L, or L-selectin, is responsible for trafficking of T cells into spleen and LN (Arbones et al., 1994). We investigated whether the total cDKO T cell population were intact but CD62L^{low} T cells were merely retained in peripheral tissues and excluded from total T cell numbers. To test this hypothesis, the frequency and numbers of T cells were calculated in multiple tissues in cDKO mice. An extensive systemic evaluation revealed reduced frequencies and numbers of T cells throughout cDKO tissues. T cells numbers in the lung, liver, peritoneal cavity, Peyers patches, mesenteric lymph nodes, the lamina propria of the small intestine and large intestine, blood, bone marrow, and skin were detected. Greater differences were found in the fold change and number of cDKO CD4 T cells than in CD8 (Figure 3.7). Compared to Cre^{POS} controls, frequencies and numbers of cDKO CD4 and CD8 T cell were significantly reduced. However, no tissues resembled a specific cDKO CD4 or CD8 T cell reservoir, to which peripheral lymphocytes migrate in cDKO mice.

Therefore, T cells are not selectively trafficking to extra-lymphoid tissues due to dysregulated CD62L expression; instead, cDKO mice exhibit systemic T lymphopenia.

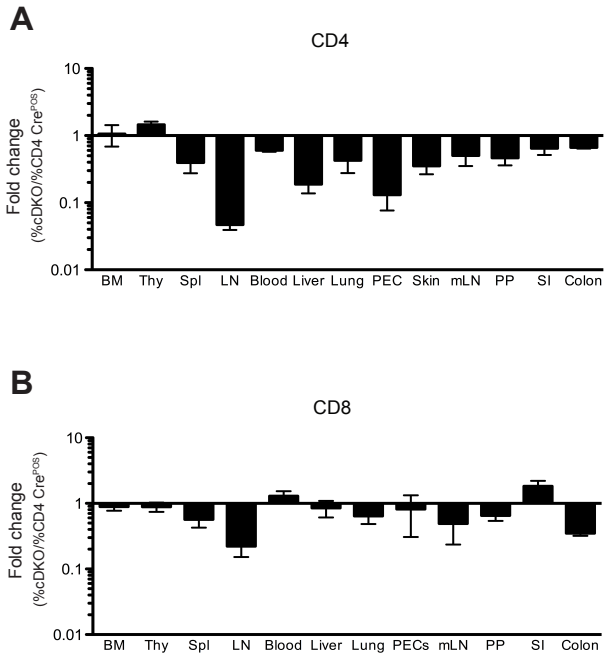


Figure 3.7. Reduced cDKO CD4 and CD8 T cells were detected in multiple tissues A-B) Percentage of conventional Foxp3- CD4 T cells (A) or (B) CD8 T cells, portrayed as fold change relative to Cre^{POS} littermate controls. Fold changes of CD4 or CD8 T cells were assessed in bone marrow (BM), thymus (Thy), spleen (Spl), pooled axillary, cervical and inguinal lymph nodes (LN), peripheral blood, liver, lung, peritoneal exudate cells (PECs), skin, pooled mesenteric lymph nodes (mLN), pooled Peyers patches (PP), small intestine lamina propria (SI), or lamina propria of large intestine (colon). Mean and standard deviation calculated from three independent experiments.

Lymphopenia can induce T cell proliferation and differentiation due to excess cytokine availability, which can stimulate the expansion of both CD4 and CD8 T cells. Expanded T cells assume a memory-like phenotype, including increased CD44 expression, similar to what was observed in $Foxp1^{cKO}$ and cDKO mice. To determine

whether cDKO lymphocytes exhibited lymphopenia-induced proliferation and were proliferating abnormally *in vivo*, we assessed turnover using Ki-67, a cytoplasmic marker of cell cycle. Frequencies of Ki-67+ CD4 and CD8 splenocytes were comparable between cDKO and Cre control mice; importantly, there was also no difference when comparing $CD44^{hi}$ populations, which typically include expanding cells (data not shown). Because Cre^{POS} mice are not lymphopenic, these data suggest $CD44^{hi}$ cDKO T cells are circulating in lymphopenic physiologic conditions, but do not exhibit increased lymphopenia-induced proliferation. However, we have not excluded other explanations for normal Ki-67 staining, such as cell-extrinsic effects or simply increased death of dividing cDKO cells. Taken together, these data indicated cDKO T cells were detected in reduced numbers and acquired altered expression of cell surface proteins associated with activation. However, double knockout cells failed to proliferate as would be expected of lymphocytes in lymphopenic conditions.

Cell-intrinsic regulation of T cell development by FoxP proteins.

In cDKO mice, young mice exhibited substantial T lymphopenia, and the remaining T cells exhibited an effector memory $CD44^{hi}CD62L^{low}$ phenotype. To assess whether the T cell phenotype in cDKO mice is caused by loss of Foxp1/4 expression, or an effect of increased lymphopenia, we generated competitive bone marrow chimeras. Briefly, bone marrow from cDKO mice and from congenically marked wild-type mice was mixed in a 90:10 ratio, respectively, to give cDKO progenitors an advantage during reconstitution of lethally irradiated congenically unique host mice (Figure 3.8A). cDKO:WT chimeras were compared to similarly generated chimeras given Cre^{POS},

Foxp1^{cKO}, or Foxp4^{cKO} bone marrow. Eight weeks after reconstitution, T cell generation in the chimera cohorts was examined.

After reconstitution, the frequency of wild-type CD45.1+ and donor-derived CD45.2+ B cells in bone marrow was determined (Figure 3.8B and data not shown). B cells are not affected by CD4Cre, and therefore should represent wild-type- and donor-derived populations from grafted bone marrow progenitors. Across three independent experiments the ratio of CD45.1+ and CD45.2+ B cells and other myeloid populations in bone marrow and spleen were approximately 90:10, allowing further examination of the effects of Foxp1 and Foxp4 deletion on T cell development in a competitive system.

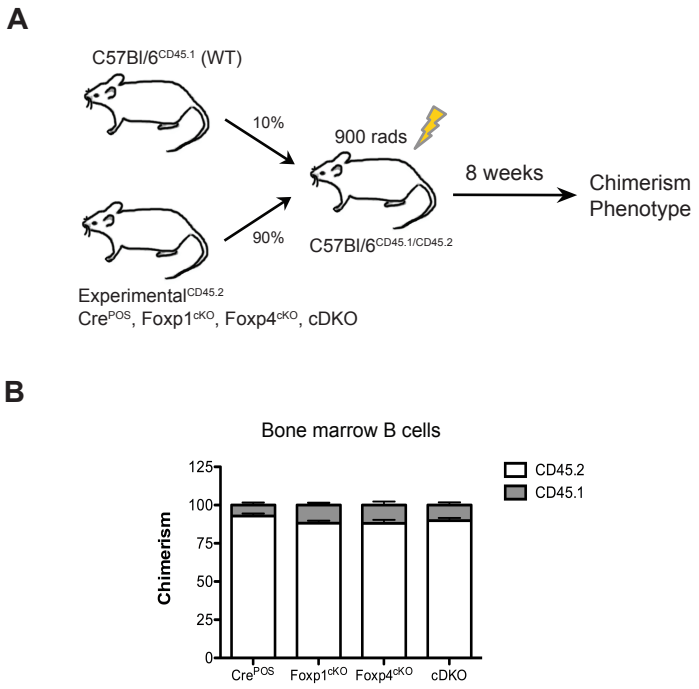


Figure 3.8. Schematic of generation of competitive bone marrow chimeras. A) Schematic diagram of mixed bone marrow chimera generation. B) Chimerism of CD45.2+ (white) and CD45.1+ (gray) cells gated from CD19+ B cells in bone marrow of host mice reconstituted with Cre^{POS}, Foxp1^{cKO}, Foxp4^{cKO}, or cDKO bone marrow in Compiled from five mice in each cohort.

Deletion of FoxP proteins did not inhibit T cell generation, as mature DP and SP thymocytes develop at approximately the expected ratio in competitive chimeras (Figure 3.9A). The relative contribution of cDKO and wild-type progenitors to DP, CD4SP and CD8SP thymocyte populations was calculated from the ratio of CD45.2+ and CD45.1+ cells in each population (Figure 3.9B). Chimerism of developing thymocyte populations was approximately the expected 90:10 ratio across all mixed bone marrow chimera cohorts.

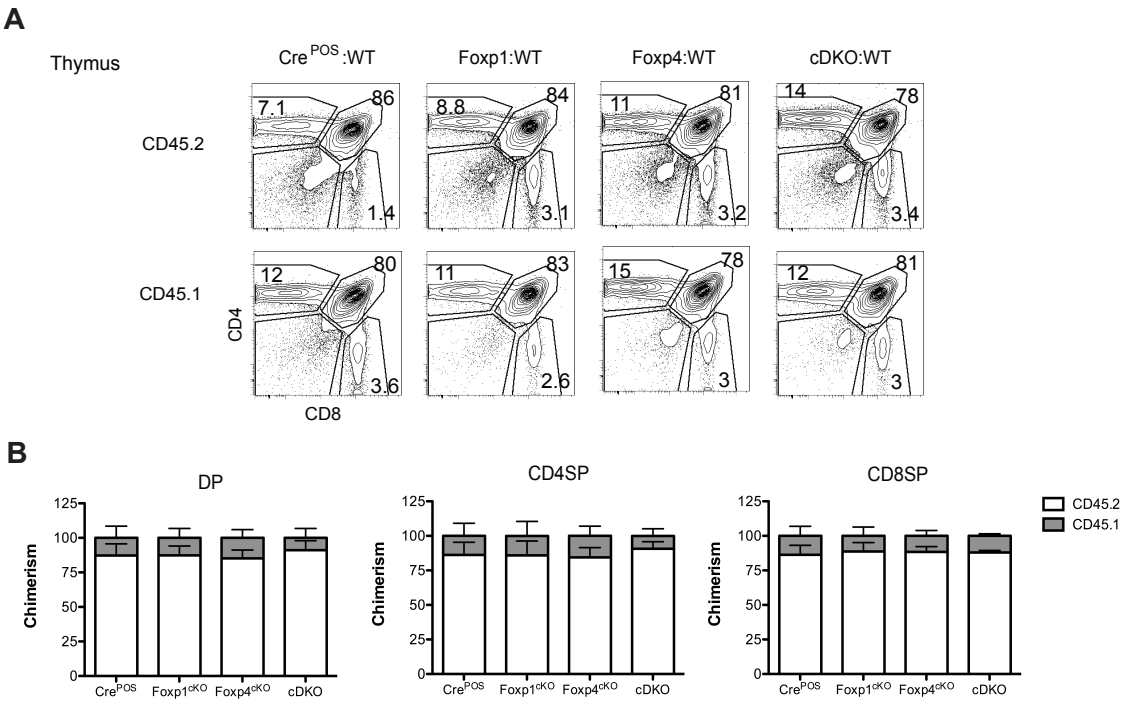


Figure 3.9. Competitive development of cDKO DP and SP thymocytes in mixed bone marrow chimeras. A) Representative plots of thymocytes gated on CD45.2+ or CD45.1+ expression, then for CD4 and CD8 expression to determine DP, CD4SP and CD8SP populations. Numbers indicate frequency of gated cells. B) Compiled chimerism of indicated populations. Average and standard deviation of the frequency of CD45.2+ and CD45.1+ thymocytes comprising total DP, CD4SP or CD8SP populations.

Although SP thymocytes exhibited comparable chimerism, Foxp1 or Foxp4 deletion conferred competitive disadvantages in peripheral lymphoid tissues of mixed chimeras. CD45.2+ CD4 and CD8 T cells were detected in peripheral tissues of single (Foxp1- or Foxp4-deficient) and double knockout mixed chimeras. However loss of Foxp1 or Foxp4 resulted in significantly reduced frequencies compared to Cre^{POS} controls (Figure 3.10A, B). Cre^{POS} CD4 and CD8 splenic T cells develop normally, but loss of either Foxp1 or Foxp4 resulted in decreased competitive chimerism from 9:1 to a 1:1 ratio of CD45.2+ $Foxp1^{cKO}$ or $Foxp4^{cKO}$ to CD45.1+ WT. Combined deletion of Foxp1 and Foxp4 resulted in the greatest disadvantage. Cre^{POS} chimeras exhibited approximately 3:1 Cre^{POS}:WT T cells in splenic populations, perhaps due to the effects of Cre expression, which can become toxic. In contrast, cDKO chimeric splenic ratios were inverted, and averaged approximately 1:3 cDKO:WT T cells.

Similar to findings in non-chimeric mice, CD45.2+ cDKO T cells are found in lower frequencies in chimeric LN than in spleen. (Figure 3.10C, D). Inguinal, axillary, and brachial lymph nodes were pooled to assess the contribution of cDKO T cells in LN from cDKO:WT chimeras. cDKO CD4 T cells were vastly outnumbered by WT lymphocytes. In contrast to the 3:1 ratio of CD45.2+:CD45.1+ T cells in control chimeric LN, CD4 T cells averaged 1:12 cDKO:WT (Figure 3.7C). Similarly, cDKO CD8 T cells constitute only 10%-12% (or ~1:10) of all LN CD8s, while Cre^{POS} controls averaged >70% chimerism (Figure 3.10D). Therefore, CD4 and CD8 cDKO T cells are not only reduced compared to endogenous WT populations, but also compared to cDKO T cells in chimeric spleens. These data recapitulate reduced cDKO cell numbers in the LN and

spleen of non-chimeric mice, and demonstrate cell-intrinsic FoxP-protein regulation of competitive T cell development and maintenance.

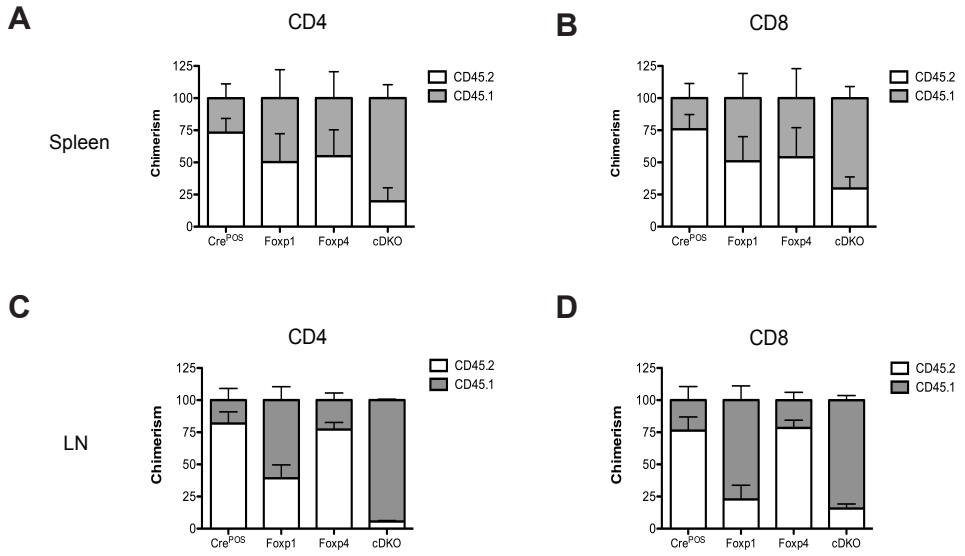


Figure 3.10. Foxp1^{cKO}- or cDKO-derived CD4 and CD8 T cells have a developmental disadvantage in peripheral lymphoid tissues in competitive bone marrow chimeras. A-B) Average chimerism of splenic CD4 (A) or CD8 (B) T cells. Frequency of CD45.2+ and CD45.1+ cells gated comprising chimeric populations was averaged from 15 Cre^{POS}, 10 Foxp1^{cKO}, 10 Foxp4^{cKO}, and 15 cDKO bone marrow chimeras. Standard deviation denoted by +/- error bars. C-D) Average chimerism was similarly calculated as A-B) from T cell populations in pooled axillary, brachial, and inguinal lymph nodes from mixed bone marrow chimeras. Mean and standard deviation is shown.

Cell-intrinsic regulation of T cell central and effector memory phenotype

To assess whether CD44 was intrinsically regulated in cDKO cells, chimeric thymi were stained for cell surface expression of phenotypic markers. CD45.2+ Cre^{POS} and Foxp4^{cKO} thymocytes are CD44^{low}, comparable to the congenically-marked wild-type thymocytes. Foxp1^{cKO}- or cDKO-derived CD4SP and CD8SP thymocytes are CD44^{hi}, similar to CD44 expression observed in non-chimeric mice (Figure 3.11A). In

Foxp1^{cKO}:WT and cDKO:WT chimeras, endogenous wild-type-derived thymocytes remained CD44^{low}, which demonstrates the dysregulation of CD44 in Foxp1^{cKO} and cDKO mice is specific to T cells lacking Foxp1. Therefore, dysregulated CD44 expression is a cell-intrinsic finding in Foxp1-deficient cells, which occurs independently of Foxp4 expression.

In competitive bone marrow chimeras, lymphopenia does not develop. Developing wild-type cells simultaneously fill the homeostatic niche in peripheral lymphoid tissues, which prevents the development of lymphopenia. Generation of cDKO:WT bone marrow chimeras allowed us to assess whether the CD62L^{lo} effector memory T cell phenotype observed in non-chimeric cDKO mice was caused by T lymphopenia and lymphopenia-induced proliferation. The cell surface phenotype of peripheral T cells in mixed bone marrow chimeras was compared to endogenous WT competitor cells and to CD45.2+ cells in other cohorts.

Like non-chimeric mice, Foxp1^{cKO} and cDKO T cells in the spleens and LN of chimeric mice are CD44^{hi}. These data demonstrated that cDKO T cells are not merely lymphopenia-induced memory-like cells, but maintain the CD44^{hi} phenotype observed in non-chimeric mice. Furthermore, cDKO CD4 and CD8 T cells express reduced CD62L, as observed in non-chimeric cDKO mice (Figure 3.11B). cDKO-derived CD4 T cells are consistently 90-95% CD44^{hi}CD62L^{low} in cDKO:WT chimeras, and 40-60% of chimeric cDKO CD8 T cells are CD62L^{low}. Moreover, T cells in peripheral LN, blood, bone marrow and mesenteric LN retained an EM phenotype (data not shown). Therefore, combined deletion of Foxp1 and Foxp4 results in an effector memory T cell phenotype, and significant defects in peripheral lymphocyte competition. The phenotypic and competitive

defects described in this section are caused by cell intrinsic dysregulation and by loss of transcriptional regulation by Foxp1 and Foxp4.

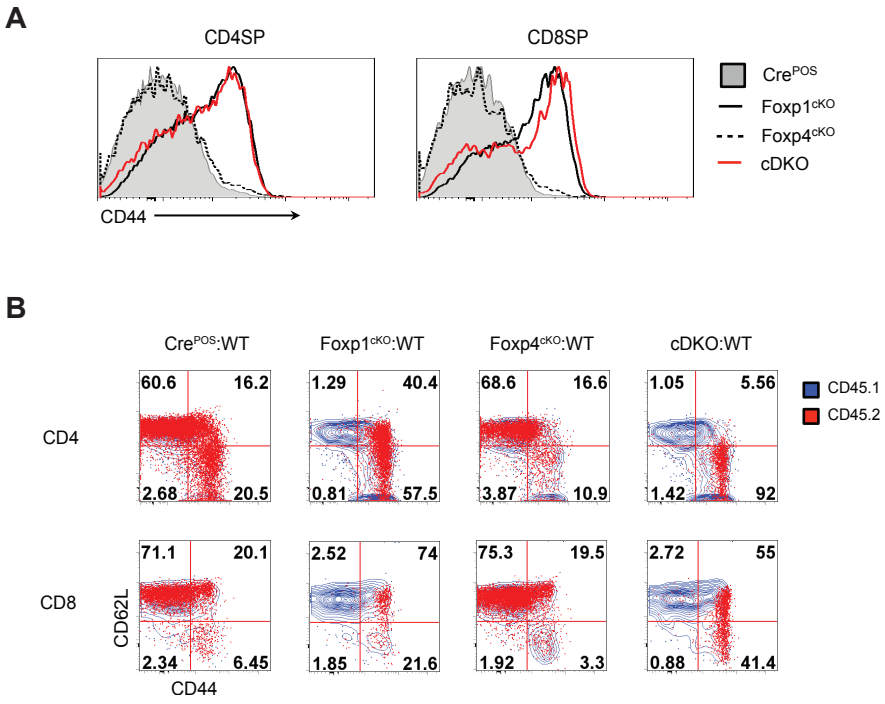


Figure 3.11. Expression of effector memory cell surface markers are intrinsically regulated by Foxp1 and Foxp4. A) Representative staining of CD44 expression on CD4SP (left) and CD8SP (right) from CD45.2+ Cre^{POS} (gray histogram), Foxp1^{cKO} (black line), Foxp4^{cKO} (dotted line), or cDKO (red line). Representative of at least 5 chimeras in each cohort. B) Representative staining of CD44 and CD62L expression on CD4 (top panels) or CD8 (bottom) splenic T cells from Cre^{POS}:WT, Foxp1^{cKO}:WT, Foxp4C+:WT or cDKO:WT competitive bone marrow chimeras. CD45.2+ experimental CD4 or CD8 T cells were gated and overlaid with respective endogenous CD45.1+ wild-type T cells from each mouse. Flow plots representative of 15 Cre^{POS}:WT, 9 Foxp1^{cKO}:WT, 10 Foxp4^{cKO}:WT, and 15 cDKO:WT chimeras.

Foxp1 regulates T cell effector functions.

The CD44^{hi} memory phenotype is associated with increased effector activity in T cells (Goldrath et al., 2000; van Leeuwen et al., 2009). Cytokine production is a key effector function of the adaptive immune system. To investigate how Foxp1 and Foxp4

regulate cytokine production in $CD44^{hi}$ CD4 and CD8 T cells, splenocytes from cDKO mice and littermate controls were stimulated with phorbol myristate acetate (PMA) and ionomycin immediately *ex vivo*. Monensin was added to prevent cytokine export. After a four-hour stimulation, cells were stained intracellularly for production of interferon gamma (IFNγ), tumor necrosis factor alpha (TNFα), interleukin-2 (IL-2), and interleukin-17 (IL-17).

Foxp1 regulates cytokine responses in CD4 T cells.

We first assessed cytokine production in stimulated T cells. To control for frequencies of $CD44^{hi}$ T cells in $Foxp1^{cKO}$ and cDKO mice, all samples were previously gated on $CD44^{hi}$ T cells. $Foxp1^{cKO}$ CD4 T cells exhibited significantly decreased IFNγ production, and three-fold increased frequencies of IL-2-producing CD4 T cells (Figure 3.12A-C). These findings agree with the initial studies of Foxp1, which demonstrated this forkhead protein represses IL-2 gene expression by directly binding the *il2* promoter (Wang et al., 2003). Subsequent work by *Feng, et al*, first demonstrated IFNγ production was dysregulated in Foxp1-deficient CD4 T cells (Feng et al., 2010). Abnormal IFNγ and IL-2 production by $Foxp1^{cKO}$ CD4 T cells was also observed throughout our studies.

These findings were extended to include TNFα and IL-17 production. The frequency of TNFα-producing CD4 T cells from $Foxp1^{cKO}$ mice is also increased two-fold relative to Cre^{POS} and $Foxp4^{cKO}$ CD4 T cells (Figure 3.12A, compiled data not shown). Additionally, the frequency of TNFα+IL2+ T cells is increased compared to Cre controls (Figure 3.14A bottom panels, and data not shown), suggesting $Foxp1^{cKO}$ T cells exhibit increased polyfunctionality.

Production of IFNγ and IL-17 is regulated by independent transcription factors, which antagonize one another. We reasoned loss of IFNγ production in Foxp1^{cKO} T cells could be caused by dysregulated differentiation into IL-17-producing T helper 17 (Th17) CD4 T cells. In Th17 cells, RORγT silences Th1 transcription factors responsible for IFNγ production. To test whether the majority of Foxp1^{cKO} CD4 T cells have acquired Th17 function, we assessed IL-17 production immediately *ex vivo*. Similar to IFNγ production, IL-17 responses are significantly reduced in Foxp1^{cKO} mice. Therefore, Foxp1 does not appear to suppress Th17 differentiation in CD4 T cells, and loss of IFNγ production is not a result of aberrant T helper differentiation.

In contrast, Foxp4 deletion alone did not produce the same defects. CD44^{hi} Foxp4^{cKO} CD4 T cell cytokine responses were similar to Cre^{POS} controls, indicating initial regulation of effector functions does not require Foxp4-dependent transcriptional regulation (Figure 3.12A-B). Furthermore, there were no significant differences between CD44^{hi} CD4 T cell responses from Cre^{POS}, cDHET, or 1H4K mice (data not shown).

Reduced IL-17 production in Foxp4^{cKO} T cells relative to Cre^{POS} controls was also observed (Figure 3.12D). In Chapter 2 we assessed Th17 lineage commitment in polarized Foxp4^{cKO} T cells, and found indications that Foxp4 may repress IL-17 and IL-2 production. In contrast, these experiments represent the frequency of natural Th17 cells immediately *ex vivo*. Together, these data indicate FoxP members may be important for generation of natural Th17 cells as well as Th17 differentiation of effector cells *in vitro*.

Whether Foxp4 contributed to effector gene regulation in Foxp1-deficient T cells remained unclear. To assess the effects of Foxp4 gene dosage on Foxp1^{cKO} T cell effector functions, stimulated splenocytes from Foxp1^{cKO}, 1K4H, and cDKO mice were compared (Figure 3.12B-D). Deletion of Foxp1 in combination with loss of one or both

alleles of Foxp4 does not alter the Foxp1^{cKO} profile of cytokine production. cDKO T cell cytokine responses resemble Foxp1^{cKO} patterns. 1K4H cells reiterated the same dysfunctional cytokine profile as Foxp1^{cKO} and cDKO cells. These data suggest that Foxp4 does not compensate for loss of Foxp1 in the regulation of cytokine programs. Foxp1 deletion elicits a dominant effector phenotype, regardless of Foxp4 expression.

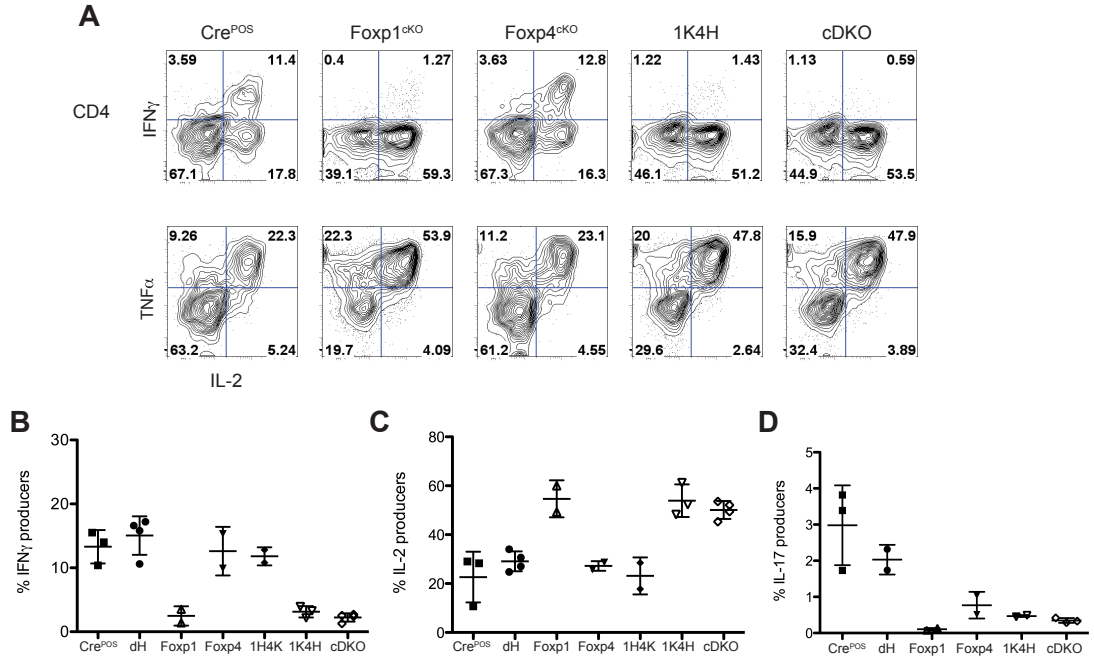


Figure 3.12. Foxp1, but not Foxp4, regulates IL-2 and IFNγ production by CD4 T cells. A) Representative plots of splenic CD4 T cells from cDKO and littermate controls. Splenocytes were stimulated in vitro with PMA and Ionomycin and stained intracellularly for cytokine production. B-D) Compiled frequencies of CD4 T cells producing B) IFNγ, C) IL-2, or D) IL-17 immediately *ex vivo*. Results from three independent experiments.

To determine whether altered cytokine production is caused by cell intrinsic defects in Foxp1-deficient lymphocytes, splenocytes from mixed bone marrow chimeras were stimulated *ex vivo* as described above. In competitive chimeras, nearly the same

pattern of cytokine expression develops. A higher frequency of CD45.2+ CD4 Foxp1- or cDKO-derived T cells produced TNFα, while IFNγ+ cells were essentially undetected (Figure 3.13A, and data not shown). Frequencies of IFNγ-producing CD4 T cells were comparable in CD45.2+ Cre^{POS}, $Foxp4^{cKO}$, and respective CD45.1+ wild-type controls (Figure 3.13B and data not shown). These findings indicate Foxp1 regulation of IFNγ is part of a cell-intrinsic program. IL-2 regulation is also an intrinsic effect of Foxp1 regulation. The frequency of IL-2-producing cDKO-derived CD4 T cells was increased two-fold compared to Cre^{POS} wild-type endogenous CD4 T cells. (Figure 15C, and data not shown).

Interestingly, TNFα expression may reflect cytokine regulation by extrinsic pressure in non-chimeric cDKO mice. In mixed chimeras, the proportion of TNFα+ cDKO T cells was increased compared to endogenous wild-type cells, but not as strikingly as observed in non-chimeric responses. Moreover, CD45.2+ cDKO TNFα production was relatively comparable to that of Cre^{POS} cells in Cre^{POS}:WT chimeras (Figure 3.13D and data not shown). Therefore, TNFα production may be regulated by non-cell-autonomous mechanisms in $Foxp1^{cKO}$ and cDKO mice.

Therefore, these data suggest Foxp1 normally represses IL-2 production, and induces IFNγ and IL-17. Together with non-chimeric cDKO CD4 T cell responses, we can conclude that loss of Foxp1, and not Foxp4, is responsible for dysregulation of several cytokine genes in CD4 effector cells.

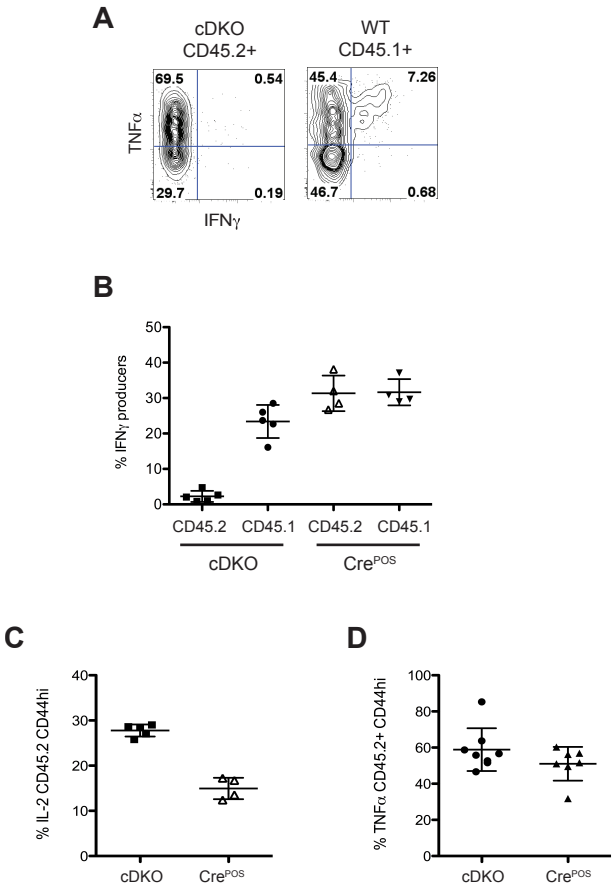


Figure 3.13. Altered CD4 T cell cytokine production reflects cell-intrinsic Foxp1-dependent regulation of IFNγ and IL-2. A) A) Representative plots of chimeric splenocytes stimulated immediately *ex vivo* and stained intracellularly for IFNγ and TNFα production. Plots are gated on CD45.2+ cells from a representative Cre^{POS}:WT or cDKO:WT competitive chimera. Numbers in each quadrant represent relative frequency. B) Relative frequency of IFNγ-producing CD4 T cells from Cre^{POS}:WT or cDKO:WT chimeras, compiled from two independent experiments. Each point represents cells gated on CD45.2+ (Cre^{POS} or cDKO) or CD45.1+ (endogenous wild-type cells) from each chimera. C-D) Compiled relative frequencies of C) IL-2-producing or D) TNFα-producing CD45.2+ cDKO or Cre^{POS} CD4 splenic T cells from chimeric mice. Each point represents one competitive chimera. Combined from two independent experiments.

Effects of Foxp1 and Foxp4 deletion on cytokine responses in CD8 T cells.

We next determined how Foxp1 and Foxp4 deletion affected CD8 T cell cytokine production. Stimulation of CD8 T cells reveals substantial differences in FoxP family

member regulation, compared to CD4 T cell function. In Cre^{POS}, $Foxp4^{cKO}$, 1H4K, and cDHET controls $CD44^{hi}$ CD8 T cells exhibited similar production of IFNγ, IL-2, TNFα, or IL-17 (Figure 16A and data not shown). Unlike $Foxp1^{cKO}$ responses in CD4 T cells, IFNγ and IL-2 production by $Foxp1^{cKO}$ CD8 T cells is comparable to controls (Figure 3.14A, C). Lastly, Foxp1- or Foxp4-deficient CD8 T cells are not IL-17 producers, demonstrating that Th17 regulation in CD8 T cells is intact without Foxp1 or Foxp4 regulation (Figure 3.14D). Taken together with CD4 cytokine responses, these findings emphasize the differences in Foxp1 regulation between CD4 and CD8 T cells. IFNγ and IL-2 gene regulation is vastly different in these two T cell subsets.

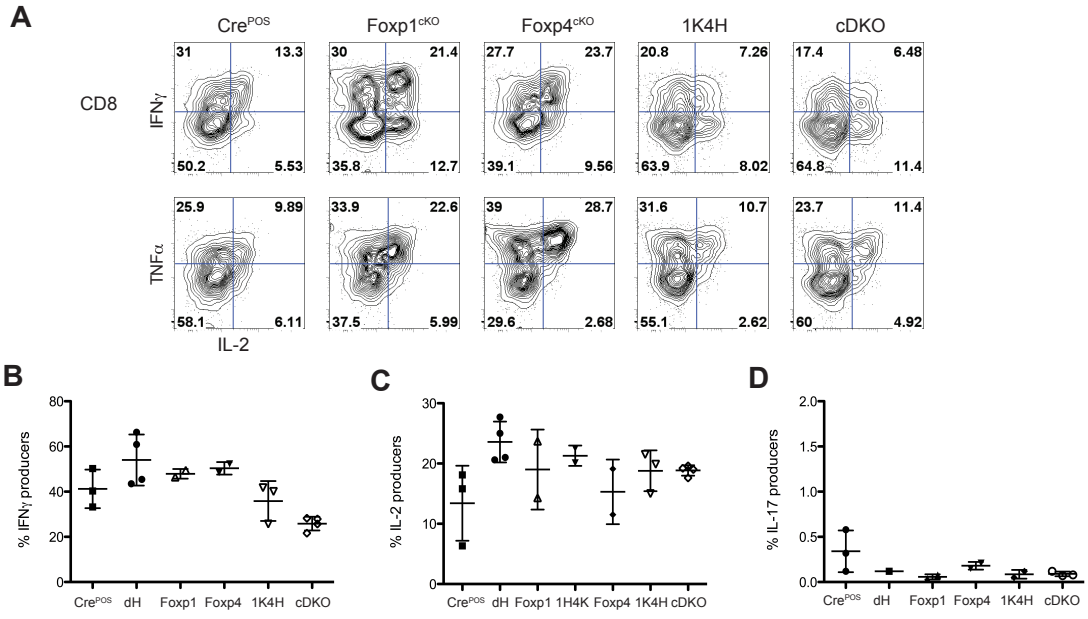


Figure 3.14. Decreased frequencies of cDKO CD8 T cells produce IFNγ. A) Representative plots of splenic CD8 T cells from cDKO and littermate controls. Splenocytes were stimulated *in vitro* and stained as in Figure 14, and gated on CD8 T cells. B-D) Compiled frequencies of CD8 T cells producing B) IFNγ, C) IL-2, or D) IL-17 immediately *ex vivo*. Results from three independent experiments.

While IL-2 and IL-17 responses did not seem grossly affected, deletion of both Foxp1 and Foxp4 resulted in reduced frequencies of IFNγ+ CD8 T cells. A dose-dependent effect of Foxp4 deletion in Foxp1-deficient CD8s results in fewer IFNγ-producing T cells in both 1K4H and cDKO mice (Figure 3.14A-B). cDKO CD8 T cell production of TNFα was also diminished relative to littermates. One possibility was that IFNγ and TNFα are gene targets of Foxp1/4 in cDKO CD8 T cells.

To determine whether TNFα and IFNγ production was altered due to cell intrinsic effects of Foxp1/4 deletion, cytokine responses by CD45.2+ Cre^{POS}, single cKO, or cDKO CD8 T cells from bone marrow chimeras were compared. As observed in non-chimeric responses, IL-2 and IL-17 production were not significantly different across cohorts or compared to endogenous WT cells (data not shown). However, in contrast to non-chimeric CD8 T cell functions, TNFα and IFNγ responses were not significantly reduced in mixed chimeras (Figure 3.15). Frequencies of cytokine-producing CD45.2+ CD8 T cells and wild-type CD45.1+ cells from cDKO:WT mixed chimeras were similar (Figure 3.15A-B, and data not shown). Differences between non-chimeric and chimeric experiments may indicate selective pressure on effector CD8 T cells for survival.

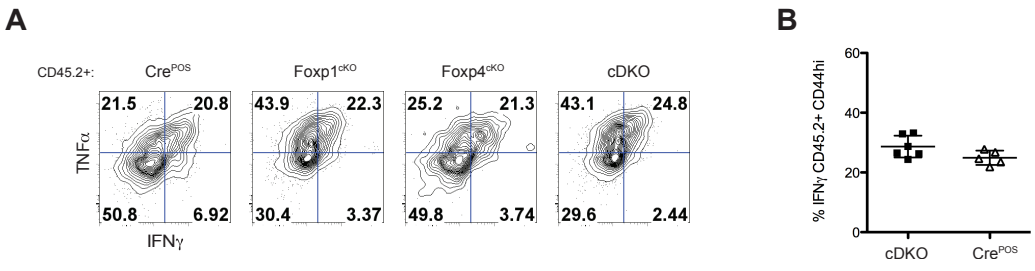


Figure 3.15. Competitive chimeras reveal comparable frequencies of cDKO CD8 T cells produce IFNγ. A) Representative plots of chimeric splenocytes stimulated immediately *ex vivo* and stained intracellularly for IFNγ and TNFα production. Plots are gated on CD45.2+ cells from each chimeric cohort. Numbers in each quadrant represent

relative frequency. B) Compiled relative frequencies of IFNγ-producing CD45.2+ cDKO or Cre^{POS} CD8 splenic T cells from chimeric mice. Each point represents one competitive chimera. Combined from two independent experiments.

Taken together, $Foxp1^{cKO}$ CD4 and CD8 T cell function indicates how Foxp1 orchestrates a complex network of effector gene regulation. A simplified summary of cDKO CD4 and CD8 T cell cytokine responses is included below (Table 3.2). This network is significantly different in CD4 and CD8 T lineage programs, and invites several avenues of investigation. Foxp4 expression does not appear to be required for effector cytokine production, and cannot compensate for loss of Foxp1 in 1K4H or cDKO CD4 T cells. Thus, Foxp1 and Foxp4 maintain non-overlapping regulatory roles in CD4 and CD8 T cell effector function.

CD4 T cell

	Increase/ Decrease (Relative to Cre^{POS})	Cell intrinsic
IFNγ	Decreased	Yes
IL-17	Decreased	Yes
IL-2	Increased	Yes
TNFα	Increased	No

CD8 T cell

	Increase/ Decrease (Relative to Cre^{POS})	Cell intrinsic
IFNγ	Decreased	No
IL-17	No change	Yes
IL-2	No change	Yes
TNFα	No change	Yes

Table 3.2. Summary of cDKO CD4 and CD8 T cell cytokine production *in vitro*.

FoxP transcription factors regulate IL-7R expression and cytokine signaling.

In both non-competitive and chimeric conditions, we determined cDKO mice possessed reduced T cell numbers. Lower numbers of T cells could be a result of impaired development, poor survival, or both. Decreased numbers of T cells under

lymphopenic conditions generally consume reduced quantities of cytokine than an intact T cell compartment, resulting in increased availability of pro-survival factors, including IL-7. However it was possible that cDKO T cells not only developed abnormally, but also failed to detect survival and growth factors *in vivo*.

Recently, elegant studies of Foxp1-deficient T cells revealed how Foxp1 and another forkhead transcription factor, Foxo1, regulate a common target: IL-7Rα (Feng et al., 2011). Competition between Foxp1 and Foxo1 for forkhead consensus binding sites on the IL-7Rα promoter controls a critical cytokine signaling process that participates in naïve T cell maintenance. Because Foxp4-deficient T cells express normal levels of IL-7R, it appeared IL-7Rα was not a target of Foxp4 regulation (Figure 2.4E). But it was unclear whether increased IL-7R expression in Foxp1^{cKO} mice required any participation by Foxp4. Furthermore, it was unknown what the effect of combined Foxp1/4 deletion would be on IL-7R expression and subsequent responses to IL-7 signals.

To determine the combined effect of Foxp1/4 deletion on IL-7Ra expression, cDKO T cells from the thymus and spleen of non-chimeric mice were stained for IL-7Rα and compared to Foxp1^{cKO}, Foxp4^{cKO} and Cre^{POS} controls. DP thymocytes in Cre^{POS}, Foxp1^{cKO}, Foxp4^{cKO} or cDKO thymi do not express IL-7Rα, demonstrating that IL-7R is dysregulated only after SP development (data not shown). In agreement with published findings, Foxp1^{cKO} CD8SP expressed the highest levels of IL-7R. Once Foxp1^{cKO} CD4SP and CD8SP upregulate IL-7R, it remains at abnormally high levels on peripheral lymphocytes.

Surprisingly, IL-7R is not as highly expressed on cDKO T cells as observed in Foxp1^{cKO} controls (Figure 3.16A-B). Foxp1/4-deficient CD4SP and CD8SP thymocytes exhibited increased IL-7Rα, relative to Cre^{POS} and Foxp4^{cKO} mice. However, IL-7R

expression was not as increased on cDKO thymocytes as observed in Foxp1^{cKO} thymi (Figure 3.16A). Peripheral cDKO CD4 T cells expressed similar levels of IL-7R compared to Cre^{POS}, but CD8 T cells in cDKO mice maintained a slight increase in IL-7R over Cre^{POS} CD8 T cells. Like thymic CD8SP populations, peripheral cDKO CD8 T cells expressed significantly less IL-7Rα protein than Foxp1^{cKO} CD8 T cells (Figure 3.16B, bottom panel).

We considered that IL-7R complexes are internalized and removed from the cell surface after IL-7 ligation. We reasoned it was possible increased levels of IL-7 cytokine in lymphopenic cDKO mice could trigger increased IL-7R internalization, and cause the appearance of lower IL-7Rα expression. Initially, expression of another member of the IL-7R complex was examined to investigate whether total IL-7R complexes were internalized. Expression of the common gamma chain (γc chain) is necessary for IL-7R complex formation and IL-7 signaling (Figure 3.16B right panels, and data not shown). cDKO, single cKO, and Cre^{POS} T cells expressed normal levels of γc chain on the cell surface. It appeared altered IL-7Rα expression was not necessarily due to significantly increased internalization of the entire IL-7R complex.

cDKO T cells exhibit increased proximal STAT5 signals but poor survival.

IL-7R signaling is paramount to naïve T cell survival and maintenance. Early onset of lymphopenia in young cDKO mice, as well as failure to compete for survival in mixed chimeras, suggests poor maintenance of cDKO T cells. Like Foxp1^{cKO} mice, cDKO mice do not develop naïve T cells, and exhibit dysregulated IL-7R expression

compared to WT controls, but it was unclear whether cytokine signaling was intact in cDKO T lymphocytes.

Following IL-7:IL-7R ligation, intracellular signaling events initiate Janus kinase (JAK) and Signal Transducer and Activator of Transcription (STAT) phosphorylation. The JAK/STAT pathway is activated by γc chain signaling cytokines, including IL-7. Trans-phosphorylation of STAT5 protein dimers results in nuclear translocation and transcriptional regulation of STAT5 gene targets. In lymphocytes, STAT5 phosphorylation is measured to assess proximal IL-7 signaling. Other signaling events downstream of IL-7R ligation include kinase activity of PI-3K and downstream effects on both the Erk and Akt pathways; however, the studies described here focused on JAK/STAT signals.

To assess whether IL-7 cytokine signals were intact in cDKO T cells, we measured levels of phosphorylated STAT5 (pSTAT5) in thymocytes and splenocytes stimulated *in vitro.* pSTAT5 was compared Cre control cells treated similarly, as well as unstimulated cDKO T cells. Frequencies of pSTAT5+ cells were significantly increased in cDKO conventional CD4 and CD8 T cells stimulated with IL-7, but not in IL-7R^{NEG} DP (Figure 3.16C-D, and data not shown). Additionally, pSTAT5 levels on a per cell basis were also increased, suggesting more cDKO T cells responded to IL-7 stimulation and each cell exhibited increased levels of pSTAT5. Therefore, IL-7R and pSTAT5 responses are intact in cDKO T cells. Furthermore, increased pSTAT5 signaling is maintained in stimulated T cells from mixed chimeras (Figure 3.16E). IL-7 signaling in mixed cDKO and wild-type cells demonstrated increased intracellular levels of pSTAT5 in cDKO CD4 and CD8 T cells over endogenous WT control cells (data not shown).

Therefore, cDKO T cells exhibit increased proximal signaling to IL-7 stimulation, compared to Cre control cells.

IL-7 signals support lymphocyte survival by inducing anti-apoptotic proteins and protecting T cells from cell death. Theoretically, increased STAT5 signaling in cDKO T cells should induce increased Bcl-2 expression, and inhibit cell death. We assessed the ability of cDKO T cells to survive for 72 hours *in vitro* with either IL-7 or in unsupplemented media.

Increased STAT5 responses to γc chain cytokines did not protect cDKO T cells from cell death. In fact, cDKO T cells demonstrated poor survival after 24 hours in culture, and total live lymphocytes were dramatically reduced after 48 and 72 hours (Figure 3.16F). Precipitous loss of live T cell numbers indicated increased incidence of apoptosis. Levels of intracellular Bcl-2 protein in harvested cDKO T cells after 24 or 72 hours in culture were measured. cDKO T cells expressed substantially reduced Bcl-2, even though cDKO pSTAT5 levels were increased in previous studies (Figure 3.16G). These data suggest FoxP proteins may be important for linking STAT5 signals to Bcl-2 gene regulation, or possibly to stabilize expression of Bcl-2 protein.

Together these data suggest Foxp1 is required for normal IL-7Rα expression, but loss of Foxp1 and Foxp4 results in expression of relatively lower levels of IL-7Rα. However, cDKO T cells maintain higher IL-7Rα expression compared to Cre^{POS} controls. Responses to IL-7 stimulation indicate a proportionately increased pSTAT5 response, but failure to induce or maintain Bcl-2 expression leaves cDKO T cells vulnerable to apoptosis.

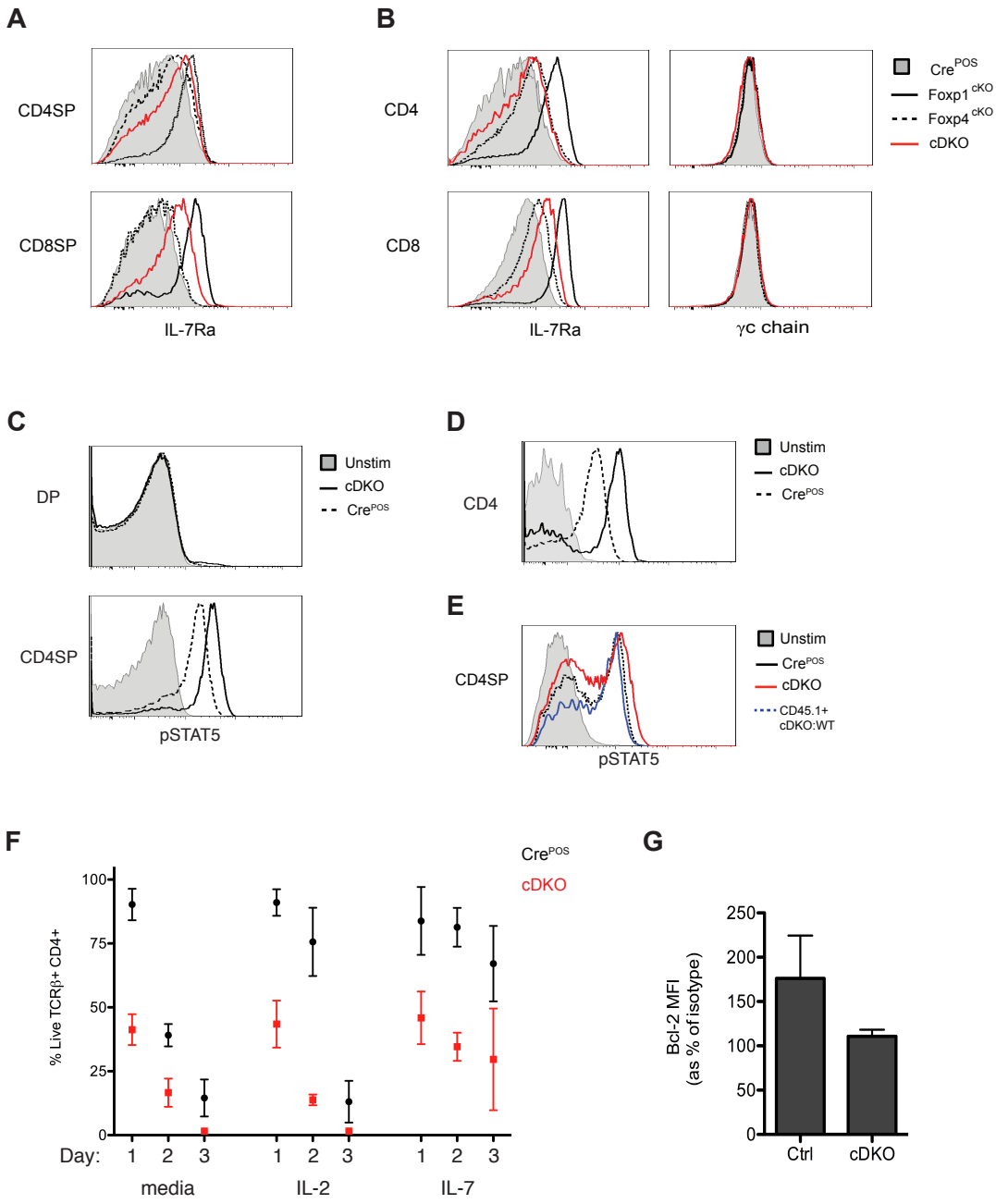


Figure 3.16. cDKO CD4 T cells exhibit increased STAT5 phosphorylation but decreased survival. A) Representative staining of IL-7Rα expression by CD4SP and CD8SP T cells. B) IL-7Rα expression by splenic CD4 and CD8 T cells (left panels). Right panels: Common gamma chain expression on splenic CD4 (top) and CD8 (bottom) T cells. C-D) Levels of phosphorylated STAT5 in thymocytes from Cre^{POS} (dotted line) or cDKO (black line) stimulated with IL-7 *in vitro*, or left unstimulated (gray histogram. C) Plots depict pSTAT5 responses by DP and CD4SP. D) pSTAT5 levels in CD4 splenocytes stimulated as in C). E) Levels of phosphorylated STAT5 in CD4SP thymocytes from Cre^{POS}:WT or cDKO:WT competitive chimera thymi. Whole unfractionated thymocytes were stimulated *in vitro* with IL-7, and stained intracellularly

for pSTAT5. CD4SP were gated out to compare to unstimulated, rested cells (gray histogram). F) Survival of live TCRβ+ CD4+ T cells from Cre^{POS} (black) or cDKO (red) mice cultured with IL-2, IL-7 or media alone for up to 72 hours. Each point represents one well harvested at indicated time points. G) Averaged mean fluorescence intensity of intracellular staining for Bcl-2 protein in live Cre^{POS} or cDKO CD4 T cells cultured for 72 hours.

Discussion

In Chapter III, the effects of combined deletion of Foxp1 and Foxp4 in T cells were investigated. In most cases, we found defects in cDKO T cells resembled the $Foxp1^{cKO}$ phenotype. Combined deletion of Foxp1/4 resulted in a slight but consistent reduction in thymic cellularity, which correlated with decreased numbers of DP thymocytes. Thymic development of cDKO SP is similar to $Foxp1^{cKO}$ thymocytes, and the $CD44^{hi}$ phenotype is observed in all cDKO SP and peripheral T cells.

Some features of the cDKO phenotype differed from $Foxp1^{cKO}$ mice. Cell-intrinsic differences in cell surface patterns of CD62L and IL-7R indicate Foxp1/4 combined deletion affects additional gene targets. T lymphopenia is significantly worse in cDKO mice compared to age-matched $Foxp1^{cKO}$ mice. In the periphery, cDKO T cells fail to maintain normal numbers in peripheral lymphoid tissues. Considering altered IL-7R and *in vitro* survival, reduced cell numbers in non-chimeric mice or competitive chimeras may be due at least partially to altered IL-7 signals and failure to induce Bcl-2 expression.

These data indicate cDKO T cell development is significantly impaired relative to single cKO T cells. The difference between cDKO and $Foxp1^{cKO}$ T cell numbers suggests that expression of Foxp4 in $Foxp1^{cKO}$ T cells partially rescues dysregulation of peripheral T cell development and maintenance. These studies indicate Foxp4 may be able to stabilize T cell populations in the absence of Foxp1 regulation. Further studies

are required to determine whether direct or indirect Foxp4 regulation of gene targets such as IL-7R or Bcl-2 occurs in Foxp1^{cKO} T cells.

From the data described here, several trends in Foxp1/4 regulation in T cells became apparent. First, CD4 and CD8 T cell requirements for FoxP family members suggests these two T cell subsets utilize Foxp1/4 to regulate different gene programs. cDKO CD4 T cells expressed lower levels of CD62L and IL-7R than CD8 T cells in the same mice. In contrast, CD8 T cells require Foxp1 expression for normal cell numbers, and total cDKO CD8 numbers illustrate more severe defects than in CD4 T cells. In mixed chimeras, however, competition by both CD4 and CD8 cDKO T cells was equally impaired. Together with increased frequencies of CD4SP cDKO thymocytes but not CD8SP, these data suggest FoxP members may have different roles in the CD4 and CD8 lineages.

Foxp1 and Foxp4 individually appear to regulate non-redundant gene programs in T cells. Importantly, cDKO findings support conclusions from Foxp1^{cKO} and Foxp4^{cKO} studies. As described in Chapter 2, the effects of Foxp4 deletion made no difference in T cell numbers in the periphery, but here we demonstrate Foxp1/4 combined deletion resulted in reduced CD8 T cells in peripheral tissues and an abnormal T cell phenotype. From these data, it is clear that Foxp1-dependent defects produce a dominant phenotype. Comparing cDKO and Foxp1^{cKO} mice reveals Foxp4 only partially compensates for Foxp1 deletion. Inclusion of 1H4K mice throughout these studies has demonstrated that expression of one allele of Foxp1 is sufficient to rescue control of effector gene programs and suppress effector T cell differentiation. Together these data underscore the importance of non-redundant roles for Foxp1 and Foxp4 regulation in T cells.

CHAPTER IV.

The FoxP family represses CD4 expression in T lymphocytes.

Introduction

The CD4 co-receptor is a transmembrane protein expressed in developing thymocytes and, later, exclusively on T cells restricted to recognize antigen presented on major histocompatibility complex Class II (MHC II) complexes (reviewed in (Bosselut, 2004; Singer et al., 2008). CD4 expression defines the subset of MHC II-restricted T cells called T helper cells, which differentiate to produce specialized cytokines in response to ongoing infection. The other major T cell subset of T cells expresses a different co-receptor, CD8, and recognizes MHC Class I antigen presentation. These CD8+ T cells do not express CD4 and acquire cytotoxic effector functions.

Several transcription factors interact to control commitment to CD4+CD8- T helper cell lineage or CD4-CD8+ cytotoxic T lineage. The network of transcription factors that dictates CD4 expression includes ThPok, Gata3, and Runx family proteins (Bosselut, 2004; Wang and Bosselut, 2009). These transcription factors interpret cytokine and TCR signals to allow CD4 helper T cells to maintain CD4 expression and to repress CD4 expression on CD8 T cells (Tani-ichi et al., 2013). Modulation of *cd4* by transcription factors requires access to the *cd4* promoter, and two other known

regulatory elements built into the *cd4* gene sequence. An upstream enhancer element promotes CD4, while a silencer motif downstream of the promoter is essential for CD8 T cell development (Xiong and Bosselut, 2012; Zou et al., 2001).

CD4 itself participates in T cell development and commitment through a complex feedback system. CD4 co-receptor is embedded in the plasma membrane and participates in signaling events both inside and outside of CD4+ T cells. Extracellular domains of the CD4 protein interact with MHC II complexes expressed by antigen-presenting cells to stabilize TCR:MHC II ligation (Gratton et al., 2000; Janeway, 1991). The intracellular tail of CD4 associates with protein kinases that initiate proximal signals necessary for T cell activation (Gascoigne and Palmer, 2011; Veillette et al., 1989; Wiest et al., 1993). Localization of proximal kinase Lck to the plasma membrane, near intracellular signaling domains of the TCR complex, shortens the interval between TCR ligation and initiation of T cell activation (Gascoigne and Palmer, 2011; Janeway, 1991). These CD4 functions shape the TCR signals that ultimately determine whether a T cell becomes a CD4+ T helper or CD8+ cytotoxic cell, or succumbs to cell death.

While characterizing cDKO T cells (described in Chapter 3), we noted a significant change in CD4 protein levels. Known transcription factor networks do not include FoxP family members, but our data suggest these proteins participate in the regulation of the *cd4* gene in both CD4 and CD8 T cells.

Results

cDKO CD4 and CD8 T cells express increased levels of CD4.

Increased CD4 expression was detected on cDKO thymocytes early in development, concomitant with Cre-mediated deletion. DP thymocytes express higher CD4 levels compared to Cre^{POS}, $Foxp1^{cKO}$, and $Foxp4^{cKO}$ controls (Figure 4.1A left panel). Mature CD4SP thymocytes also express increased CD4 expression, relative to Cre^{POS} and $Foxp1^{cKO}$ or $Foxp4^{cKO}$ cells. Moreover, cDKO CD4SP express increased levels of CD4 compared to DP in the same thymi, suggesting CD4 expression is dysregulated after deletion of Foxp1 and Foxp4, and increases with SP maturation of cDKO thymocytes (Figure 4.1A right panel, Figure 3.4A, C, and data not shown). $Foxp1^{cKO}$ CD4SP thymocytes express slightly increased levels of CD4, relative to Cre^{POS} and $Foxp4^{cKO}$ cells, but this trend did not reach statistical significance. CD4 protein levels were assessed by mean fluorescence intensity, and normalized to matched Cre^{POS} controls. CD4 was significantly upregulated on DP and CD4SP thymic populations (Figure 4.1B-C, and data not shown).

Peripheral CD4 T cells in cDKO mice also expressed increased levels of CD4 (Figure 4.1D). The majority (85-90%) of cDKO CD4 T cells expressed higher levels of CD4, relative to Cre^{POS} and littermate controls. The fraction of cDKO CD4 T cells expressing CD4 at control levels mostly consisted of Foxp3+ Tregs (data not shown). Mean fluorescence intensity of CD4 on cDKO CD4 T cells was increased compared to Cre^{POS} and littermate controls (Figure 4.1E). As observed in the thymus, loss of Foxp1 also led to increased levels of CD4 on CD4 T cells; however, cDKO thymocytes exhibited significantly more CD4 expression relative to $Foxp1^{cKO}$ T cells.

In contrast to CD4 T cells, most cDKO CD8 T cells were CD8+CD4-. However, approximately 15-20% of total peripheral CD8 T cells expressed higher levels of CD4 than control CD8 T cells (Figure 4.1C). We also noted a small percentage (5-10%) of CD8 T cells in Foxp1^{cKO} mice were also CD4^{dim}CD8 T cells. These CD4^{dim}CD8 T cells were consistently observed in CD8 populations throughout Foxp1^{cKO} and cDKO mice (data not shown). It was unclear whether this population was a stable subset of CD8 T cells, or whether CD4^{dim}CD8 T cells were associated with lymphopenia induced proliferation in T lymphopenic cDKO mice. Alternatively, this population of CD8 T cells may have escaped normal thymic selection, and were in fact MHC class II-restricted. To determine whether CD4 expression on both CD4 and CD8 T cells was a cell-autonomous occurrence, we investigated CD4 levels on T cells generated under competitive conditions in mixed bone marrow chimeras.

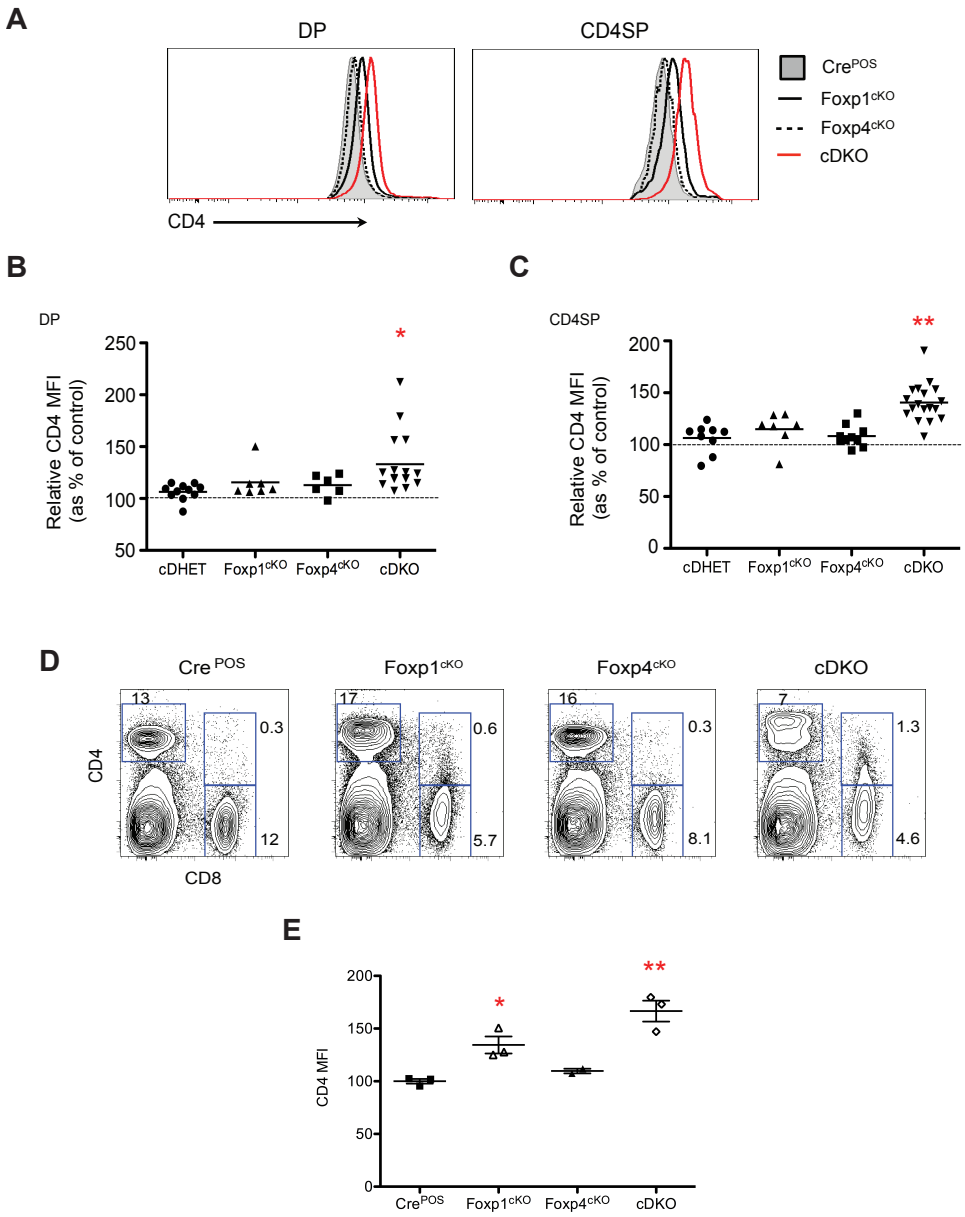


Figure 4.1. cDKO CD4 and CD8 T cells express increased levels of CD4 co-receptor. A) Representative histograms of CD4 expression on DP and CD4SP thymocytes from cDKO mice and littermate controls. Representative of 16 experiments. B-C) Mean fluorescence intensity (MFI) of CD4 staining on cDHET, Foxp1^{cKO}, Foxp4^{cKO}, and cDKO B) DP or C) CD4SP. CD4 MFI is relative to matched Cre^{POS} controls in every experiment. Data compiled from 16 independent experiments. Each point represents one mouse. D) Representative plots of CD4 expression on splenocytes from indicated mice. Relative frequency of CD4, CD8 and CD4^{dim} CD8 T cells of total, live splenocytes is indicated. E) Representative CD4 MFI from staining of CD4 splenic T cells from cDKO mice and littermate controls in one experiment. Mean and standard error of the mean is shown. Representative of 4 experiments. B-E) Statistical analyses by one-way ANOVA and Dunnett post-test. *p<0.05, **p<0.01.

CD4 is intrinsically regulated by FoxP family members.

CD4 regulation in both T lineage subsets proved to be a cell-autonomous phenomenon (Figure 4.2). Mixed bone marrow chimeras revealed significantly increased CD4 expression on Foxp1^{cKO}- and cDKO-derived DP and CD4SP (Figure 4.2A). Mean fluorescence intensity of CD4 staining illustrates specific dysregulation of CD4 expression on Foxp1-deficient thymocytes. Defects observed in cDKO-derived thymocytes were larger than in Foxp1^{cKO}-derived cells. In contrast, endogenous wild-type cells, wild-type Cre^{POS} and Foxp4^{cKO} controls expressed similar levels of CD4.

CD4 profiles of splenic cDKO-derived T cells resembled that of CD4 and CD8 T cells in non-chimeric cDKO mice. CD4^{hi} T cells and CD4^{dim}CD8 T cells were observed in CD45.2+ populations in cDKO:WT chimeras, but not in Cre^{POS}:WT or single cKO:WT chimeras, or in any CD45.1+ WT T cells (Figure 4.2C). CD4^{hi} and CD4^{dim}CD8 T cells were also observed in LN, peripheral blood, mesenteric LN, and bone marrow of cDKO:WT chimeric mice (data not shown). Together these data indicate increased CD4 is a cell-intrinsic defect caused by deletion of Foxp1 and Foxp4.

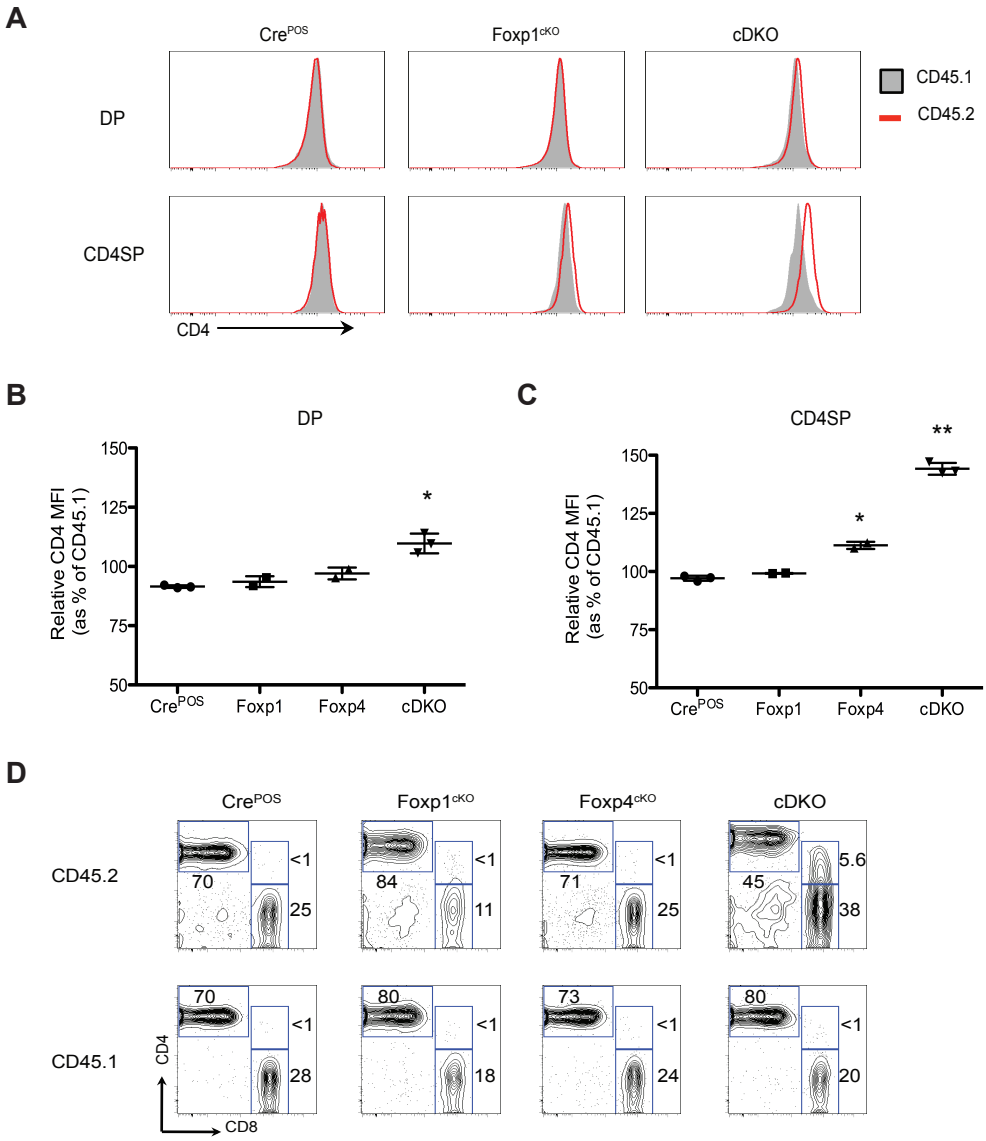


Figure 4.2. Increased CD4 expression is cell-intrinsically regulated by FoxP proteins. A) Representative histograms of CD4 expression on CD45.1+ WT (gray histogram) and CD45.2+ (red line) DP and CD4SP from mixed bone marrow chimeras. Representative of 5-10 chimeric mice in each cohort generated in 3 separate sets. B-C) Mean fluorescence intensity (MFI) of CD4 staining on CD45.2+ Cre^{POS}, $Foxp1^{cKO}$, $Foxp4^{cKO}$, or cDKO B) DP or C) CD4SP. CD4 MFI is normalized to endogenous CD45.1+ WT MFI in every chimeric mouse. Data is compiled from one experiment with 2-3 chimeras in each cohort. Mean and s.e.m. is shown. Each point represents one chimeric mouse. D) Representative plots of CD4 expression on splenocytes from chimeric mice. Plots are previously gated on live, singlet, TCRβ+, CD45.2+ (top) or CD45.1+ (bottom) lymphocytes. Relative frequency of CD4, CD8, and $CD4^{dim}$ CD8 T cells of total TCRβ+ splenocytes is indicated. Representative of 10-15 chimeric mice in each cohort.

CD4 is a transcriptional target of FoxP family regulation.

We next investigated whether total CD4 protein was increased in $CD4^{hi}$ T cells, or whether CD4 coreceptor was merely trafficked to the plasma membrane and inefficiently internalized. T cells were stained for cell surface CD4 protein, and then permeabilized to stain for CD4 intracellularly. Comparison of cells stained only for surface CD4 or for total protein indicates CD4 is not isolated to the cell surface of cDKO T cells. Intracellular and extracellular CD4 levels were higher than extracellular CD4 alone. Therefore, it does not appear that CD4 is sequestered on the cell surface. Instead, total CD4 expression in cDKO T cells was increased (Figure 4.3A).

To determine whether total levels of CD4 were increased due to increased *cd4* transcription, we assessed *cd4* mRNA transcript levels in sorted Cre^{POS}, $Foxp1^{cKO}$, $Foxp4^{cKO}$, and cDKO CD4 T cells. Relative quantification by real-time PCR demonstrated *cd4* transcript was increased 50-fold in cDKO CD4 splenic T cells (Figure 4.3B). Expression of *cd4* transcript was increased in $Foxp1^{cKO}$ or $Foxp4^{cKO}$ cells, relative to Cre^{POS} controls. $Foxp1^{cKO}$ CD4 T cells express >20-fold higher CD4 mRNA, and $Foxp4^{cKO}$ expression is 10-fold increased. Similar quantification of *cd4* transcript levels in $CD4^{dim}$CD8 T cells showed increased mRNA in cDKO cells as well (data not shown). These data demonstrate that repression of *cd4* requires FoxP family regulation. CD4 expression is most dysregulated in conventional cDKO T cells, but expression of either Foxp1 or Foxp4 can partially function to repress CD4 transcription, suggesting direct or indirect *cd4* transcriptional repression may be a shared target of these FoxP proteins.

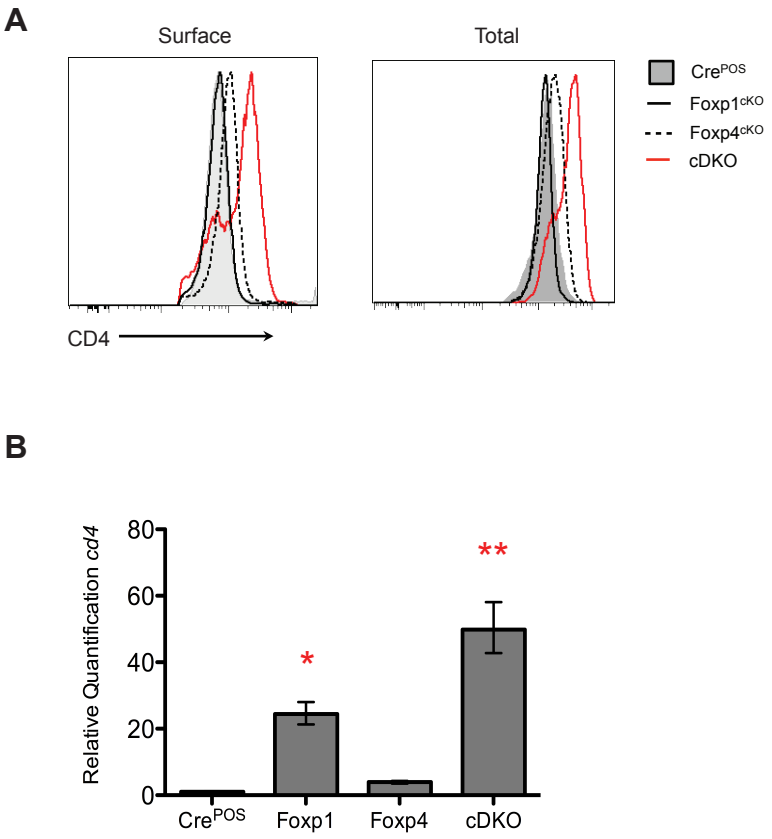


Figure 4.3. Total CD4 protein level and *cd4* transcript is increased in cDKO CD4 T cells. A) Representative histograms of surface CD4 expression and total (surface + intracellular) CD4 levels on Cre^{POS}, $Foxp1^{cKO}$, $Foxp4^{cKO}$, cDKO CD4 splenic T cells. Representative of 2 experiments including at least two mice per group. B) Relative quantification of *cd4* transcript levels by real-time PCR in sorted splenic CD4 t cells from cDKO and littermate control. CD4 mRNA levels are set relative to Cre^{POS} samples, and normalized to β-actin levels. Samples were tested in triplicate, mean and standard deviation is shown. *p<0.05, **p<0.001.

Discussion

In this chapter we have demonstrated that FoxP family members regulate *cd4* transcription in T cells. Loss of Foxp1 and Foxp4 resulted in increased CD4 levels in the majority of CD4 T cells, and a fraction of CD8 T cells. In CD4 T cells, deletion of Foxp1 resulted in altered CD4 expression. Further deletion of Foxp4 in CD4 T cells exacerbated de-repression of CD4 expression. Because Foxp3+ Tregs did not exhibit increased levels of CD4 expression, it appears expression at least one FoxP protein can repress CD4 in MHC II-restricted T cells. Conventional T cells require expression of at least Foxp1 or Foxp4 to maintain relatively normal CD4 expression.

CD4 T cells require Gata3 and ThPok to induce CD4 expression, and one possible cause of increased expression of CD4 in cDKO T cells is unrestricted activity of one or both of these factors. FoxP proteins may be required to repress expression or activity of factors that induce *cd4* expression. Another hypothetical mechanism is dysregulated repression at this locus. These studies did not address whether FoxP proteins bind directly to the *cd4* locus, so it remains unclear whether Foxp1/4 can antagonize *cd4* expression by physically obscuring consensus binding sites.

In contrast to CD4 T cells, only a subset of cDKO CD8 T cells exhibited dysregulated CD4 expression. Frequencies of $CD4^{dim}$CD8 T cells were substantially higher in cDKO mice than in littermate controls. In cDKO:WT mixed bone marrow chimeras, the detection of $CD4^{dim}$CD8 T cells indicated cell-intrinsic dysregulation of *cd4* silencing. However, we were unable to determine whether $CD4^{dim}$CD8 cells develop in the thymus, as that CD4 profile resembles DP thymocytes. Furthermore, these studies

did not address whether $CD4^{dim}$CD8 T cells were in fact MHC I-restricted, or whether this population represents thymocytes that escaped normal T cell selection.

Runx family member, Runx3, controls CD4 expression in CD8 T cells by regulating the silencer element in the *cd4* locus (Collins et al., 2009). Runx3 recruits chromatin-remodeling factors that methylate histones in the *cd4* locus, rendering the gene inaccessible to transcription factors (Taniuchi et al., 2002). Because cDKO CD8 cells failed to silence CD4 expression, these studies suggest FoxP family members regulate *cd4* silencer activity either directly or indirectly. Foxp3 is known to interact with Runx family proteins (Hu et al., 2007), so one possible mechanism of FoxP protein repression of *cd4* is through cooperation with Runx3. Alternatively, Runx3 itself may be a target of FoxP family members, and downstream *cd4* regulation is an effect of altered Runx3 levels.

The studies described here demonstrate CD4 expression is regulated by FoxP family transcription factors. Dysregulated CD4 expression holds implications for thymic selection, MHC-restriction, and T cell signaling, but further work is required to understand how CD4 levels are regulated and why it may be important to maintain specific regulation at this locus in mature T cells.

CHAPTER V.

Foxp1 and Foxp4 regulate Foxp3+ Treg cell development.

Introduction

Regulatory T cells (Tregs) are critical for the maintenance of self-tolerance and the prevention of autoimmunity (Ochs et al., 2007; Ziegler, 2006). During infection, Tregs are necessary for controlling immune responses and suppressing effector T cell responses following pathogen clearance. This subset of immunomodulatory lymphocytes is a subset of CD4+ T cells that express interleukin-2 receptor (IL-2R) and the forkhead transcription factor Foxp3 (Rudensky, 2011; Sakaguchi et al., 2006). Foxp3 governs transcriptional programs and modifies gene expression, enabling Tregs to acquire effector-like characteristics, migrate to sites of inflammation, and suppress immune responses (Campbell and Koch, 2011; Ding et al., 2012; Feuerer et al., 2009; Josefowicz et al., 2012).

Treg development typically occurs in the thymus during thymic selection. Thymic Treg (tTreg) generation requires T cell receptor- and cytokine-generated signals to induce Foxp3 expression. How these signals cooperate to induce tTreg development remains unclear. Recent studies have suggested a model in which a two-step developmental process requires T cell receptor (TCR) signals to prime a population of CD4 single positive (CD4SP) thymocytes termed preTregs (Burchill et al., 2008; Lio and

Hsieh, 2008). This precursor population upregulates cytokine receptors, and subsequent STAT5 signaling induced by cytokine signals results in expression of Foxp3 (Burchill et al., 2008; Lio and Hsieh, 2008). A similar combination of TCR and cytokine stimulation can induce Foxp3 expression in conventional CD4 T cells from peripheral tissues, causing these lymphocytes to differentiate into inducible Tregs (iTregs) *in vitro*.

Generation of preTregs requires selection of a limited repertoire of immature thymocytes. The quality and quantity of TCR signaling appear to be critical for Treg development. Weak TCR response to self-peptide is not sufficient to select Tregs precursors, while overpowering TCR signals result in negative selection and deletion (Jordan et al., 2001). Downstream cascades of TCR ligation also play an integral role in Treg development by inducing transcription factors AP-1, NFAT, and NFκB, which regulate Foxp3 expression (Huehn et al., 2009; Ruan and Chen, 2012). Conversely, phosphorylation of Akt by PI3K, and subsequent activation of mammalian target of rapamycin (mTOR) directly interferes with Foxp3 induction (Liu et al., 2009; Merkenschlager and von Boehmer, 2010; Sauer et al., 2008).

Cytokine signaling is also necessary for Foxp3 induction, and homeostasis of Tregs. (Turka and Walsh, 2008; Vang et al., 2008; Wuest et al., 2008). Specifically, Tregs depend on signaling through complexes containing the common gamma chain (γc), which is a subunit necessary for signaling of several cytokine receptor, including IL-2, IL-7, and IL-15. The γc chain pairs with different complementary chains that lend specificity to cytokine signaling. Cytokine signal transduction occurs when receptor complexes embedded in the cell membrane bind soluble cytokines. Extracellular ligation translates to intracellular signaling. Receptor-associated Janus kinase (JAK) tyrosine kinases inside the cell are trans-phosphorylated. Through a series of phosphorylation

events, transcription factors called Signal Transducer and Activator of Transcription 5 (STAT5) are also phosphorylated. Phosphorylated STAT5 (pSTAT5) forms homodimers, and translocates to the nucleus. Transcriptional regulation by STAT5 dimers supports T cell survival by inducing expression of anti-apoptotic proteins, such as Bcl-2 (Lord et al., 2000; Van Parijs et al., 1999). Intracellular levels of Bcl-2 protein correlate with survival and homeostasis of T lymphocytes. STAT5 dimers are also required to bind directly to the *foxp3* promoter to induce Foxp3 expression (Burchill et al., 2007; Passerini et al., 2008; Snow et al., 2003). Cytokines important for Foxp3 induction and Treg homeostasis include IL-2, IL-7, and IL-15 (Cheng et al., 2011; Le Campion et al., 2012).

Forkhead-domain containing FoxP transcription factors are necessary for the development and function of T lineage memory cells (Feng et al., 2010; Feng et al., 2011; Wiehagen et al., 2012). Despite similar primary sequences and expression in lymphocytes, T cell-specific deletion has demonstrated that Foxp1 and Foxp4 are not redundant in T cells. CD4-Cre mediated deletion of Foxp1 at the double positive (DP) stage of thymic development results in dysregulated homeostasis of peripheral T cells (Feng et al., 2010). In contrast, T cell-specific deletion of Foxp4 does not alter thymocyte development, peripheral T cell activation, or differentiation (Wiehagen et al., 2012). Importantly, neither CD4-Cre mediated deletion of Foxp1 nor Foxp4 alone impairs Treg development (Feng et al., 2010; Feng et al., 2011; Wiehagen et al., 2012).

The FoxP family proteins dimerize to interact with target gene sequences and participate in protein-protein interactions. Previous studies have shown Foxp1 and Foxp3 bind to one another, as well as Foxp1 to Foxp4 (Li et al., 2004a; Song et al., 2012). Furthermore, Foxp3 can occupy the promoters of Foxp1 and Foxp4, and Foxp3 represses Foxp1 transcription (Rudra et al., 2012). Gene array data demonstrates that

Foxp1 expression is downregulated in peripheral wild-type Foxp3+ Tregs (Heng and Painter, 2008; Rudra et al., 2012). In addition to FoxP protein-protein binding, several of the transcription factors required for Treg generation are known to interact with FoxP proteins (Bettelli et al., 2005; Hu et al., 2007; Sauer et al., 2008; Wu et al., 2006; Zhang et al., 2008). These data generated the hypothesis that FoxP family members Foxp1 and Foxp4 participate in Treg development. Thus, the comparatively normal Treg generation in $Foxp1^{cKO}$ and $Foxp4^{cKO}$ mice may suggest Foxp1 and Foxp4 compensate for one another.

To better understand the roles of Foxp1 and Foxp4 in Treg development, we generated mice in which both Foxp1 and Foxp4 were conditionally deleted using a CD4-Cre transgenic. Surprisingly, while loss of either Foxp1 or Foxp4 alone did not alter thymic Treg (tTreg) numbers, combined loss resulted in a significant decrease in Treg numbers. cDKO CD4 splenocytes do not differentiate into inducible Tregs, indicating multiple defects in Treg development. cDKO Tregs exhibited an abnormal cell surface phenotype, and reduced capacity to suppress conventional CD4 T cells *in vitro*.

The decrease in tTregs was a cell-intrinsic effect of loss of Foxp1 and Foxp4. Under competitive conditions, double-knockout Tregs exhibited impaired cytokine-induced signaling and were at a significant developmental disadvantage relative to wild-type competitors. These findings establish that expression of Foxp1 or Foxp4 is required for normal generation of tTregs, and that more than one member of the FoxP family contributes to immunological tolerance.

Results

Foxp1 and Foxp4 are expressed in regulatory T cells.

A defining feature of regulatory T cells is expression of FoxP family member Foxp3. To characterize expression of other FoxP family members, steady-state levels of *foxp1* and *foxp4* transcript were assessed in highly purified wild-type thymic Tregs using real-time PCR (Figure 5.1A). Similar to findings in peripheral Tregs, Foxp1 expression is reduced in tTregs relative to conventional Foxp3- CD4SP T cells. In contrast, Foxp4 transcript expression was 2-3 fold increased (Figure 5.1A, right panel). These data indicate FoxP family members are individually regulated in Foxp3+ Tregs.

Foxp1 and Foxp4 are required for normal Treg generation.

In Chapter 3, initial characterization of cDKO mice revealed that the total cellularity of thymi from cDKO mice was lower compared to Cre^{POS}, $Foxp1^{cKO}$, and $Foxp4^{cKO}$ mice, due to reduced DP numbers (Figure 3.2B, 3.3A-B). Although total numbers of cDKO CD4SP were normal, we noted a higher frequency of CD4SP cells within cDKO thymi (Figure 3.4C). Increased frequencies of cDKO CD4 cells did not correlate with increased Foxp3+ Tregs, however. Instead, a modest but statistically significant reduction was observed in Foxp3+ tTregs, relative to either total thymocytes or total CD4SP (Figure 5.1B-C and data not shown). Despite reduced frequencies, the total numbers of tTregs were not significantly different across any cohorts (Figure 5.1D, and data not shown).

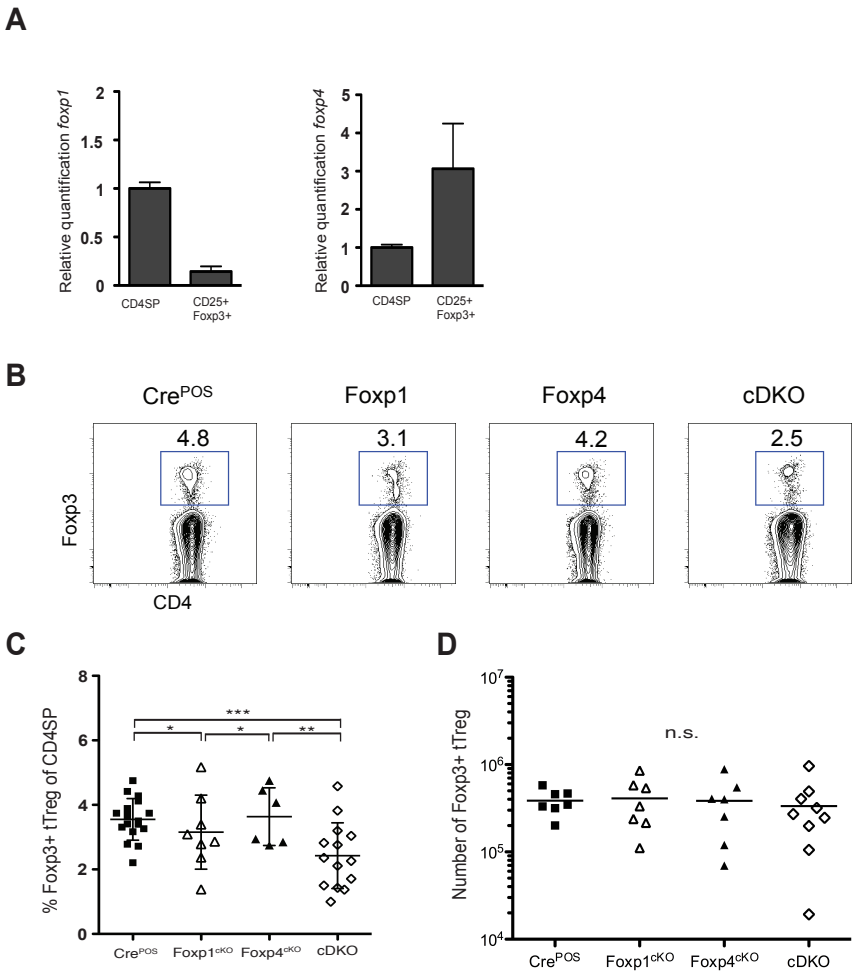


Figure 5.1. Conditional deletion of Foxp1 and Foxp4 alters thymic Foxp3+ tTreg frequencies. A) Relative levels of stead-state *foxp1* or *foxp4* transcript in sorted wild-type tTregs as determined by real-time PCR. tTreg expression compared to CD4SP thymocytes, and normalized to β-actin levels in each population. B) Representative plots previously gated on CD4SP thymocytes from four-week-old Cre positive (Cre^{POS}), single knockout $Foxp1^{cKO}$ and $Foxp4^{cKO}$, and cDKO mice. Relative frequencies of Foxp3+ cells are indicated. Results are representative of 12 experiments. C) Frequency of Foxp3+ tTreg as a percentage of CD4SP. Data is combined from twelve independent experiments. Each point represents an individual mouse. Statistical analysis generated with one-way ANOVA with Dunnett post-test. * $p<0.05$. n.s. = not significant. D) Absolute numbers of Foxp3+ tTreg in cDKO and littermate controls. Mean is shown. Each point represents one mouse. Compiled from 6 independent experiments. Statistical analysis indicates $p>0.05$ for all comparisons.

As discussed in Chapter III, CD4SP thymocyte numbers were comparable to littermate controls, but CD4 T cell numbers in the spleen and LN were significantly diminished (Figure 3.5). When we quantified Tregs in peripheral tissues, the total numbers of cDKO Foxp3+ cells were significantly reduced in the spleen and LN compared to Cre^{POS}, $Foxp1^{cKO}$, and $Foxp4^{cKO}$ mice (Figure 5.2A-B). Splenic Treg numbers were significantly decreased only in cDKO mice, while quantification of Tregs in pooled LN demonstrated significantly fewer Foxp3+ cells in both cDKO and 1K4H mice (Figure 5.2B). These data suggest cDKO Tregs, like conventional T cells, are present in lower numbers in peripheral lymphoid tissues, and that defects in Treg cellularity is more severe in LN compared to the spleen. Quantification of the Treg population in multiple tissues of the cDKO mouse demonstrated that Treg numbers were diminished throughout the animal. Peritoneal tissues, skin, mesenteric LN, and Peyers patches contained reduced numbers of Tregs compared to Cre^{POS} mice (Figure 5.2C). These data suggest decreased numbers of Tregs contribute to the total T lymphopenia observed in cDKO mice discussed in Chapter III.

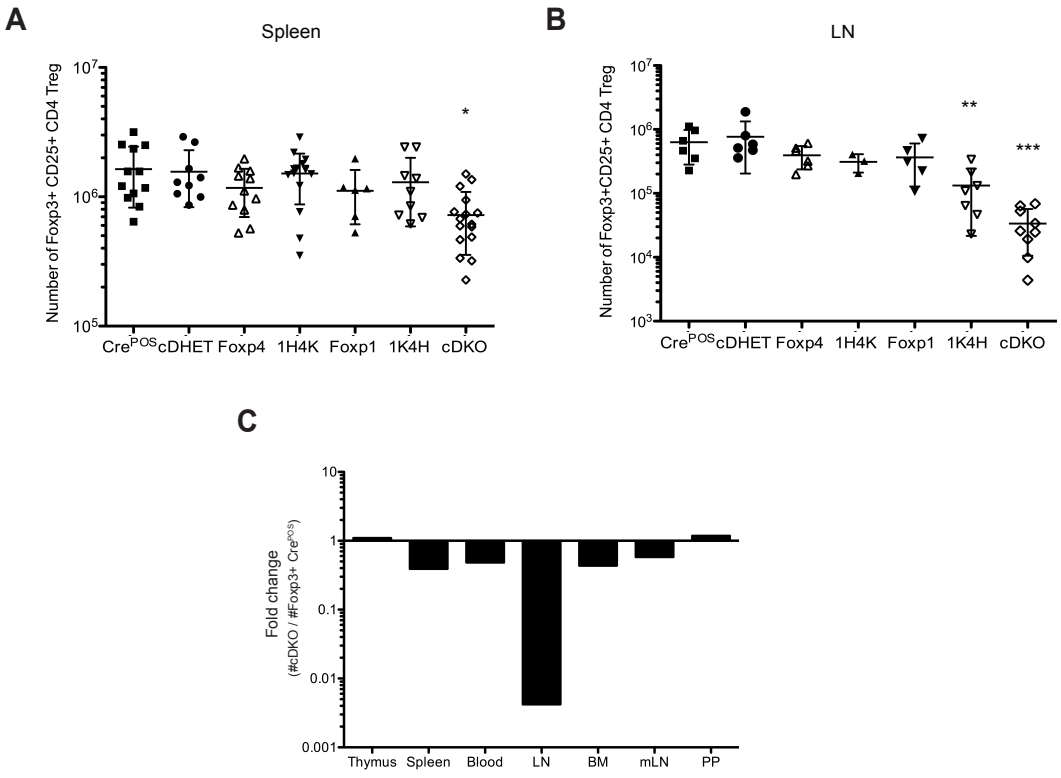


Figure 5.2. Foxp1/4 deletion results in significantly reduced numbers of peripheral Tregs. A-B) Absolute numbers of Foxp3+ CD4+ T cells in the A) spleens or B) pooled LN of cDKO mice and littermate controls. Mean and standard deviation are shown. Each point represents one mouse. Compiled from 10 individual experiments. Statistics generated from one-way ANOVA with Bonferroni post-test. Spleen: * $p<0.001$. LN: ** $p<0.001$, *** $p<0.0001$. C) Fold change in Foxp3+ numbers in indicated tissues of cDKO mice, relative to Cre^{POS} controls.

In contrast to cell numbers, the relative frequency of Foxp3+ Treg of total CD4 T cells was significantly increased in cDKO mice. In the spleen, an average 15% of cDKO CD4 T cells were Foxp3+ Tregs. Cre^{POS} mice typically maintained 10% Tregs within the total CD4 population (Figure 5.3A-B). Percentages of Tregs in spleens of $Foxp4^{cKO}$, cDHET and 1H4K mice were not significantly different, but loss of Foxp1 caused a trend towards slightly increased frequencies. However, frequency of Tregs in cDKO LN was substantially increased compared to all littermate controls (Figure 5.3A,C). Almost 40%

of cDKO CD4 T cells in LN were Foxp3+ Tregs, compared to an average 12% of Tregs in Cre^{POS} mice.

To determine whether Tregs were selectively enriched in the spleen and LN of cDKO mice, we assessed the relative percentage of Foxp3+ Tregs of total CD4 T cells in multiple tissues. Treg frequencies were set relative to frequencies of Tregs in Cre^{POS} controls to determine the fold change in cDKO Treg percentages. The frequency of cDKO Tregs was increased in all tissues examined except bone marrow, skin, and thymus (Figure 5.3D and data not shown). Together, these data indicate cDKO Tregs are relatively spared compared to conventional CD4 T cells throughout the cDKO mice, as a higher frequency of Tregs comprise total CD4 populations.

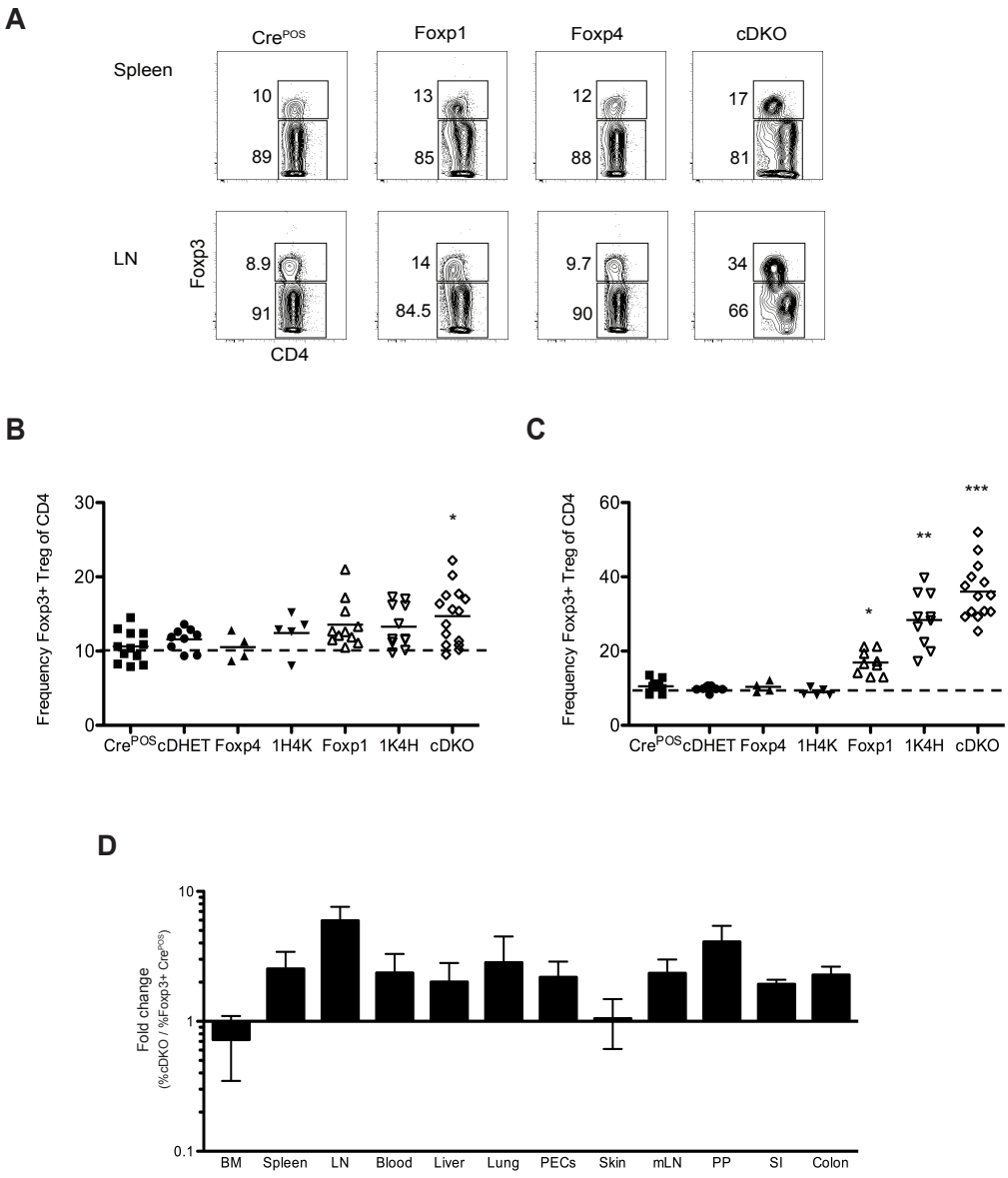


Figure 5.3. Frequencies of cDKO Tregs are significantly increased in spleen and LN. A) Representative plots of Foxp3+ tTreg in total splenic or LN CD4 T cells. Relative frequencies of Foxp3+ Treg and Foxp3- conventional CD4 T cells are indicated. Results representative of 12 experiments. B-C) Compiled frequencies of Foxp3+ Tregs in B) spleen or C) pooled LN. Data combined from 12 experiments. Each point represents an individual mouse. Statistical analysis generated with one-way ANOVA with Dunnett post-test to Cre^{POS} controls. Spleen: * $p<0.05$. LN: *$p<0.05$, **$p<0.001$, ***$p<0.0001$ D) Fold change in Treg frequency, relative to Cre^{POS} littermate controls, assessed in bone marrow (BM), thymus (Thy), spleen (Spl), pooled axillary, cervical and inguinal lymph nodes (LN), peripheral blood, liver, lung, peritoneal exudate cells (PECs), skin, pooled mesenteric lymph nodes (mLN), pooled Peyers patches (PP), small intestine lamina propria (SI), or lamina propria of large intestine (colon). Mean and standard deviation calculated from 3 experiments.

cDKO Tregs phenotypically resemble memory Tregs

Analysis of cell surface marker expression of cDKO Foxp3+ tTregs revealed abnormal expression patterns compared to Foxp3+ Tregs from littermate control mice (Figure 5.4A). In the absence of Foxp1 and Foxp4 expression, $Foxp3^{+}$ Tregs in the thymus express higher levels of CD44 and glucocorticoid-induced TNF-receptor-related protein (GITR), compared to $Foxp1^{cKO}$, $Foxp4^{cKO}$, or Cre^{POS} tTregs. Other markers associated with Treg activation were also increased. PD-1, KLRG1, and α4β7 integrin (CD103) are highly expressed only on cDKO Tregs. L-selection (CD62L) was downregulated on Tregs, similar to findings in Foxp3- conventional cDKO CD4 T cells reviewed in Chapter III.

Elevated expression of CD44, GITR, CD103, KLRG1 and ICOS has been previously reported on Tregs in the gut, sites of immunologic activity, and under lymphopenic conditions (Cheng et al., 2012; Feuerer et al., 2010). We reasoned abnormal cDKO Treg development could be a direct consequence of loss of Foxp1 and Foxp4 transcriptional regulation or due to recirculation of peripheral Tregs, expanding in lymphopenic conditions. Foxp3+ tTregs are present in two-day-old cDKO neonates (data not shown), a developmental time point where few if any CD4+CD25+ lymphocytes have exited the thymus (Asano et al., 1996; Kelly and Scollay, 1992). The presence of Foxp3+ cells indicates that tTregs can develop in the absence of recirculation, but does not formally rule out a role for recirculating Tregs under homeostatic conditions in the adult thymus.

Like thymic Tregs, peripheral cDKO Tregs express phenotypic markers of activation and differentiation (Figure 5.4B). Expression of another TNF family receptor,

OX40, was slightly increased on splenic and LN cDKO Tregs, and neuropilin-1 (Nrp-1) was decreased. These markers correlate with expression of GITR, CD103, KLRG1, and PD-1 to establish a phenotype reminiscent of activated or memory Tregs. There are significantly reduced frequencies of $CD62L^{hi}$ Tregs compared to littermate controls, suggesting Foxp1/4 deletion alters the expression of CD62L on Tregs as well as conventional T cells. However, neither CD4SP nor peripheral CD4 T cells expressed GITR, CD103, or other markers observed on cDKO tTregs (data not shown). Therefore, combined deletion of FoxP family members elicited a phenotype restricted to cDKO Tregs. Further, this phenotype was unique from cells in which either Foxp1 or Foxp4 was deleted.

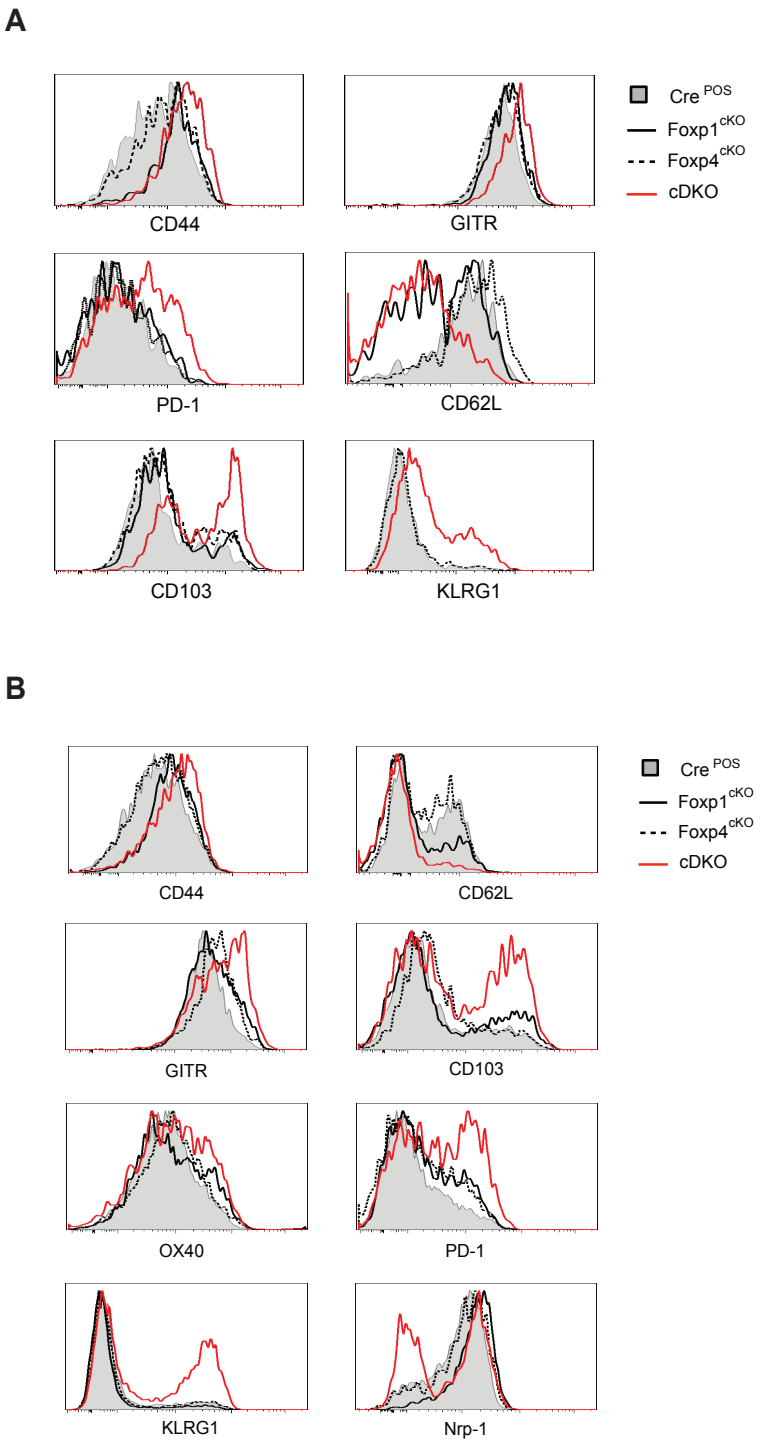


Figure 5.4 cDKO Tregs express an altered cell surface phenotype. A-B) Representative histograms of A) tTreg or B) splenic Treg expression of markers of activation. Plots are previously gated on Live, singlet, CD4SP Foxp3+ cells. Representative of six independent experiments.

cDKO Tregs exhibit loss of suppressive function in vitro.

The cell surface phenotype of cDKO Tregs suggested these cells may have improved effector function. To assess whether the increased expression of cell surface markers associated with activation also correlated with increased suppressive function, sorted cDKO Tregs were assessed using an *in vitro* suppression assay. Briefly, titrated numbers of Tregs were cultured with stimulated wild-type (WT) conventional CD4 T cells (Tconv). In this assay, suppressive activity correlates with how well Tregs restrain Tconv proliferation. Despite expression of an activated phenotype, cDKO Tregs exhibited reduced suppression of WT Tconv. Compared to controls, Tconv cells plated with cDKO Tregs demonstrated increased proliferation (Figure 5.5A). Enumeration of divided Tconv cells in cDKO wells also shows increased total number compared to controls (Figure 5.5B). Numbers of live cDKO Tregs at the end of the assay were also significantly lower than numbers of control Tregs, despite identical starting numbers (data not shown), perhaps indicating cDKO Tregs do not survive in culture to suppress Tconv proliferation. Taken together, these data suggest cDKO Tregs exhibit reduced suppressive function, despite having an activated cell surface phenotype.

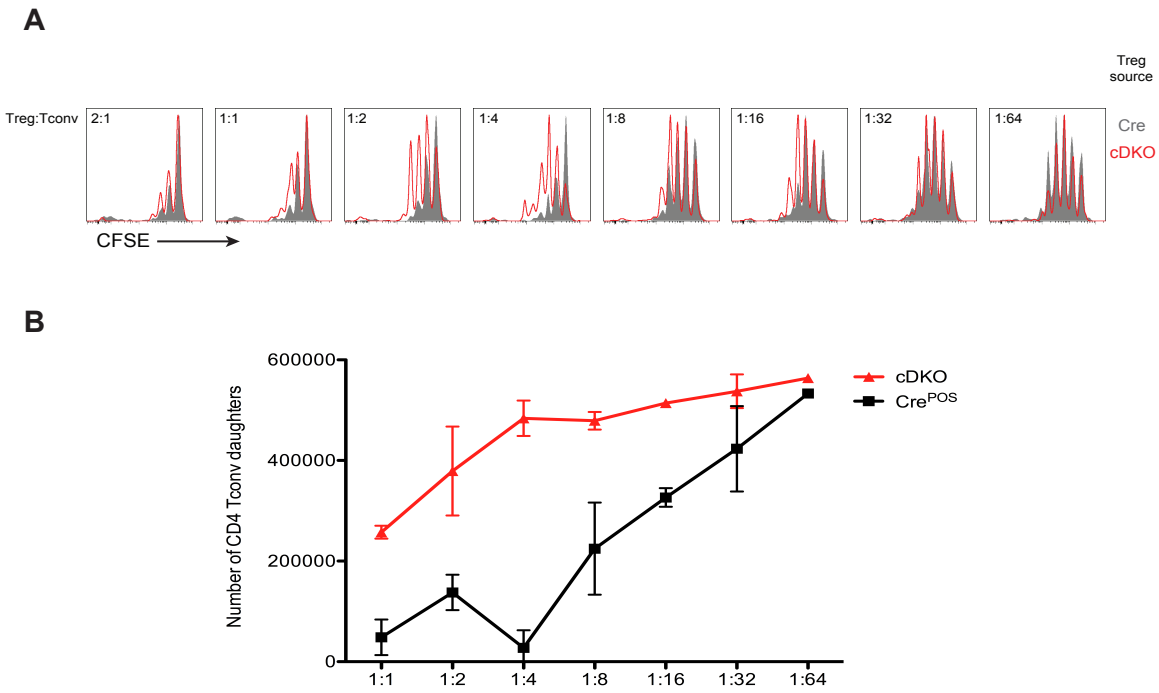


Figure 5.5. cDKO Tregs exhibit reduced suppressive ability. A) Representative plots of CFSE dilution of sorted WT Foxp3- CD4 cells stimulated *in vitro* with titrated numbers of cDKO or Cre control Tregs. Plots depict gray histograms of cell division of cells suppressed by sorted Cre control Tregs, and red histograms of cells cultured with cDKO Tregs. Ratio of Treg:Tconv is noted in each plot. Representative of 2 independent experiments. B) Numbers of expanded daughter cells calculated from CFSE dilution frequency of cells in each peak. Data compiled from 2 experiments.

FoxP members regulate adaptive Treg development.

Peripheral CD4 T cells can convert to Tregs given appropriate cytokine and TCR signals. We next assessed the ability of cDKO CD4 T cells to differentiate into inducible Tregs. Splenic CD4 T cells were highly purified from WT, Foxp1^{cKO}, Foxp4^{cKO}, or cDKO mice, and cultured with TGFβ, IL-2, and anti-CD3 and anti-CD28 stimulation. The concentration of TGFβ was titrated to measure iTreg conversion at low and high doses of cytokine. After culture, WT and Foxp4^{cKO} CD4 T cells converted into Foxp3+ Tregs

(Figure 5.6A). High levels of TGFβ drove increased frequencies of Foxp3+ Tregs in both WT and Foxp4-deficient cells (Figure 5.7B). In contrast, Foxp1^{cKO} and cDKO CD4 T cells failed to induce Foxp3 expression in culture, even when cultured with the highest concentrations of TGFβ. Excess TGFβ (5ng/mL) also did not result in cDKO iTreg development (data not shown).

Both Foxp1^{cKO} and cDKO CD4 T cells are CD44^{hi} and exhibit an activated phenotype. WT and Foxp4^{cKO} CD4 T cells are >80% CD44^{low} naïve T cells. To control for initial phenotype differences in the starting populations of CD4 T cells, WT CD44^{hi} CD4 T cells were sorted and cultured. Similar to Foxp1^{cKO} and cDKO results, WT CD44^{hi} CD4 T cells failed to convert into iTregs, even at the highest concentrations of TGFβ (Figure 5.6B). CD44^{hi} Foxp4^{cKO} T cells also did not generate frequencies of iTregs comparable to naïve CD4 T cell controls. Together, these data indicate that while naïve CD4 T cells can induce Foxp3 *in vitro* and convert to iTregs, CD44^{hi} effector-like CD4 T cells from WT or mutant mice are unable to convert into iTregs. Therefore, both natural and inducible Treg development is altered in cDKO mice.

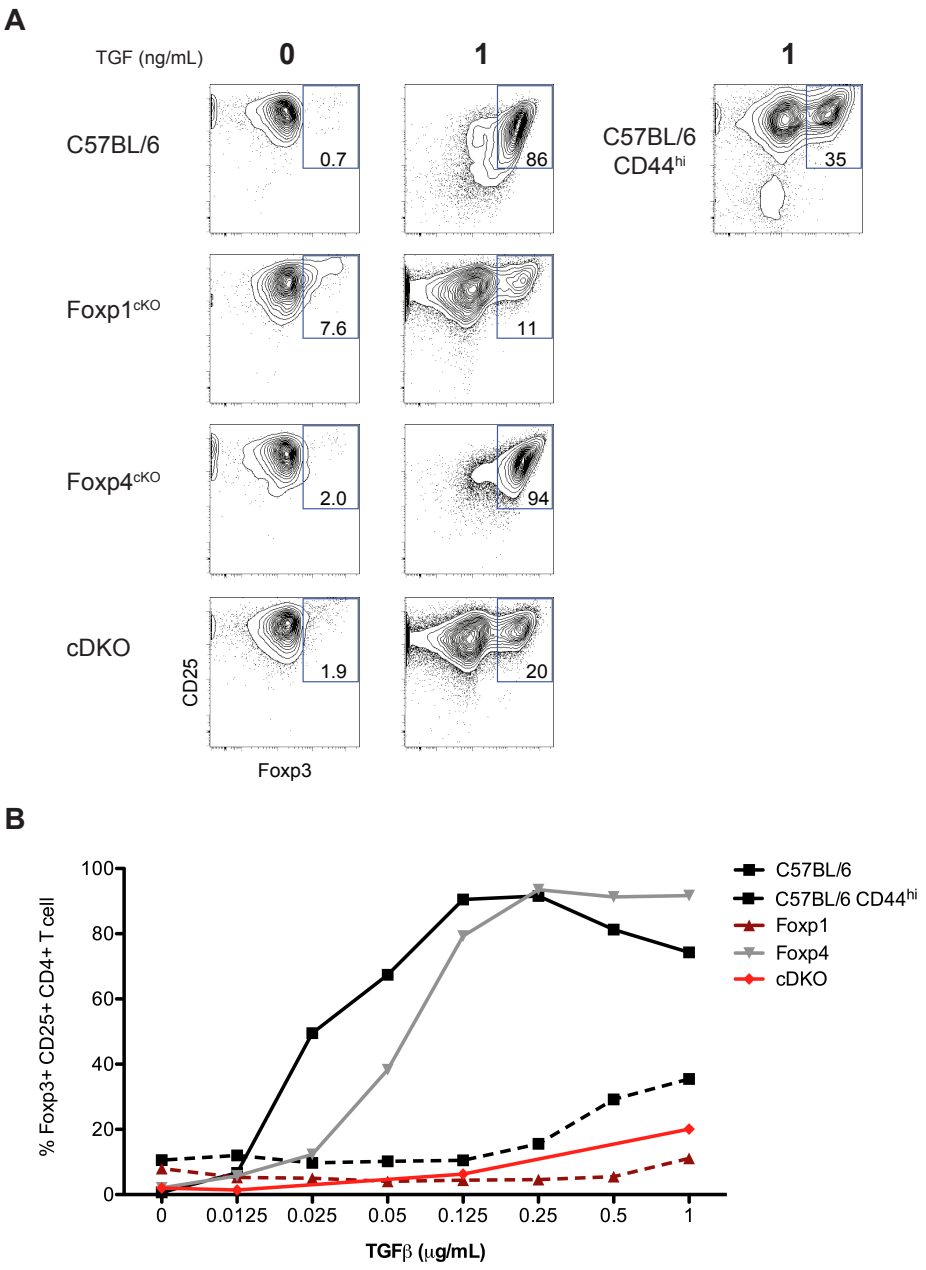


Figure 5.6. Inducible Treg differentiation requires Foxp1 expression. A) Representative plots of CD4 T cells sorted from cDKO mice and controls and cultured with IL-2, anti-CD3 and anti-CD28 stimulation, and 0 or 1 ng/mL TGFβ. B) Frequencies of Live, TCRβ+ CD4+ Foxp3+ iTregs generated *in vitro* as in A) with titrated concentrations of TGFβ. Data representative of 3 independent experiments.

Defective cDKO Treg development is cell intrinsic.

To assess whether the altered phenotype and frequencies of cDKO tTregs are cell-intrinsic, we investigated Treg development in competitive bone marrow chimeras. Bone marrow from cDKO (CD45.2+) and wild-type CD45.1+ congenic mice was mixed in a 9:1 ratio (Figure 5.7A). We reasoned that this combination would give cDKO progenitors an advantage during reconstitution. cDKO:WT chimeras were compared to those similarly generated with Cre^{POS}, $Foxp1^{cKO}$, or $Foxp4^{cKO}$ bone marrow. Eight weeks after reconstitution, tTreg generation and cell surface phenotype were evaluated.

DP and SP thymocyte development was grossly normal in cDKO:WT chimeras (Figure 3.9B, Figure 5.7B, far left panels). Chimerism in the DP compartment was also similar to that found in the DN and bone marrow B cell compartments (Figure 5.7C, Figure 3.8B, and data not shown). Frequencies of total (CD45.1 plus CD45.2) CD4SP and Foxp3+ Tregs were similar across all cohorts (Figure 5.7B and data not shown). Within the CD4SP population, approximately 80%-90% of thymocytes were derived from CD45.2+ experimental progenitors, regardless of the genotype used for CD45.2+ bone marrow, indicating that even under competitive conditions, CD4Cre-mediated deletion had no adverse effect on development of this population (Figure 5.7B). In contrast, deletion of Foxp1 and Foxp4 resulted in a striking defect in tTreg generation. CD45.2+ cDKO-derived cells comprised less than 10% of the total tTreg pool (Figure 5.7B, far right panels, and 5.7C). Reduced chimerism of tTregs required loss of both Foxp1 and Foxp4, as tTreg development in $Foxp1^{cKO}$ or $Foxp4^{cKO}$ competitive chimeras was comparable to Cre^{POS} controls. These results suggest that either Foxp1 or Foxp4 is sufficient for tTreg generation and that expression of at least one of these transcription factors at the DP stage or later is required for tTreg development.

Previous studies have described a population of $CD25^{+}$ $GITR^{+}$ Foxp3- CD4SP thymocytes, which are precursors to thymic Tregs (Burchill et al., 2008; Lio and Hsieh, 2008). To determine whether cDKO tTreg generation is altered at an earlier developmental stage or limited to maintenance of mature tTreg, we assessed chimerism of the CD25+GITR+$Foxp3^{NEG}$ preTreg population. In cDKO:WT chimeras, the frequency of cDKO CD4SP preTregs was approximately 60% of expected, while thymocytes in control chimeras exhibited normal preTreg development (Figure 5.7C). Together, these data demonstrate that the early stages of tTreg differentiation are dysregulated without expression of Foxp1 and Foxp4, but expression of either Foxp1 or Foxp4 is sufficient for preTreg and Treg generation.

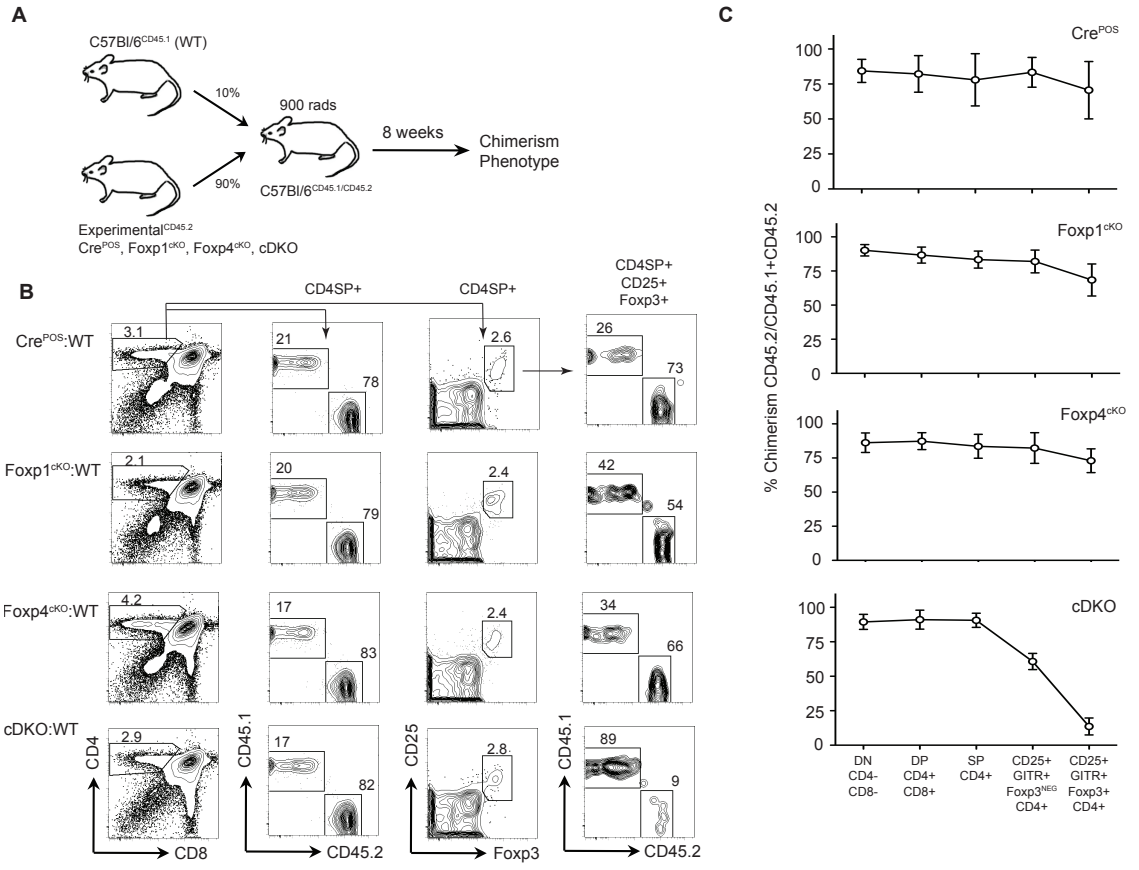


Figure 5.7. Foxp1 or Foxp4 expression is necessary for normal tTreg generation. A) Schematic diagram of mixed bone marrow chimera generation. B) Representative plots of chimeric thymocytes. Total thymi (Left panel), CD4SP gated chimerism (Center left panel), total Foxp3+ CD25+ tTreg (Center right panel), and Foxp3+ CD25+ tTreg gated chimerism (Right panel). Data is representative of two individual sets of chimeras with 5 mice of each genotype per set. C) Summarized chimerism of developing thymocytes from CD45.2+ double negative (DN) cells through DP selection and tTreg development. Chimerism is shown as the frequency of CD45.2 (Cre^{POS}, $Foxp1^{cKO}$, $Foxp4^{cKO}$ or cDKO) cells in each given thymic population. Each point represents a mean +/- s.d.

We next assessed Treg chimerism in the spleens of chimeric mice. Total numbers of splenic Tregs are consistent across chimeric cohorts, as WT cells are able to develop normally (data not shown). Loss of Foxp1 or Foxp4 results in decreased frequency of CD45.2+ Tregs in respective chimeric cohorts. We compared the relative percentage of CD45.2+ Tregs of total CD45.2+ CD4 T cells across cohorts (Figure 5.8A, top panels). The frequency of $Foxp4^{cKO}$ Tregs is approximately 50% lower than the relative frequency of Cre^{POS} Tregs in Cre^{POS}:WT chimeras. Foxp1 deletion results in nearly 75% fewer CD45.2+ Tregs in $Foxp1^{cKO}$:WT spleens relative to Cre^{POS}. Furthermore, the absolute number of CD45.2+ $Foxp1^{cKO}$ Tregs is 10-fold lower than numbers of Cre^{POS}-derived Tregs (Figure 5.8B). Deletion of both Foxp1 and Foxp4 results in the most significant defect. In cDKO:WT chimeras, less than 1% of CD45.2+ cDKO CD4 T cells are Foxp3+ (Figure 5.8A, far right panels). Total cDKO-derived splenic Treg numbers are 100-fold lower than Cre^{POS} Tregs (Figure 5.8B). Foxp3 staining of CD45.1+ endogenous WT cells demonstrates the majority of Tregs detected in $Foxp1^{cKO}$:WT and cDKO:WT chimeras are WT Tregs.

In LN of chimeric mice, the same trend was observed, with loss of Foxp4 or Foxp1 resulting in reduced CD45.2+ chimerism. However, cDKO-derived Tregs were nearly undetectable in pooled chimeric LN (data not shown). These findings reproduce

the same trends observed in non-chimeric mice, in which T cell maintenance in spleen and LN were significantly different. Taken together, these data demonstrate development defects and competitive disadvantages of Foxp1- or Foxp4-deficient Tregs. However, deletion of both Foxp1 and Foxp4 results in the most severe defects, suggesting that expression of at least one FoxP family member is necessary for Treg generation.

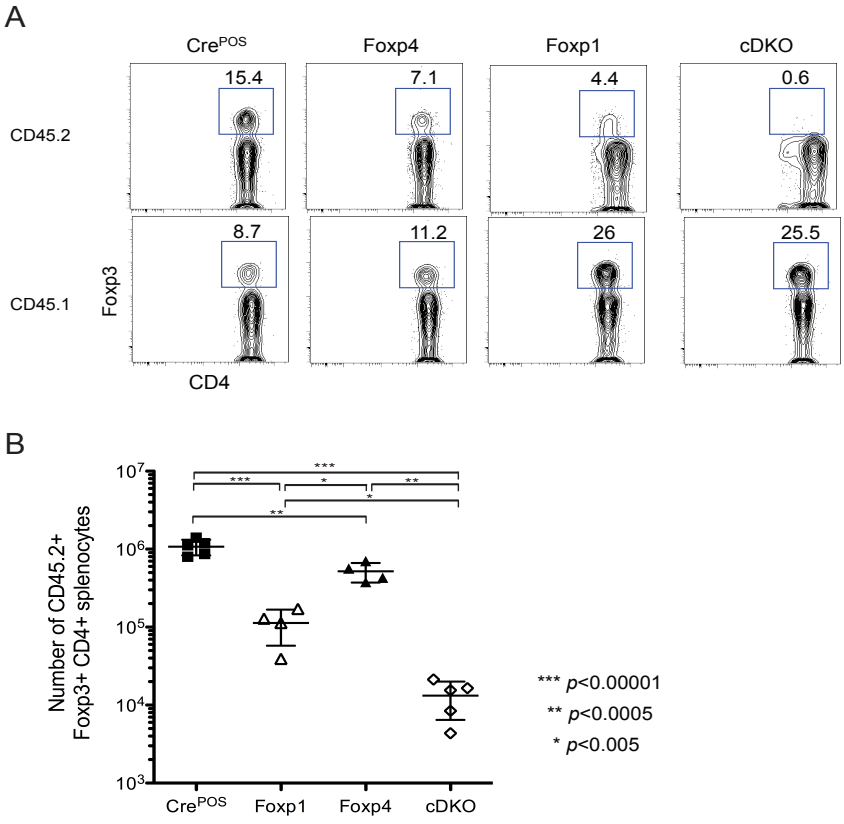


Figure 5.8. CD45.2+ cDKO Tregs are nearly absent in peripheral lymphoid tissues of competitive chimera mice. A) Representative plots previously gated on CD45.2+ or CD45.1+ CD4SP thymocytes from competitive chimeras in each cohort. Relative frequencies of Foxp3+ cells in each population are indicated. Results are representative of 10-15 chimeras in each cohort, in 3 independent experiments. B) Absolute numbers of CD45.2+ Tregs in the spleens of chimeric mice. Each point represents on chimeric

mouse. Mean +/- SD are shown. Statistical analyses generated from a one-way ANOVA, and Bonferroni post-test.

cDKO thymocytes signal abnormally through the T cell receptor.

To begin to address what defects in cDKO cells hinder Treg generation, our focus returned to the thymus, where the first defects in competitive chimerism were observed. We first investigated signals in the populations from which preTregs develop: DP and CD4SP. Because suitable TCR signals are vital for Foxp3- precursor generation, we determined whether maturing DP and CD4SP thymocytes expressed normal levels of signaling receptors. cDKO DP thymocytes expressed slightly higher levels of CD4, TCRβ, and CD5 immediately *ex vivo*, suggesting higher TCR signaling in these cells (Figure 3.3 and data not shown). CD4SP cells also express increased CD4 levels, but TCRβ, CD28 and CD5 were comparable to single cKO controls (Figure 4.1A, C, and data not shown).

Next, we assessed whether cDKO thymocytes responded to stimulation appropriately *in vitro*. Resting thymocytes were stimulated with anti-CD3, or anti-CD3 and anti-CD28 to measure phosphorylation of downstream signaling proteins, Akt and Erk1/2, by flow cytometry. DP and CD4SP cells from cDKO thymi responded more vigorously to CD3 and CD28 stimulation compared to Cre^{POS} thymocytes (Figure 5.9A-B and data not shown). Erk1/2 phosphorylation was consistently reduced in CD4SP cells stimulated with anti-CD3 alone, but CD3 and CD28 stimulation resulted in significantly increased levels of phosphorylated Akt (pAkt) and phosphorylated Erk (pErk).

To determine whether cDKO cells exhibit constitutively higher TCR signaling, resting levels of pAkt and pErk1/2 were measured. As determined by relative mean

fluorescence intensity of pAkt and pErk in cDKO and control thymocytes, resting levels of these signaling proteins were not significantly different in DP or CD4SP (Figure 5.9, and data not shown). Therefore, these data demonstrate significantly increased activity of Akt and Erk1/2 pathways when cDKO thymocytes are stimulated with anti-CD3 and anti-CD28, but relatively normal steady-state kinase activity.

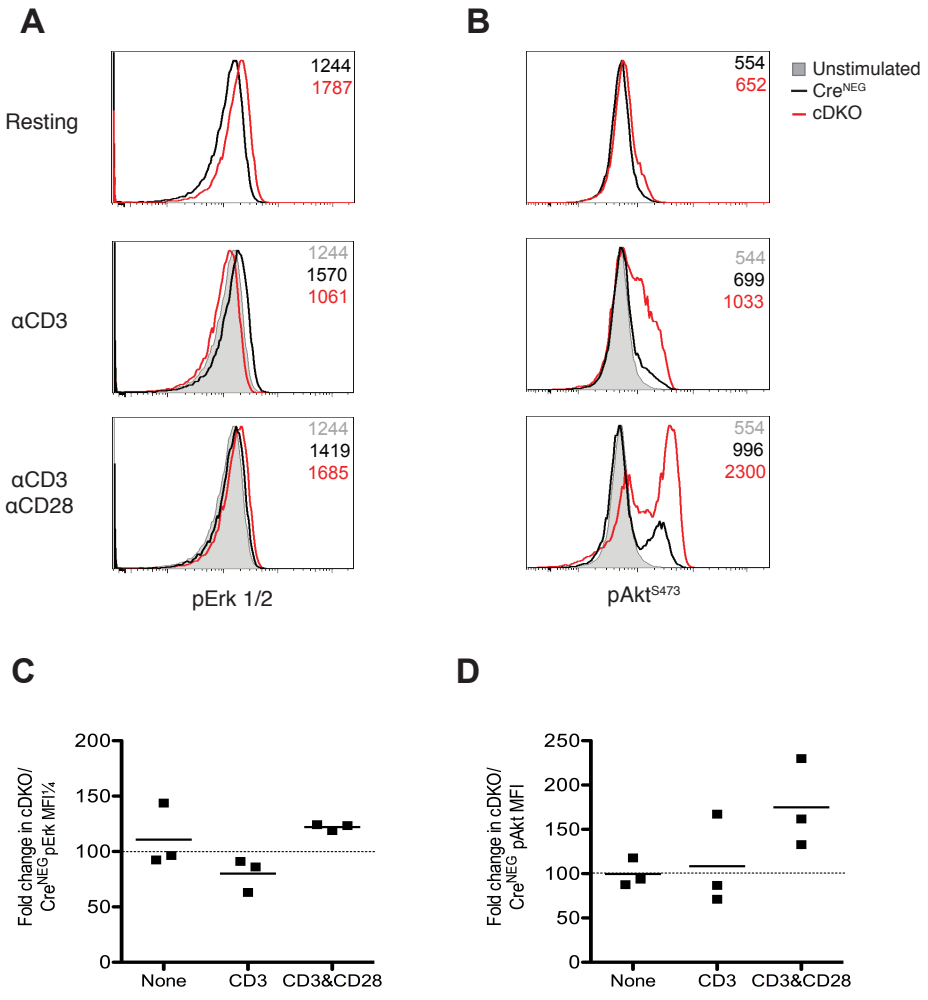


Figure 5.9. cDKO CD4SP T cells display increased signaling to anti-CD3 and anti-CD28 stimulation *in vitro*. A-B) Representative plots of phosphorylated A) Erk1/2 or B) Akt in CD4SP Foxp3- thymocytes rested for 3 hours, or rested and stimulated *in vitro* in wells coated with anti-CD3 or anti-CD3 and anti-CD28. MFI of pErk1/2 or pAKt is noted in each plot. C-D) Compiled MFI of C) pErk1/2 or D) pAkt levels in each culture

condition. Mean is indicated MFI is set relative to MFI of WT controls (at 100%) in each experiment. Compiled from 3 experiments.

STAT5 signals are dysregulated in cDKO Tregs.

Development of tTregs requires cytokine signals for induction of Foxp3 expression (Burchill et al., 2007; Lio and Hsieh, 2008; Turka and Walsh, 2008; Vang et al., 2008; Zorn et al., 2006). We evaluated expression of cytokine receptors on the cell surface of cDKO preTregs and tTregs. In the thymus, cDKO Foxp3+ tTregs, but not Foxp3- preTregs, express higher levels of the IL-2Rα chain (CD25), IL-7Ra chain (CD127), and the common gamma chain (CD132) (Fig. 5.10A). These data suggested cDKO Tregs possess dysregulated regulation of cytokine receptor genes due to loss of Foxp1/4 repression. Alternatively, a selection event occurred between preTreg and Treg development in which Tregs expressing the highest levels of cytokine receptors were selectively maintained at the expense of Tregs expressing lower levels of cytokine receptors.

We next assessed whether cytokine receptors on cDKO Tregs were functional. Levels of phosphorylated STAT5 (pSTAT5) are indicative of γc cytokine signaling responses in stimulated cells. We treated thymocytes from cDKO and Cre^{POS} controls with individual common gamma chain cytokines to measure pSTAT5 levels. cDKO thymic Tregs levels of pSTAT5 after stimulation with IL-2, IL-15 and IL-7 are comparable to Cre controls, suggesting proximal cytokine signaling is intact (Figure 5.10B).

As a result of pSTAT5 translocation and transcription, expression of anti-apoptotic protein Bcl-2 is induced. To test distal signaling after cytokine treatment and Bcl-2 induction in Tregs, we tested survival *in vitro*. To guarantee a highly purified

population of starting Tregs, we utilized cDKO mice that had been crossed to the Foxp3GFP reporter mice (Bettelli et al., 2006). We sorted live GFP+ CD4 T cells from cDKO or Cre^{POS} Foxp3GFP mice and cultured these cells in the presence of γc cytokines IL-2 or IL-7, or media alone. We compared survival and Bcl-2 levels of cDKO and Cre^{POS} cells and found a striking defect in cDKO Treg survival (Figure 5.10C). Common gamma chain signals partially rescued viable cell numbers, but we found cDKO Tregs incubated with IL-2 or IL-7 did not express comparable levels of Bcl-2 after 24 hours (data not shown). As early as 24 hours after plating, cDKO Tregs were detected in decreased numbers compared to controls, regardless of excess cytokine signals.

STAT5 signals generated by common gamma chain (γc) cytokines IL-2, IL-7 or IL-15 regulate induction and steady-state protein levels of Foxp3. We examined the expression of Foxp3 in cDKO tTregs compared to littermate controls. Variable levels of Foxp3 protein was detected in cDKO tTreg compared to controls with a trend toward reduced levels (data not shown.) These data together suggest cDKO tTregs are selected for the cells expressing the highest levels of cytokine receptor, but pSTAT5 signals fail to induce normal levels of Foxp3 and Bcl-2 expression.

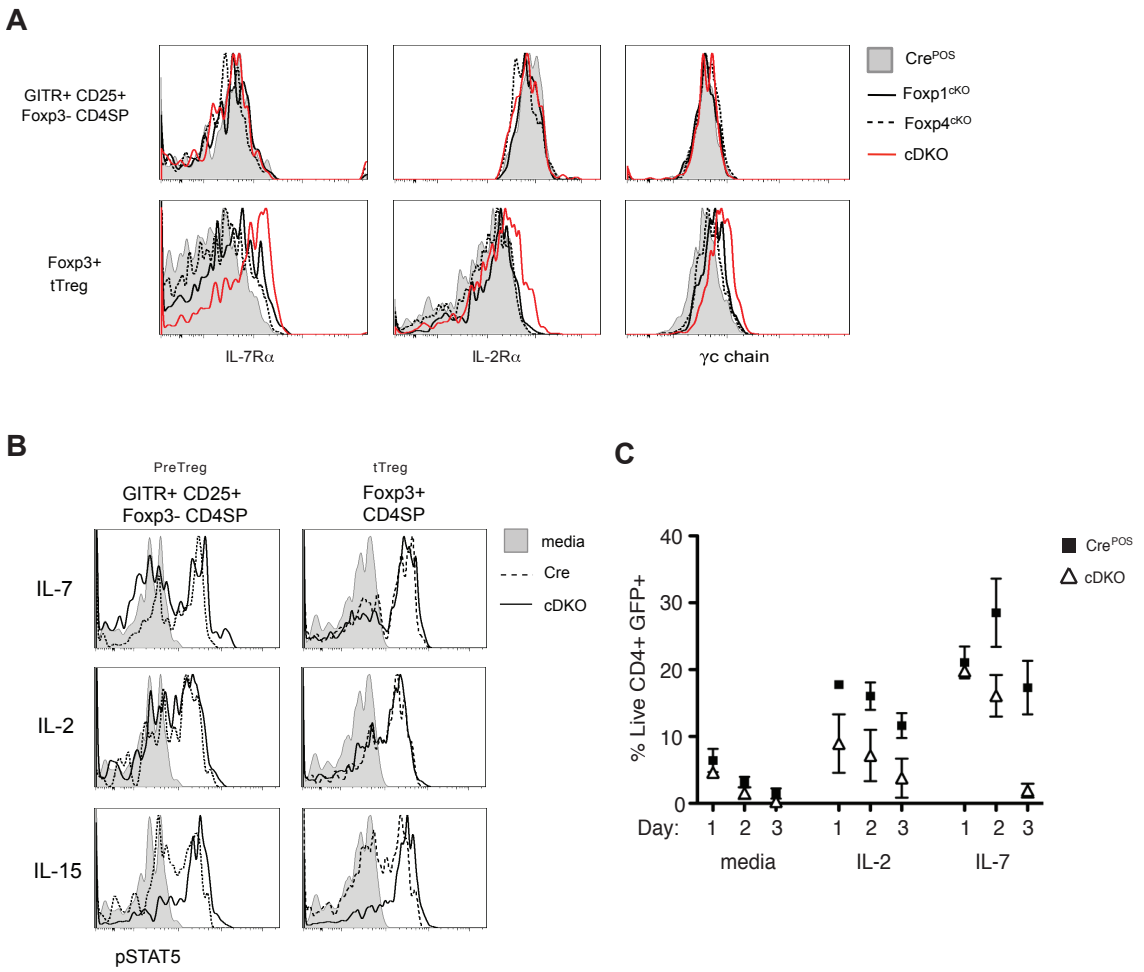


Figure 5.10. cDKO tTregs express increased cytokine receptors but exhibit impaired survival. A) Representative plots of preTreg (top) and tTreg (bottom) cytokine receptor expression. Representative of 6 independent experiments. B) pSTAT5 levels in preTreg (left) and tTreg (right) stimulated *in vitro* with IL-7, IL-2 or IL-15. Thymocytes were stimulated and stained intracellularly for pSTAT5 protein. preTreg plots are previously gated on Live, singlet, GITR+ CD25+ Foxp3- CD4 T cells, and tTreg are similarly gated on Foxp3+ cells. C) Survival of live TCRβ+ CD4+ GFP+ T cells from Cre^{POS} (black) or cDKO (red) mice expressing the Foxp3-GFP reporter. Sorted GFP+ cells were cultured with IL-2, IL-7 or media alone for up to 72 hours. Each point represents one well harvested at indicated time points.

Impaired cytokine signaling and Foxp3 induction is an intrinsic defect in cDKO thymocytes.

In contrast to normal tTreg numbers in non-chimeric cDKO, competitive chimeras demonstrate developmental disadvantages early in tTreg differentiation. To begin to understand the mechanism underlying the lack of cDKO-derived tTregs in competitive chimeric mice, we assessed cytokine receptor expression and function. CD45.2+ tTregs from competitive chimeric cohorts were compared for expression of CD25, CD127, CD122 (IL-15Rα/IL-2Rβ), and CD132 (γc). cDKO-derived Foxp3+ tTregs and preTregs expressed reduced levels of CD25 and CD127 as compared to endogenous CD45.1+ competitor wild-type cells (Figure 5.11A and data not shown). Expression of CD122 and CD132 were not significantly different in cDKO tTregs compared with endogenous wild-type cells (Figure 5.12A bottom panels, and data not shown). Furthermore, CD25, CD122, CD127 and CD132 expression by preTregs and tTregs in Foxp1^{cKO}:WT or Foxp4^{cKO}:WT chimeras was comparable to Cre^{POS} controls (data not shown). Together, these data demonstrate that Foxp1 or Foxp4 may be required for normal expression of the high affinity IL-2 and IL-7 receptors on developing tTregs. Furthermore, these findings support a model in which Tregs were selected for increased cytokine receptor expression under non-competitive conditions.

To test whether decreased cell surface receptor levels translated to a functional signaling defect, we assessed whether cDKO thymocytes from bone marrow chimeras received normal signals in response to cytokine stimulation. Thymocytes from Cre^{POS}:WT and cDKO:WT competitive chimeras were stimulated *in vitro* with either IL-2 or IL-7 and pSTAT5 levels were measured (Figure 5.11C-D, and data not shown). IL-2 stimulation elicited substantially reduced phosphorylation of STAT5 in both cDKO-

derived Treg precursors and Tregs. Similarly, IL-7 stimulation resulted in decreased pSTAT5 in both pre-Tregs and Tregs compared to endogenous WT competitors and Cre^{POS} thymocytes (data not shown). Titration of IL-2 or IL-7 did not rescue pSTAT5 responses, as stimulation with higher doses failed to compensate for reduced receptor expression (data not shown). These data demonstrate that cDKO thymocytes have a defect in cytokine-induced phosphorylation of STAT5 and suggest that a defective response to cytokine signals is a potential mechanism for the developmental disadvantage observed in cDKO preTregs and tTregs.

We next determined Foxp3 protein levels by mean fluorescence intensity (MFI) of Tregs from chimeras (Figure 5.11E). cDKO tTregs from cDKO:WT chimeras expressed significantly lower levels of Foxp3, relative to CD45.1+ wild-type tTregs. In contrast, $Foxp4^{cKO}$ Tregs and Cre^{POS} controls expressed comparable levels of Foxp3 protein. $Foxp1^{cKO}$ tTregs express an intermediate amount of Foxp3 which is significantly lower than Cre^{POS} and $Foxp4^{cKO}$ controls, but higher than that found in the cDKO (Figure 5.11D). Therefore, deletion of both Foxp1 and Foxp4 under competitive conditions results in fewer cells expressing Foxp3 with reduced levels expressed on a per cell level.

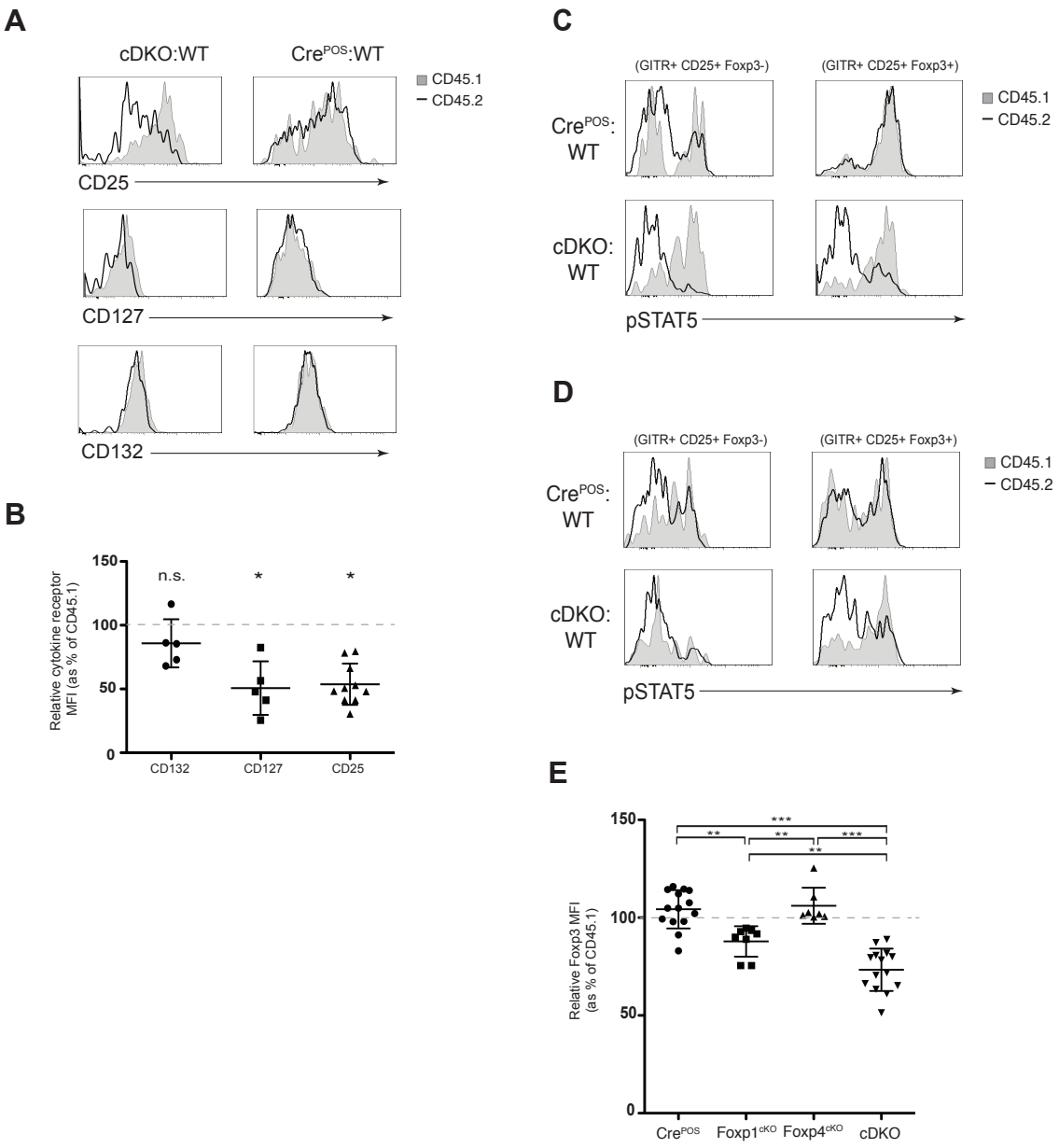


Figure 5.11. cDKO thymocytes have intrinsic cytokine signaling defects. A) Representative staining of cytokine receptor expression, gated on Foxp3+ CD4SP thymocytes. Gray histograms are gated on CD45.1+CD45.2-. Black lines are CD45.1-CD45.2+ gated cells. Data are representative of 4 experiments. B) Relative MFI of CD132, CD127 and CD25 from CD45.2+ Foxp3+ cDKO compared with CD45.1+ endogenous wild-type cells from the same mouse. Each point represents an individual mouse. CD132 and CD127 comparison is compiled from 5 cDKO:WT chimeras from 2 experiments, and CD25 levels are compiled from ten chimeras from four independent experiments.*p<0.005. C-D) Levels of phosphorylated STAT5 following stimulation with C) IL-2 or IL-7) in gated CD4SP GITR+CD25+Foxp3^{NEG} (left panels), and CD4SP Foxp3+ tTregs (right panels). Gray histograms gated on CD45.1+CD45.2-. Black lines

are CD45.1-CD45.2+ cells. E) Relative levels of Foxp3 expression in CD45.2+ tTregs as determined by ratio of MFI of CD45.2+/CD45.1+. **p<0.01, ***p<0.0001.

Discussion

Foxp3-related forkhead family members Foxp1 and Foxp4 are transcription factors that homo- and heterodimerize with each other and with Foxp3. Expression patterns of Foxp1 and Foxp4 in Tregs demonstrate increased Foxp4 and decreased Foxp1 expression. These data suggest that Tregs may not rely on regulation by Foxp1 and Foxp4 equally. However, while loss of either Foxp1 or Foxp4 transcriptional regulation in T cells has little effect on thymic Tregs, combined deletion results in abnormal Treg generation.

Under competitive conditions, reduced frequencies and numbers of CD45.2+ cDKO Tregs were detected. In the thymus, single cKO Tregs constituted slightly reduced frequencies of total Tregs, compared to Cre^{POS} cells. In the spleen and LN, we found reduced numbers of $Foxp1^{cKO}$- or $Foxp4^{cKO}$-derived Tregs. These data suggest Foxp1 or Foxp4 deletion results in subtle defects in Treg generation, potentially due to impaired development or competition.

We investigated Treg generation in cDKO mice to assess the contribution of Foxp1 and Foxp4 expression in Foxp3 regulation. Altered development of cDKO Tregs is immediately evident by expression of markers of activation on tTregs. The spleen and LN contained significantly reduced numbers of cDKO Tregs, and these cells also demonstrate reduced suppressive activity and survival.

The lack of CD45.2+ thymic Treg in cDKO-derived mixed chimeras contrasts with non-chimeric cDKO mice in which relatively normal numbers of tTreg develop. In cDKO

mice, ample “space” provided by growth factors may allow cDKO thymocytes to differentiate into Foxp3+ Tregs, survive, and expand to fill a Treg niche *in vivo*. Competition with wild-type thymocytes in chimeras limits available cytokine and survival signals required for persistence, turnover, and tTreg commitment. Our data cannot distinguish whether poor cytokine signaling is the cause of reduced survival of cDKO Treg precursors or Foxp3+ thymocytes, or both. Alternatively, impaired Foxp3 expression may be the primary cause for reduced tTreg development. Understanding the precise nature of either a survival or developmental defect will specify how FoxP family members affect the course of Treg generation.

This work suggests several potential molecular mechanisms of Foxp1 and Foxp4 in tTreg development. Deletion of both genes results in decreased Foxp3 induction or stability, and/or tTreg survival. Altered TCR and cytokine signaling in cDKO thymocytes may interfere with normal Foxp3 expression or survival of tTregs. Increased pAkt signaling is known to hamper *foxp3* induction (Haxhinasto et al., 2008; Sauer et al., 2008), and dysfunctional downstream STAT5 regulation may prevent assembly of the transcriptional complex required for *foxp3* expression. Alternatively, tTreg development may require Foxp1 and/or Foxp4 directly to bind the *foxp3* locus. Known interactions with histone deacetylases also suggest Foxp1 and/or Foxp4 may participate in chromatin remodeling to permit optimal *foxp3* transcription. Additionally, FoxP members may be required in Foxp3-complexes to preserve stability and function in maintaining a Treg phenotype. Consistent with this model, recent studies have demonstrated Foxp1 and Foxp4 in Foxp3 oligomeric complexes (Rudra et al., 2012).

Another possibility is that Foxp1 and Foxp4 may regulate gene programs independently of Foxp3. Regulation by FoxP proteins may be required at multiple loci to

support preTreg and tTreg maturation and survival. Foxp1 and Foxp4 may have dual or exclusive roles in regulating chromatin remodeling and gene targets, including potentially CD25 and CD127. Similarly, Foxp1-dependent regulation of naïve T cell development may also affect normal Treg development. Loss of naïve CD4 T cells in both Foxp1^{cKO} and cDKO mice appeared to impair iTreg differentiation *in vitro.* Failure of CD44^{hi} CD4SP cDKO thymocytes to induce cytokine receptor and/or Foxp3 expression in the thymus may further constrict Foxp3+ Treg numbers. In this way, both Foxp1 and Foxp4 expression impact Treg development, but as an effect of larger genomic regulation and histone modification that supports preTreg and tTreg differentiation. Whether FoxP proteins function cooperatively or autonomously, these studies demonstrate that FoxP family members are necessary for normal Treg development and maintenance of tolerance.

CHAPTER VI.

Discussion

Introduction

The studies described in this thesis have demonstrated novel roles for FoxP family proteins in T cell development, differentiation, and function. Previous reports demonstrated the necessity for Foxp1 and Foxp3 in T cells: Foxp1 regulates development of naïve T cells, and Foxp3 controls development and function of regulatory T cells. Known roles for FoxP regulation in T cells, as well as sequence homology between FoxP proteins, generated our initial hypothesis that FoxP family member Foxp4 also regulates gene programs in the T lineage. Similarity between Foxp1 and Foxp4 suggested these two proteins may function similarly by recognizing related gene targets. FoxP heterodimerization through the conserved leucine zipper domain implied Foxp1/3/4 proteins may co-regulate gene targets, and be required in combination for normal conventional and regulatory T cell development or function. A summary of the findings presented in this thesis, and discussion of recurring themes and remaining questions, will be provided in this chapter.

I. Foxp4 regulation in effector and memory T cells

To investigate the hypotheses surrounding Foxp4-specific transcriptional regulation, we examined the effects of Foxp4 deletion on normal T cell generation and function. From studies of Foxp4^{cKO} CD4Cre mice, we present evidence that Foxp4 is not required for development of T cells, but Foxp4 regulation is necessary for normal effector/memory T cell functions. Throughout these studies, we found Foxp4^{cKO} CD4 and CD8 T cells develop normally, and Foxp3+ regulatory T cells were detected in normal numbers and frequencies. Furthermore, lack of autoimmunity suggested normal suppressive function of Foxp4^{cKO} Tregs, and that Foxp4 was not necessary for the maintenance of self-tolerance in conventional T cells.

The most notable changes we observed in Foxp4^{cKO} mice were in activated effector CD4 T cells and antigen-experienced memory T cells. Expression of Foxp4 was transiently increased in activated T cells and suggested a role for Foxp4 in activation programs. Furthermore, Foxp4 expression was increased in wild-type Th17-polarized CD4 T cells. Our data suggest Foxp4-deficient CD4 T cells more readily differentiate into Th17 T cells, and indicate Foxp4 may normally repress Th17 programs. A connection between the FoxP family and Th17 differentiation was established from studies showing Foxp3 and Th17-lineage transcription factor RORγT antagonize one another. Given similarities in FoxP proteins, a similar antagonistic function of RORγT and Foxp4 is possible. Like Foxp3, Foxp4 may suppress RORγT regulation and moderate Th17 responses. In the absence of Foxp4, RORγT is free to enforce Th17 gene expression.

However, unlike normal Th17 CD4 T cells, increased frequencies of Foxp4^{cKO} Th17-polarized T cells produce IL-2. IL-2 typically drives Treg differentiation and inhibits Th17 commitment, so Th17 production of IL-2 is counter-productive. IL-2 is a known

target of Foxp1 suppression, and one possible reason for unusual IL-2 production in Foxp4^{cKO} Th17 cells may be a requirement for FoxP protein expression to limit *il2* transcription. Foxp4 upregulation in Th17 cells indicates this T cell population may require Foxp4 specifically for dual roles in moderating IL-2 and IL-17. Further studies to determine how Foxp4 regulation alters Th17 commitment may prove Foxp4 is another FoxP family member necessary for maintaining the balance between tolerogenic and inflammatory CD4 T cell responses.

The second Foxp4-specific defect we observed was reduced memory T cell function. Infection with either viral or intracellular parasitic pathogens was initially controlled by Foxp4^{cKO} T cell responses, but cytokine production was reduced following rechallenge of memory T cells. From these studies it was unclear which cells required Foxp4 expression. LCMV studies suggested CD4 T cells are primarily affected by loss of Foxp4 expression, but *T. gondii* experiments did not distinguish defects in CD4 or CD8 T cells, or whether all memory T cells performed poorly to rechallenge with cognate antigen. It is possible, therefore, that only cytokine-producing CD4 T cells are affected without Foxp4 expression in both models, and cripple effector immune cells that require CD4 T cell help. Another possibility is that different infections cause different outcomes for CD4 and CD8 T cells. Perhaps LCMV and *T. gondii* cause different effects in memory T cells. Setting aside for the moment whether Foxp4-specific regulation was limited to one T cell population or globally affects antigen-specific cells, reduced recall responses can be explained by several defects potentially caused by Foxp4 deletion.

First, our data do not suggest antigen specific memory cells die in LCMV-infected Foxp4^{cKO} mice, as tetramer reagents detected similar frequencies and numbers of gp61-specific CD4 T cells after clearance of the pathogen. However, we did not determine

whether gp61-specific CD4 T cells were turning over to account for cell death, or whether *T. gondii*-specific cells persisted normally in chronically infected cohorts. Abnormal survival in either case can contribute to overall loss of effector cytokine responses. To determine whether cell death is increased, future experiments are needed to assess longevity of memory T cell populations. Pulse-chase assays with an intracellular marker, such as bromodeoxyuridine (BrdU), measure loss of labeled T cells over time. This approach may reveal a change in the rate of cell death by comparing changes in numbers of BrdU+ cells.

Alternatively, Foxp4 expression may be necessary for preventing exhaustion in memory T cell populations. Particularly in chronic infection, continued TCR stimulation by antigen can cause T cells to lose effector function and transition into an anergic state. Reduced IFNγ production in response to STAg or to gp61 stimulation could be a consequence of antigen specific T cell exhaustion. It also remains unclear whether memory T cell functions are reduced across a spectrum of infections. These data suggest anti-viral and anti-parasite responses may be impaired, but challenge with fungal, bacterial, or chronic viral pathogens can determine whether the defect in IFNγ recall responses are the only affected memory functions in Foxp4^{cKO} T cells.

Because we assessed memory T cells in peripheral lymphoid tissues or in the CNS in *T. gondii* infection, it is also possible memory T cells simply migrated elsewhere, causing the appearance of overall reduced responses. Cell homing and migration defects can be interrogated by performing adoptive transfers of Foxp4^{cKO} memory T cells into similarly infected host mice, to control for inflammatory conditions. Short-term homing assays will determine how transferred populations migrate to peripheral lymphoid tissues or sites of inflammation. These data, along with investigation of T cell

exhaustion and cell death, are necessary to further define the role of Foxp4 in long-term memory responses.

Taken together, our findings in the Foxp4^{cKO} mouse suggests Foxp4 expression is not necessary for normal T cell development, but effector and memory T cells require Foxp4 to preserve effector functions, including cytokine production. Expression data suggest Foxp4 is maintained until activation of T cells, when Foxp4 expression is upregulated. In activated CD4 T cells, Foxp4 regulates T helper cell differentiation. In memory T cells, Foxp4 is required to maintain long-term immunity against recurrent infections.

Future Directions: Foxp4 regulation in lymphocyte development

In Chapter II, we demonstrated Foxp4 expression in developing thymocytes, peripheral T cells, recently activated T cells, and in polarized CD4 T cells. Immunohistochemistry indicated increased Foxp4 expression in cortical regions of the thymus, which correlated with patterns of Foxp4 mRNA detected in real-time PCR assays. The highest levels of *foxp4* transcript were detected in sorted DN3 cells, which develop in the thymic cortex before migrating into the medulla. Upregulation of Foxp4 early in thymic development suggests a critical role for this FoxP family member during TCR checkpoints in DN cells. Alternatively, upregulation of Foxp4 in DN3 cells may not be part of a developmental program, but a consequence of successful β rearrangement. Mature T cells stimulated through the TCR exhibit increased Foxp4 levels. β signaling also induces TCR signals, which may alter Foxp1 levels and induce Foxp4 expression.

To determine whether Foxp4 is required for T cell development, Cre-mediated deletion of the $Foxp4^{FLOX}$ locus can be induced at an earlier stage of development. Preliminary studies introduced Cre expression under the *lck* promoter. In $Foxp4^{cKO}$ LckCre mice, Foxp4 is deleted in the T lineage early in the DN stages of thymic development. Preliminary data showed decreased thymic cellularity was detected in $Foxp4^{cKO}$ LckCre mice, and decreased numbers of developing thymocytes compared to Cre^{NEG} littermates. However, similar effects on thymic development were observed in $Foxp4^{cHET}$ LckCre mice, suggesting a Cre-specific effect. Alternatively, loss of one allele of Foxp4 in $Foxp4^{cHET}$ LckCre mice may be sufficient to alter development. Further experiments may prove the maturation from DN to DP to be a critical stage for Foxp4 expression.

We also induced Foxp4 deletion in hematopoietic precursor populations by introducing Cre under the *vav* promoter. Vav proteins are guanine nuclear exchange factor for proteins in the Rho family, and Vav expression in mature T and B cells plays a critical role in activated lymphocyte signaling cascades (Ogilvy et al., 1998). Because Vav is expressed in hematopoietic precursors, Vav-mediated deletion affects both T and B cells (de Boer et al., 2003; Ogilvy et al., 1999). In preliminary studies of $Foxp4^{cKO}$ VavCre mice, we detected signs of defective T and B cell development. Numbers of $Foxp4^{cKO}$ VavCre total thymi were decreased compared to $Foxp4^{cHET}$ VavCre controls, and defects extended to both DN and SP thymocytes. B cell development appeared impaired in the generation of immature B cells. FoxP family member Foxp1 is known to be important for B cell development (Hu et al., 2006). Therefore, defects in Foxp4-deficient B cell development fits with known FoxP family member functions. Further investigation of both T and B cells from VavCre mice will likely demonstrate Foxp4-specific defects not observed in $Foxp4^{cKO}$ CD4Cre mice.

II. Foxp1 and Foxp4 in the regulation of lymphocyte development.

In Chapters III-V, we reported the results of the first studies of conditional Foxp1/4 double knockout T cells. Unlike Foxp4 conditional deletion studies, the consequences of Foxp1 and Foxp4 deletion were observed in both T cell development and function. CD4SP and CD8SP thymocytes in both Foxp1^{cKO} and cDKO mice expressed abnormally high levels of CD44, but developed in normal numbers despite decreased numbers and frequencies of DP cells. Peripheral lymphocytes maintained an activated cell surface phenotype, and persisted in reduced numbers. Both CD4 and CD8 populations appeared to acquire effector functions and undergo abnormal effector differentiation. Defective development appeared to occur upon deletion of FoxP proteins in developing thymocytes, suggesting thymocyte selection or maturation is altered without Foxp1 expression. Consequences of abnormal naïve T cell generation extended into CD4 and CD8 T cell number, function, and survival.

cDKO thymocytes exhibit altered T cell receptor signaling.

In Chapter V, developing thymocytes were stimulated with anti-CD3 and anti-CD28 to assess signaling pathways involved in Treg differentiation. We found cDKO thymocytes have significantly increased levels of phosphorylated Akt and Erk1/2 proteins immediately after stimulation *in vitro*. During thymic selection, strong TCR-generated signals drive CD4SP clones with self-reactive TCR complexes out of the T cell repertoire by inducing apoptosis (Gascoigne and Palmer, 2011; Hsieh et al., 2012; Jordan et al., 2001; Kishimoto and Sprent, 2000). It is thought thymic Tregs may develop

from CD4SP clones receiving strong TCR signals that do not quite reach the threshold for clonal deletion (Jordan et al., 2001). Although it is still unknown how strong TCR signals directly induce Treg differentiation, one model is that TCR signals drive differentiation of a Treg precursor population, called preTregs (Burchill et al., 2008; Lio and Hsieh, 2008). In cDKO CD4SP thymocytes, thymic Treg precursor generation may be intact due to strong TCR signaling.

The implications for strong TCR signals for selection of Foxp3- CD4SP and CD8SP clones are unclear, however. Although the TCR signaling studies described in this thesis focused on cDKO thymocytes, previously published work has demonstrated abnormal Erk1/2 signaling in Foxp1^{cKO} peripheral T cells (Feng et al., 2011). Therefore, it may be reasonable to assume Foxp1 deletion somehow alters Erk1/2 signaling process in both Foxp1^{cKO} and cDKO T cells. Our findings do not extend to other TCR signaling pathways that may be dysregulated in cDKO thymocytes. Further work to determine whether c-Jun N-terminal kinase (JNK) activity, NFκB, NFAT or calcium signaling is also increased in mutant thymocytes would clarify if total TCR signals are altered in cDKO mice, and whether thymic selection of a protective T cell repertoire is intact.

Defects in cDKO T cells suggest Akt/Foxo1 dysregulation.

We find similarities between Foxp1^{cKO} and cDKO Erk1/2 activity, but whether Foxp1 deletion is also associated with Akt signaling remains unknown. Levels of phosphorylated Akt (pAkt) were significantly increased in cDKO thymocytes compared to Cre^{POS} thymocytes, which may explain why Foxo1 gene targets appeared dysregulation in cDKO T cells. TCR signaling induces Akt phosphorylation of Foxo1, which prevents Foxo1 from translocating into the nucleus (Merkenschlager and von Boehmer, 2010).

When Foxo1 is sequestered in the cytoplasm, this forkhead factor is unable to induce expression of gene targets including the IL-7Rα chain, and transcription factor Kruppel-like Factor 2 (KLF2). KLF2 directly induces expression of CD62L, and chemokine receptors including CCR7 (Kerdiles et al., 2009; Kerdiles et al., 2010). Both CD62L and CCR7 support lymphocyte trafficking to peripheral lymphoid tissues including the lymph nodes.

In cDKO T cells, we find reduced expression of CD62L expression and significantly reduced frequencies and numbers of CD4, CD8, and regulatory T cells in LN, relative to lymphocytes detected in the spleen. Further evidence for altered KLF2 regulation was generated from preliminary data to assess expression of another KLF2 gene target, *s1pr1*, which encodes sphingosine 1-phosphate receptor type 1, which is another cell surface receptor important for lymphocyte trafficking. In cDKO T cells, *s1pr1* were decreased relative to Cre^{POS} controls (data not shown). Therefore, one possible mechanism of cDKO T cell migration and IL-7 sensitivity is altered Foxo1 activity, and impaired KLF2 expression.

However, one caveat of this model is that increased pAkt levels were observed after strong, direct TCR stimulation, and it is unclear whether baseline, homeostatic signals generated by interactions with MHC:self-peptide complexes also induced increased pAkt. cDKO lymphocytes characterized throughout these studies were harvested and analyzed without TCR stimulation, but still expressed an altered cell surface phenotype. Furthermore, resting thymocytes expressed minimal defects in pAkt levels, which were not significantly different from levels in resting Cre^{POS} thymocytes. Therefore, future work may demonstrate increased pAkt levels are a phenomenon limited to stimulated cDKO T cells.

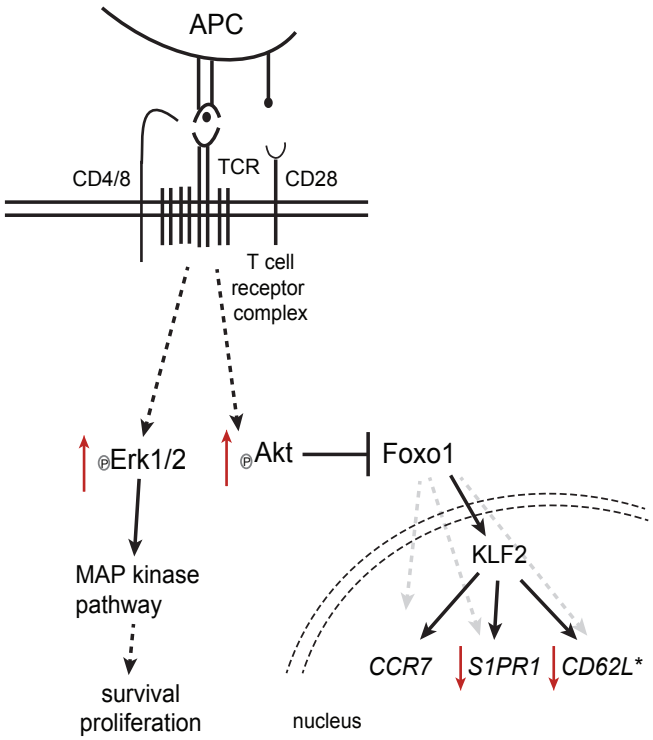


Figure 6.1. Model of TCR signaling defects in cDKO T cells. General model of signaling through the TCR complex in cDKO T cells. Increased pErk1/2 and pAkt and relative changes in downstream targets are indicated by red arrows. *Altered CD62L expression may be caused by transcriptional and/or post-translational regulation.

Instead of Akt-dependent Foxo1 sequestration, a possible alternative explanation for cDKO cell surface phenotype is a requirement of FoxP protein co-regulation of Foxo1 gene targets. Forkhead factors Foxp1/4 and Foxo1 may be required to bind the promoters of KLF2, or CD62L itself, to induce transcription. Foxp1 and Foxp4 may share redundant functions at these loci, accounting for relatively normal KLF2 target expression in Foxp1^{cKO} or Foxp4^{cKO} T cells.

Characterization of cDKO T cells suggests altered regulation of Foxo1 and KLF2 targets. Taken together with pAkt levels downstream of TCR stimulation, it is possible that Foxo1 activity is impaired by Akt-dependent phosphorylation, preventing nuclear

translocation in cDKO T cells. But how does the loss of FoxP family members affect total TCR signaling pathways? One possibility is that Foxp1/4 regulates expression of negative regulators important for modulating TCR signals. For example, phosphatase and tensin homologue (PTEN) is a negative regulator of Akt activity in T cells (Suzuki et al., 2001; Wang et al., 2000). No evidence exists for direct or indirect FoxP protein regulation of PTEN or other phosphatases involved in TCR signal downregulation, however, so the grounds for this mechanism are purely hypothetical. Therefore, extensive studies are required to investigate whether Foxp1, and possibly Foxp4, negatively regulate TCR signaling, to prevent downstream activation of developing thymocytes. Or whether Foxp1 maintains a broader program of T cell quiescence and chromatin regulation that prevents TCR signaling during thymic selection from causing activation and abnormal differentiation of thymocytes.

Foxp1 and Foxp4 regulation of IL-7 receptor signaling and survival.

Cytokine signaling mechanisms in cDKO T cells also appeared dysregulated. T cell lymphopenia in cDKO mice and competitive disadvantages in mixed bone marrow chimeras suggested FoxP proteins are required for normal persistence of both CD4 and CD8 T cells. Further studies of CD4 T cells cultured *in vitro* demonstrated poor survival, despite addition of excess cytokine. These findings contradicted our predictions that increased IL-7R and pSTAT5 levels in cDKO T cells would provide increased survival signals. Instead, both TCR and cytokine pathways exhibit significant defects without Foxp1/4 expression, suggesting the FoxP family is critical for T cell development and maintenance.

Although IL-7Rα expression may be altered due to multiple mechanisms, including Foxo1 dysregulation, it was unclear why cDKO T cells also exhibited defects in STAT5 regulation. Although cDKO T cells exhibited increased pSTAT5 levels after cytokine stimulation, it appeared STAT5 failed to elicit normal Bcl-2 expression and survival. It is also unknown whether IL-7R signaling and survival is disconnected in all T cells, or whether CD4 T cells and Foxp3+ Tregs are solely affected by loss of Foxp1/4 expression. Further work to understand how Foxp1/4 regulation affects cytokine receptor expression and regulation of STAT5 gene targets may reveal important roles for FoxP proteins in T cell homeostasis.

Future Directions: Foxp1 and Foxp4 regulation of immune responses in vivo.

During an immune response, both antigenic and interleukin signals are vital for T cell function. Cumulative defects in both TCR and cytokine signaling pathways suggested cDKO T cells may not perform normally during infection *in vivo.* Preliminary experiments in which cDKO mice were infected with *T. gondii* demonstrated T cell responses were significantly impaired. A cohort of mice was sacrificed 10 days after infection with Me49 cysts. T cell numbers were significantly reduced compared to Cre^{POS} controls, and both CD4 and CD8 T cells exhibited altered cytokine responses *in vitro*. From these studies it was unclear whether effector T cells exhibited impaired survival, altered expansion, failed to differentiate, or were susceptible to some combination of these defects. Most strikingly, cDKO mice infected with *T. gondii* did not survive longer than 21 days.

The studies described in this thesis have focused on naïve, uninfected cohorts of mice, but future work using model pathogens in single cKO and cDKO mice will provide

further insights into how Foxp1 and Foxp4 regulation are required for T cell-specific immune responses in acute and chronic infections. Furthermore, Foxp1 and Foxp4 may be required for maintenance of memory T cell populations. Cytokine signals support long-term survival of antigen-specific memory T cells, which protect the host from recurrent infections. It will be interesting to learn whether cDKO mice are capable of maintaining CD4 and CD8 memory recall responses and surviving rechallenge. *In vivo* models of infection may demonstrate additional roles for Foxp1 and Foxp4 regulation in T cell biology.

III. Foxp1 and Foxp4 regulation of Foxp3+ Treg generation

Altered generation of regulatory T cells was one of the most profound defects we found in the cDKO mouse model. Tregs are fundamental to maintaining peripheral tolerance (Gambineri et al., 2003; La Cava, 2009; Ochs et al., 2007; Sakaguchi et al., 2006; Ziegler, 2006). Regulatory T cell development function requires expression of transcription factor Foxp3. Induction of Foxp3 expression requires both TCR- and cytokine-generated signals, and participation by multiple downstream transcriptional regulators (Hori et al., 2003; La Cava, 2008; Lio and Hsieh, 2008; Merkenschlager and von Boehmer, 2010; Passerini et al., 2008; Sauer et al., 2008; Turka and Walsh, 2008; Vang et al., 2008; Wuest et al., 2008). Because FoxP proteins can interact through leucine zipper domains, we were particularly interested in Treg development in models of FoxP protein deletion in T cells. However, previous reports of Foxp1^{cKO} mice and our own findings in Foxp4^{cKO} models demonstrated Foxp3+ Tregs developed and functioned normally in single cKO mice.

Further investigation of Treg development in cDKO mouse models demonstrated FoxP proteins do play an important role in the maintenance of Treg populations. cDKO mice exhibited reduced frequencies of thymic Treg, and abnormal expression of cell surface markers associated with Treg activation. Furthermore, competitive bone marrow chimeras revealed significant developmental disadvantages in cDKO Tregs. Putative GITR+CD25+Foxp3- thymic Treg precursors exhibited competitive defects in mixed bone marrow chimeras, suggesting thymic development of Tregs was impaired at the earliest stages of differentiation.

Under competitive conditions, cDKO thymic Treg development was significantly impaired. In bone marrow chimeras, cDKO-derived preTreg and tTreg expressed decreased IL-2Rα (CD25), which correlated with reduced pSTAT5 signaling. Signaling through γc chain receptors is required for normal induction of Foxp3. IL-2 signaling in particular is a critical regulator of Treg development and maintenance. Loss of IL-2 signaling likely contributed to significantly reduced chimerism in tTreg and peripheral Treg populations.

Future Directions: Rescuing cDKO Treg development

Questions remain regarding the regulation of CD25 in cDKO Tregs. Altered CD25 may be caused by several defects: loss of Foxp1 and Foxp4 directly affects CD25 transcription, abnormal TCR signals in cDKO T cells results in abnormal CD25 expression, or Foxp3 itself fails to maintain increased CD25 levels. Abnormal expression of CD25 on Foxp3- preTreg populations suggests initial induction of CD25 is impaired. TCR signaling in CD4SP is thought to induce expression of CD25 on preTreg populations. Subsequent IL-2 signaling induces Foxp3 expression. TCR signals regulate

CD25 expression through activity of transcription factors, NFAT and AP-1 (Wu et al., 2006). FoxP family members interact with NFAT, but it remains unclear whether Foxp1/4 bind the *il2ra* promoter to induce CD25 expression. Further studies to examine Foxp1/4 binding of the *il2ra* promoter using chromatin immunoprecipitation should clarify direct or indirect FoxP family regulation of cytokine signaling pathways.

Because IL-2 signaling appears deficient in cDKO Tregs, supplying STAT5 signals artificially may rescue defects in cDKO Treg cellularity and survival. Here we propose infection of cDKO T cells with retroviral transgenic vectors encoding constitutively active STAT5 (CA-STAT5) to rescue Treg development. If the main defect in Treg development is loss of normal IL-2 signals, CA-STAT5 expression should rescue Treg numbers. Additionally, similar defects in STAT5 signaling may cause T lymphopenia and poor survival of conventional cDKO T cells. Parallel studies with artificial STAT5 or transgenic Bcl-2 expression in conventional cDKO T cells may repair cytokine signaling defects and produce improved cDKO T cell numbers and survival.

Secondly, it remains unknown whether Foxp3 itself is a target of Foxp1/4 regulation. Decreased expression of Foxp3 was detected in Tregs from both cDKO mice and cDKO:WT bone marrow chimeras. These data suggest loss of Foxp1/4 results in altered Foxp3 expression and/or maintenance. While cDKO T cells appear capable of inducing Foxp3 expression, it is possible that stabilization of Foxp3 protein requires Foxp1/4 protein interaction. Co-immunoprecipitation of Foxp3 and FoxP family members indicates these proteins can bind, but the purpose of these interactions remains unclear. Alternatively, Foxp3 does not require direct binding with Foxp1/4 proteins, but with transcription factors regulated by FoxP family members. Runx family members Runx1 and Runx3, as well as FoxO proteins, Foxo1 and Foxo3, play vital roles in the

expression and stability of Foxp3 (Kerdiles et al., 2010). FoxP family interactions with FoxO and Runx proteins have already been reported (Feng et al., 2011; Kerdiles et al., 2010; Klunker et al., 2009; Merkenschlager and von Boehmer, 2010; Zhang et al., 2008), but how Foxp1 and Foxp4 may interact with these transcription factor families in Tregs has not been investigated.

Future Directions: Investigation of Foxp1/4 regulation of Treg maintenance

Studies described in this thesis are limited to CD4Cre-mediated deletion of both Foxp1 and Foxp4. cDKO Treg development under competitive conditions demonstrated defects in cytokine signaling and Foxp3 expression. Further investigation of how Foxp1/4 regulates Treg maintenance may be potentially complicated by combined defects in generation and homeostasis. To limit our focus to Foxp1/4 regulation in Treg maintenance, we have bred $Foxp1^{FLOX}Foxp4^{FLOX}$ mice to Foxp3Cre mice, which express Cre under the *foxp3* promoter (Rubtsov et al., 2008). In these mice, T cells develop normally, until Foxp3 expression is induced during Treg generation. Cre-mediated deletion is limited to Foxp3+ regulatory T cells, which allows us to study Foxp1/4-deficient Tregs without the complications of lymphopenic conditions and activated T cells in cDKO CD4Cre mice. Furthermore, Foxp3Cre activity in Tregs can be determined by YFP reporter expression (Rubtsov et al., 2008).

Preliminary results from cDKO Foxp3Cre mice suggest cDKO YFP+ Tregs develop in reduced frequencies and numbers compared to undeleted Tregs in the same mouse. Similarly altered patterns of cell surface markers are expressed, but it remains unclear whether that is a cell-intrinsic event. Together with cDKO CD4Cre data, these findings suggest Foxp1/4 regulation is not only important generation of Tregs, but also

for Treg survival and maintenance. In addition to future directions in cDKO CD4Cre mice described above, further investigation of Treg maintenance in cDKO Foxp3Cre mice will expand models of Foxp1 and Foxp4 regulation in Foxp3+ Tregs.

Future Directions: Runx:FoxP family interactions

The Runx (Runt-related transcription factor) family of transcription factors are critical for T lineage development and function (Collins et al., 2009; Egawa et al., 2007; Grueter et al., 2005; Tani-ichi et al., 2013; Wong et al., 2011a). The Runx family expresses the highly conserved RUNT DNA-binding domain. Three proteins (Runx1-3) comprise the Runx family, but only Runx1 and Runx3 are expressed in T cells (Collins et al., 2009; Egawa et al., 2007; Wong et al., 2011a). Runx1 is highly expressed in CD4 T cells, and Runx3 expression is highest in CD8 T cells and CD4 T cells polarized to the T helper 1 (Th1) subset (Collins et al., 2009; Wang and Bosselut, 2009; Wong et al., 2012). In both CD4 and CD8 T cells, Runx family members control lineage commitment, cell cycle and proliferation, and differentiation (Blyth et al., 2005). Previous studies have also demonstrated Runx1/3 regulation is necessary for expression of Foxp3 and stability of Foxp3 protein in regulatory T cells (Klunker et al., 2009; Rudra et al., 2009). As discussed earlier, abnormal Treg development and altered expression of Foxp3 protein on a per cell basis may correlate with reduced Runx1 regulation in the CD4 T lineage.

Characterization of cDKO T cells revealed further defects that closely resemble dysregulation observed in Runx family knockout T cell models. Known interactions between Foxp3 and Runx family members Runx1 and Runx3 further support models of FoxP:Runx protein interactions in T cells (Bruno et al., 2009; Klunker et al., 2009; Ono et al., 2007). However, it must be asserted that the FoxP:Runx models discussed below

are purely speculative, and intended to generate hypotheses. Similarity between Runx and FoxP findings are simply correlative and require extensive work to prove causation.

Runx1 regulation in developing thymocytes provides anti-apoptotic signals, and Runx1-deficient DP thymocytes were detected in reduced numbers (Egawa et al., 2007; Wong et al., 2012). Runx1-/- DP exhibited increased expression of Fas and Bim, pro-apoptotic proteins, and poor survival (Wong et al., 2012). In Chapter III, we determined reduced thymic cellularity was due to reduced numbers and frequencies of DP thymocytes. A possible explanation for decreased DP populations in cDKO thymi may be loss of Runx-dependent survival signals.

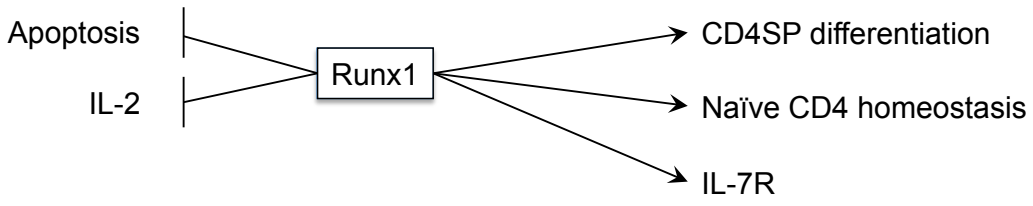


Figure 6.2. Schematic of Runx1-regulated gene targets in the CD4 T lineage. Runx1 expression in the CD4 T lineage promotes CD4SP thymocyte development and homeostasis of peripheral CD4 T cells, partially through regulating IL-7R expression. Runx1 inhibits apoptosis of double positive thymocytes during selection, and impedes IL-2 production by naive CD4 T cells.

Runx1^{cKO} T cells exhibit the CD44^{hi}CD62L^{lo} phenotype described in cDKO T cell populations (Wong et al., 2012). Separate studies with Runx1^{cKO} cells expressing the Bcl-2 transgene were designed to prevent cell death and assess populations of peripheral Runx1-deficient CD4 T cells (Wong et al., 2012). Runx1^{cKO} Bcl-2Tg CD4 and CD8 T cells acquired an activated phenotype, including increased expression of CD44 and decreased CD62L, similar to that observed in cDKO cells (Wong et al., 2012). This

defect proved to be a cell intrinsic effect of losing Runx1 regulation. This phenotype mirrors CD4 T cells in cDKO mice, and mixed bone marrow chimeras demonstrated this phenotype is also a cell-intrinsic phenomenon. However, activated Runx1-deficient T cells caused pathology in the lungs of Runx1^{cKO} mice (Wong et al., 2012). We do not see similar signs of pathology in cDKO mice. One reason for the lack of pathology in cDKO mice may be because cDKO T cells do not survive long enough to cause substantial pathology. cDKO T cells are not supported by Bcl-2 expression and may experience a shorter lifespan relative to Runx1^{cKO} Bcl2Tg populations.

Effector T cell functions also require Runx1 expression (Wong et al., 2011a; Wong et al., 2012; Wong et al., 2011b; Zhang et al., 2008). Runx1 represses IL-2 production in CD4 T cells (Wong et al., 2011b). Loss of Runx1 and FoxP regulation may explain abnormally high IL-2 production by cDKO T cells *in vitro*. Furthermore, Runx1 participates with RORγT to upregulate IL-17 production and support Th17 commitment (Zhang et al., 2008). In Foxp4^{cKO} mice, slightly increased Th17 effector functions were observed in polarized CD4 T cells. In cDKO cells, the frequency of IL-17-producing effector T cells immediately *ex vivo* was reduced. Further work is required to investigate whether cDKO T cells are incapable of mounting Th17 responses.

Th1 differentiation of CD4 T cells also requires either Runx1 or Runx3 regulation (Lee et al., 2009; Wong et al., 2011a; Wong et al., 2012; Zhang et al., 2008). Runx1/3 interact with T-bet, the prototypical regulator of Th1 gene programs (Lee et al., 2009). Together, these factors are potent inducers of IFNγ production. Stimulation of both CD4 and CD8 cDKO T cells revealed decreased IFNγ responses. CD4 T cells expressed nearly undetectable levels of IFNγ, suggesting complete loss of IFNγ regulation. But without Th1 polarizing studies, it remains unclear whether Foxp1/4-deficient CD4 T cells

are capable of differentiating into any T helper subsets. Therefore, future work is required to assess how cDKO T cells react to polarization. Further studies to observe whether Foxp1/4-deficient CD4 T cells are resistant to differentiation, or whether multiple Runx-dependent pathways are dysregulated, will refine or refute this model.

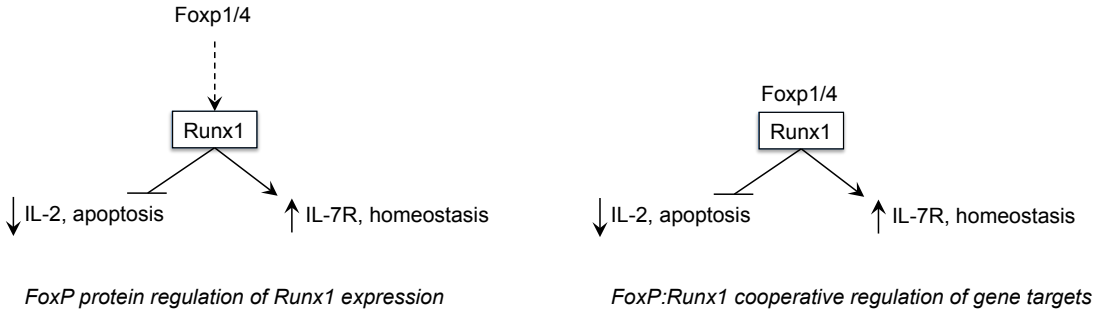


Figure 6.3. Models of Foxp1/4 and Runx regulation of selected gene targets in CD4 T cells. (Left) FoxP proteins induce Runx1 expression, indirectly regulating downstream targets and gene programs. (Right) Foxp1/4 bind to Runx1 gene targets to co-regulate Runx targets. Loss of Foxp1/4 in either case results in loss of Runx1 gene target regulation.

Perhaps the best evidence for Runx family dysregulation in cDKO T cells is abnormal expression of CD4 protein, as discussed in Chapter IV (Collins et al., 2011; Egawa et al., 2007; Taniuchi et al., 2002; Wang and Bosselut, 2009). CD4 levels are increased on both CD4 and CD8 T cells in cDKO mice. These data suggested dysregulation of Runx-dependent regulation of CD4. Runx3 is required for silencing of the *cd4* locus in CD8 T cells (Taniuchi et al., 2002; Wang and Bosselut, 2009). The population of $CD4^{dim}$CD8 T cells observed in cDKO mice correlates with loss of *cd4* silencing. Runx3 directs chromatin remodeling that normally mutes CD4 expression on MHC Class I-restricted CD8 T cells (Taniuchi et al., 2002). However, it is unclear whether FoxP family members directly participate in CD4 silencing, or indirectly control CD4 levels through regulating Runx1/3 expression.

Several caveats permeate this model, however. Runx1 also promotes CD4SP differentiation. Whereas other findings suggest loss of Runx1 activity, we observe increased frequencies of CD4SP cells in cDKO thymi. Inconsistencies in CD8 T cell findings also suggest Runx regulation is not the entire solution to altered cDKO T cell development.

In CD8 T cells, Runx3 regulation suppresses CD4SP development and promotes differentiation of CD8SP thymocytes (Collins et al., 2009; Grueter et al., 2005; Wang and Bosselut, 2009). Deletion of Runx3 causes disruption of the CD4:CD8 T cells ratio observed in both thymus and peripheral lymphoid organs (Djuretic et al., 2009; Grueter et al., 2005). cDKO mice do not appear to have the same phenotypic changes as $Runx3^{cKO}$ mice. It has been reported that Runx1 and Runx3 can compensate for one another (Collins et al., 2009; Wong et al., 2011a; Wong et al., 2012), but we must just as quickly accept Runx3 function may be normal in cDKO CD8 T cells.

In conclusion, several questions surround the interactions between Forkhead transcription factors and Runx family proteins. However, the correlation between several reported $Runx1^{cKO}$ defects and the cDKO phenotype in T cells provokes further investigation.

The relationship between Foxp1 and Foxp4 in T cells

Several features of the relationship between Foxp1 and Foxp4 became apparent from these studies. Setting aside the specific effects of single or combined FoxP protein deletion in T cells, these studies illustrate important lessons about the behavior of Foxp1 and Foxp4. Firstly, Foxp1 and Foxp4 regulate both sovereign and common gene targets in the T lineage. Secondly, Foxp1 and Foxp4 regulation is different in CD4 and CD8 T

cells. Lastly, naïve and activated T cells appear to require different FoxP family member regulation.

Previous experiments in cardiac tissues demonstrated Foxp1 and Foxp4 have non-redundant roles: Foxp1 is required for shaping normal vasculature and controlling cell growth in cardiomyocytes (Bai and Kerppola, 2011), while Foxp4 directs localization of cardiac tissues during embryogenesis and controls cell death of cardiopulmonary tissues (Li et al., 2004b). In contrast, pulmonary epithelial tissues exhibit specific requirements for Foxp2 for cell maturation and differentiation, but Foxp1 and Foxp4 serve redundant roles. Similarly, we find evidence in T cells of specific Foxp1 gene programs, such as maintaining naïve T cells, and suppressing effector-like differentiation. Foxp4 is not capable of compensating for loss of Foxp1 to maintain these gene targets.

However, we identified at least two cases in which Foxp1 and Foxp4 can partially compensate for one another: the expression and repression of CD4, and supporting cytokine receptor expression on thymic Tregs. Foxp3+ regulatory T cell development progresses relatively normally with either Foxp1 or Foxp4 expression, suggesting these proteins may be functionally redundant in this process. Therefore, Foxp1 and Foxp4 regulate specific gene targets in T cells, but some examples support redundant Foxp1/4 compensation function redundantly.

FoxP family member regulation of gene targets is different in CD4 and CD8 T cells. Differences in the frequency of $CD62L^{lo}$ CD4 and CD8 T cells suggest altered development or maintenance of Foxp1/4-deficient cells expressing effector memory or central memory phenotypes. CD8 T cells consistently included more $CD62L^{hi}$ cells than CD4 populations. Similarly, patterns of IL-7R expression in CD4 and CD8 T cells

indicate disproportionate regulation at another locus. Expression of many different transcription factors contribute to CD4 versus CD8 T cell differentiation, so the finding that FoxP members regulate targets differently in either population is perhaps obvious. Transcription factors in either the CD4 or CD8 T lineage likely shape the availability and accessibility of FoxP gene targets. However, these data strongly support the conclusion that Foxp1 and Foxp4 regulation is tailored to each cell type.

Lastly, naïve T cells appear to require Foxp1 for normal development, while activated effector or antigen-experienced memory T cells rely on Foxp4 regulation. Our data and others' support a model in which Foxp1 is highly expressed in naïve T cells to maintain a dormant, naïve state. Upon T cell activation, Foxp1 is downregulated, and our data indicate Foxp4 is upregulated (Heng and Painter, 2008; Rudra et al., 2012; Wiehagen et al., 2012). In Th17 T cells and long-lived memory T cells, Foxp4 regulates effector functions, through mechanisms that remain unclear.

In the studies described in this thesis, crossover between Foxp1 and Foxp4 regulation appears to be limited to relatively few gene targets, and Foxp1 and Foxp4 do not compensate well for one another in single cKO models. Separate expression patterns of Foxp1 and Foxp4 may prevent complete compensatory functions in the FoxP family. Additionally, Foxp1 and Foxp4 may have evolved specificity for gene targets common to naïve or activated T cells, respectively, to moderate cell fate decisions of T cells requiring diverse transcriptional regulation. Once Foxp1 is downregulated following activation, the global changes to chromatin remodeling and transcriptional activity during T cell differentiation may expose Foxp4 consensus binding sites. Foxp4 is then capable of regulating differentiation function of activated T cells. In antigen-experienced memory T cells, Foxp1 expression remains reduced, which may allow Foxp4 to control the

stabilization of cytokine gene loci and effector programs important for long-term immunity. Through these opposing patterns of expression, Foxp1 may regulate FoxP gene targets from early in development of naïve T cells, while Foxp4 assumes transcriptional control in effector and memory T cells. This division of duties between Foxp1 and Foxp4 may then provide regulation of cell fate decisions throughout the life of T cells.

CHAPTER VII.

Materials and Methods

Generation of Foxp4^{cKO} Mice

Foxp4^{FLOX} mice were generated by introducing LoxP sites flanking exons 12,13 encoding Forkhead DNA binding domain by standard recombineering (Liu et al., 2003). A 5′ LoxP site was introduced in the intron between exons 11 and 12, and a 3′ LoxP site was introduced in the intron between exons 13 and 14. Southern blot analysis with both 5′ and 3′ probes were used to verify homologous integration. Correctly targeted ES clones were injected into blastocysts to generate chimeric mice, which were further bred to C57BL/6 mice with successful germline transmission of the mutant Foxp4 allele. The neomycin resistance cassette was subsequently removed by intercrossing with Flpe transgenic mice (JAX ACTFLPe-9205Dym/J). CD4-Cre mice were purchased from Taconic (Lee et al., 2001). Foxp4^{null} mice have been described (Li, Zhou et al. 2004). All mice used were backcrossed to C57BL/6 for 1-2 generations. Mice were housed and bred in the University of Pennsylvania mouse facility. All animal experiments were performed in accordance with the University of Pennsylvania Institutional Animal Care and Use Committee guidelines.

Real-time PCR and probes

Total RNA was extracted from sorted splenocytes or thymocytes derived from adult mice with TRIZOL (Invitrogen, Karlsruhe, Germany) following the manufacturer's instructions. Five nanograms of DNase treated RNA were reverse transcribed in a 20µl reaction, using First Strand cDNA Synthesis Kit (Amersham Bioscience, Freiburg, Germany). The following TaqMan® assays and primers were employed: Mm00466368_m1 (*FoxP4*), and FAM-MGB 4352933E *(beta-Actin)*. Applied Biosystems Inc. probe sequences are as follows: Foxp4-Fwd (5' ATCGGCAGCTGACGCTAAATGAGA 3'), Foxp4-Rev (5' AAACACTTGTGCAGGCTGAGGTTG 3'). Expression levels of Foxp4 were normalized to beta-actin. Fold changes were analyzed with the $2^{-\Delta\Delta Ct}$ method.

Antibodies and Flow Cytometry

Fluorochrome-conjugated antibodies used for staining were purchased from BioLegend, eBioscience, Invitrogen, BD Biosciences, Beckman Coulter, or Caltag Laboratories. Lymphocytes from spleen were isolated after hypotonic lysis with BioWhittaker ACK solution (Lonza). For intracellular cytokine analysis, cells were stimulated in the presence of Brefeldin A with media alone, 2C11 (BioXCell, West Lebanon, NH), or with 5 ng/mL phorbol myristate acetate (PMA) and 1 µg/mL Ionomycin. After stimulation, cells were harvested and stained using a Cytofix/Cytoperm kit (Becton Dickinson) according to the manufacturer's instructions. All samples were acquired using an LSRII flow cytometer (Becton

Dickinson) and analyzed using FlowJo Version 8 (TreeStar). A cocktail of Pacific Blue-conjugated CD11b (Invitrogen), CD11c (Biolegend), Gr-1 (Biolegend), B220 (Biolegend), NK1.1 (Biolegend) and F4/80 (Caltag) was used to identify lineage negative thymocytes for sorting and real-time PCR.

Proliferation assay

Spleens were harvested and dissociated into single cell suspension between the frosted sides of two glass slides. Erythrocytes were lysed by hypotonic shock, and splenocytes were washed in T cell media (RPMI [Invitrogen] with 10% FBS, 1% penicillin/streptomycin, 10 mM HEPES, and 1×10^{-5} M 2-ME). Cell counts were performed using a hemocytometer and Trypan Blue dead cell exclusion. Cells were labeled with CFSE by washing with room temperature PBS, resuspending at 1×10^{7} cells/ml PBS, and mixing with an equal volume of PBS containing CFSE (2μM final concentration). Cells were gently mixed continuously for 3 minutes. The reaction was quenched with 100% FBS, and cells were gently mixed for an additional 2 min in the presence of quench before centrifugation. CFSE-labeled unfractionated splenocytes were plated at 1×10^{6} cells/well in 100 μl T cell media into round-bottom wells of a 96-well plate coated overnight with serial dilutions of anti-CD3 (2C11) in PBS and washed three times with PBS. On the fourth day, cells were harvested, stained, and analyzed by flow cytometry. Live cells were detected by flow cytometry by staining with LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Invitrogen).

Generation of iTreg

Single cell suspensions were prepared from spleens as described above, and cells were stained with FITC-CD4 (eBioscience), PerCPCy5.5-CD25 (BD Pharmingen), Pacific Blue-CD44 (Biolegend), and APC- or PECy7-CD62L (BD Pharmingen or Biolegend, respectively). Naïve T conventional cells, defined as $CD4^{+}CD25^{-}CD44^{lo}CD62L^{hi}$, were separated using flow cytometric sorting on Aria (BD Biosciences), and plated in a 48-well flat bottom plate. Cells were plated at 0.5×10^{5} cells/well in a total of 400µL T cell media (RPMI [Invitrogen] with 10% FBS, 1% penicillin/streptomycin, 10 mM HEPES, and 1×10^{-5} M 2-ME) including stimulus. Wells were pre-coated with 5µg/ml anti-CD3 (2C11) and cultured in the presence of 5µg/mL soluble anti-CD28, 50U/mL IL-2 (R&D) in the presence or absence of 5ng/mL TGFβ (R&D). After 4 days, wells were harvested and cells were stained for cell surface expression of CD4 and CD25, and intracellular Foxp3 protein using Invitrogen anti-Mouse/Rat Foxp3 Staining Set (APC) as per manufacturer's instructions. Live cells were detected by flow cytometry by staining with LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Invitrogen).

In vitro T cell activation marker upregulation

Whole splenocytes were isolated, counted, and suspended at 1×10^{7} cells/mL. Wells of a 96-well round bottom plate were coated overnight with 1µg/mL anti-CD3 (2C11) and washed three times with PBS before plating. 1×10^{6} unfractionated splenocytes in 100µL T cell media were plated on coated wells with 5µg/mL soluble anti-CD28, or left unstimulated in media in uncoated wells.

After a 24-hour stimulation, half of the wells were harvested and stained for PacificBlue-CD4, APCCy7-CD8, PerCP-Cy5.5- CD69, PECy7-CD127, FITC-CD44, APC-CD62L for polychromatic flow cytometry on LSRII. Four days after plating, the remaining wells were harvested and cells stained with the same cocktail.

Infection with *Toxoplasma gondii*

An i.p. injection of 20 cysts of *T. gondii* ME49 strain, isolated from brains of infected CBA/CaJ mice, was used for infection. For measurement of parasite burden, the 35-fold repetitive *T. gondii B1* gene was amplified by real-time PCR as described (Stumhofer et al., 2006). Soluble tachyzoite antigen was prepared from tachyzoites of the RH strain as described (Sharma et al., 1983). BMNCs from individual chronically infected mice were isolated in accordance with a published protocol(Wilson et al., 2005).

Infection with Lymphocytic Choriomeningitis Virus

Mice received 2×10^5 plaque-forming units (PFUs) of LCMV-Armstrong by intraperitoneal injection.

Analysis of IFNγ production by ELISA

A two-site enzyme-linked immunosorbent assay (ELISA) was employed to assay levels of IFN-γ as previously described (Cai et al., 2000; Sander et al., 1993). Briefly, spleens were dissociated in complete RPMI (10% heat-inactivated fetal

calf serum, 2 mM glutamine, 1,000 U of penicillin per ml, 10 μg of streptomycin per ml, 0.25 mg of amphotericin B [Fungizone], 10 mM HEPES [Gibco, Grand Island, N.Y.], 1 mM sodium pyruvate, 1% [vol/vol] nonessential amino acids [Gibco], 5×10^{-5} M 2-mercaptoethanol) to give a single cell suspension. Brain tissues were processed as previously described (Wilson et al., 2005). After lysis of erythrocytes, cells were washed twice and plated out at 10^5/well in a final volume of 200 μl. Cultures were stimulated with anti-CD3 or RH strain Soluble tachyzoite antigen (STAg) or in media for 24 hours. rmIFN-γ was purchased from Genzyme (Cambridge, Mass.)

Statistical Analyses

Where indicated, *p* values were determined by a 2-tailed unpaired Student t-test or one-way ANOVA. Dunnett of Bonferroni post-tests were used, as described in figure legends. *p* values <0.05 were considered statistically significant. All graphs show mean ± SD calculated in Prism software (GraphPad).

Generation of cDKO Mice. $Foxp1^{FLOX}$ and $Foxp4^{FLOX}$ have been previously described (Feng et al., 2010; Li et al., 2012; Zhang et al., 2010). CD4-Cre mice were purchased from Taconic (Lee et al., 2001). B6.CD45.1 mice were originally obtained from Taconic and were maintained in our facility. All mice used were backcrossed toward C57BL/6 for at least 1–2 generations. Mice were housed and bred in the University of Pennsylvania mouse facility. All animal experiments

were reviewed and approved by the University of Pennsylvania Institutional Animal Care and Use Committee.

Generation of Foxp3GFP reporter cDKO Mice. cDKO mice (described above) were crossed with mice expressing the Foxp3-GFP knock-in transgene as described in (Bettelli et al., 2006). Wild-type mice expressing CD4Cre were also bred as controls to express the Foxp3 reporter (Cre^{POS} Foxp3-GFP mice).

Generation of mixed bone marrow chimeras. Bone marrow harvested from wild-type CD45.1^{+} C57Bl/6, Foxp1$^{+/+}$Foxp4$^{+/+}$Cre^{+} (Cre^{POS}), Foxp1$^{Flox/Flox}$Foxp4$^{+/+}$Cre^{+} ($Foxp1^{cKO}$), Foxp1$^{+/+}$Foxp4$^{Flox/Flox}$Cre^{+} ($Foxp4^{cKO}$), and Foxp1$^{Flox/Flox}$Foxp4$^{Flox/Flox}$Cre^{+} (cDKO) mice were depleted of CD4 (GK1.5 clone) and CD8 (2.43 clone) expressing cells by magnetic separation. Cre^{POS}, $Foxp1^{cKO}$, $Foxp4^{cKO}$ or cDKO T-depleted bone marrow was then mixed in a 9:1 ratio with wild-type marrow cells, and injected intravenously into lethally irradiated wild-type CD45.1^{+}/CD45.2^{+} C57Bl/6 mice. Chimerism was determined in B cell gated bone marrow at the time of analysis.

Antibodies and Flow Cytometry**.** Fluorochrome-conjugated antibodies used for staining were purchased from BioLegend, eBioscience, Invitrogen, BD Biosciences, Beckman Coulter, or Caltag Laboratories. Thymocytes were isolated, counted, and stained in FACS buffer (1% FCS in PBS) for 30 minutes. Intracellular staining for Foxp3 was performed using a Foxp3 Staining Buffer kit

(eBioscience, cat. 00-5523-00) with Pacific Blue Foxp3 (BD Biosciences, 561182) or APC Foxp3 (eBioscience, 17-5773), according to the manufacturer's instructions. All samples were acquired using an LSRII flow cytometer (Becton Dickinson) and analyzed using FlowJo Version 8 (TreeStar).

In vitro stimulation and intracellular staining for STAT5 phosphorylation.

Freshly isolated thymocytes were rested two hours in a 37°C water bath. $5x10^6$ cells were stimulated with 100U IL-2 (Sigma) for 30 minutes, or 1ng/mL IL-7 for 20 minutes, or left unstimulated. At end of stimulation, cells were fixed in 10% paraformaldehyde for 10 minutes at room temperature, washed in PBS, and pelleted. Cells were resuspended in 90% ice-cold methanol and incubated on ice for at least 30 minutes. Cells were then washed in staining buffer and allowed to reconstitute for 30 minutes on ice in PBS with 2% FCS before incubation with antibodies to surface markers for 30 min at room temperature.

In vitro survival assay.

Spleen and LN cells were pooled from cDKO or Cre^{POS} mice. CD4+TCRβ+ Foxp3GFP- conventional T cells or CD4+TCRβ+ Foxp3GFP+ Tregs were sorted by flow cytometry using FACS Aria II. Cells were washed, counted, and plated at 50,000 cells/well. Cells were cultured at 37C. in cRPMI supplemented with recombinant IL-2 (10 ng/ml) or recombinant mouse IL-7 (10 ng/ml) (R&D Systems), or cRPMI alone. Wells were harvested 24, 48, or 72 hours after plating, and stained for Live CD4+ TCRβ+ and GFP reporter expression.

In vitro thymocyte stimulation assay for phosphorylated Akt or Erk1/2.

Whole thymocytes harvested from cDKO or Cre^{POS} mice were aliquoted at $5x10^6$ cells/mL and rested for 3 hours in eppendorf tubes at 37C. Cells were then plated on wells coated with 10μg/mL anti-CD3 and 10μg/mL anti-CD28. After 15 minutes cells were harvested and incubated in PhosFlow buffer (BD) and treated according to manufacturer's instructions. Staining for cell surface and intracellular epitopes were performed following phosphatase inactivation.

CHAPTER VIII.

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