The Role of Adenomatous Polyposis Coli in T cell biology: Development, Naïve T cell maintenance, and T cell Homeostatic Proliferation

by

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Dedication

To my loving and supportive family and friends. And to my thesis mentor, Pan, and Yang, whose supportive words and direction have hopefully made me a slightly acceptable immunologist. Finally to all of the past and present members of Zheng/Liu lab, who have put up with my messy lab bench and incessant questions

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Abstract

T cell development begins with the migration of early progenitors from the bone marrow to the thymus. Once out of the thymus, naïve T cells are held in an inactive state until they encounter their cognate antigen/MHC complex to further differentiate into effector T cells. Many studies have shown that Wnt signaling is a crucial pathway that regulates many aspects of T cell biology from development to effector T cell polarization, however, little is known about the role of Wnt in naïve T cells. In this study, we show that Adenomatous polyposis coli (APC) is critical for the maintenance of naïve T cells. Wnt signaling has also been linked to cancers, though Wnt's association with autoimmune disease is less known. Here, we show an association between ectopic dysregulation of Wnt causing a T-cell driven autoimmunity with a Crohn's disease-like phenotype.

Using an *Apc* conditional allele that has *loxP* sites inserted into introns 13 and 14 of the endogenous *Apc* gene under the control of a T cell specific cre-recombinase, CD4-Cre or Lck-cre, we can delineate the function of *Apc* in T cells. Flow cytometry, survival curves, and immunohistochemistry were used to study the T cell phenotype and autoimmune disease caused by ectopic activation of Wnt. Creation of bone marrow chimeras allowed us to investigate whether this T cell phenotype was cell-intrinsic and

whether the observed autoimmunity was linked to homeostatic proliferation. Using an exon 3 deletion of *cMyc*, we studied the impact of transcriptional activation of *cMyc*.

Though an *Apc* deficiency does not affect thymocyte development, we show that it does causes a massive cell-intrinsic loss of peripheral naïve T cells. We concluded that loss of *Apc* not only causes a loss of naïve T cells due to *cMyc*-induced apoptosis, but also results in highly activated T cells that drives the development of a Crohn's disease-like phenotypes. Our study has further linked ectopic Wnt activation to autoimmunity through a T cell specific deletion of *Apc*, suggesting a direct role of Wnt in regulating T cell driven etiology of autoimmunity.

Chapter 1

Introduction

1.1 Overview of Wnt signaling

The term “Wnt” is derived from the drosophila segment polarity gene *Wingless* (Wg) [3, 4] and the human homologue and proto-oncogene *Int1 [5-10];* a gene found to be activated in mouse mammary tumors. Wnt signaling exerts regulation on many developmental pathways, ranging from cell fate determination to maintaining the self-proliferative properties of stem cells [11-17]. Wnt signaling can be categorized into the canonical pathway or the noncanonical pathway. The canonical Wnt signaling pathway, as diagramed in Fig. 1, regulates transcriptional activity of β-catenin. The noncanonical pathways are independent to β-catenin and TCF/LEF. The WNT-Ca^{2+}, which regulates cell fate, migration, and the planar cell polarity (PCP) pathway (regulates cell polarity) are examples of the noncanonical pathways [11, 13, 16].

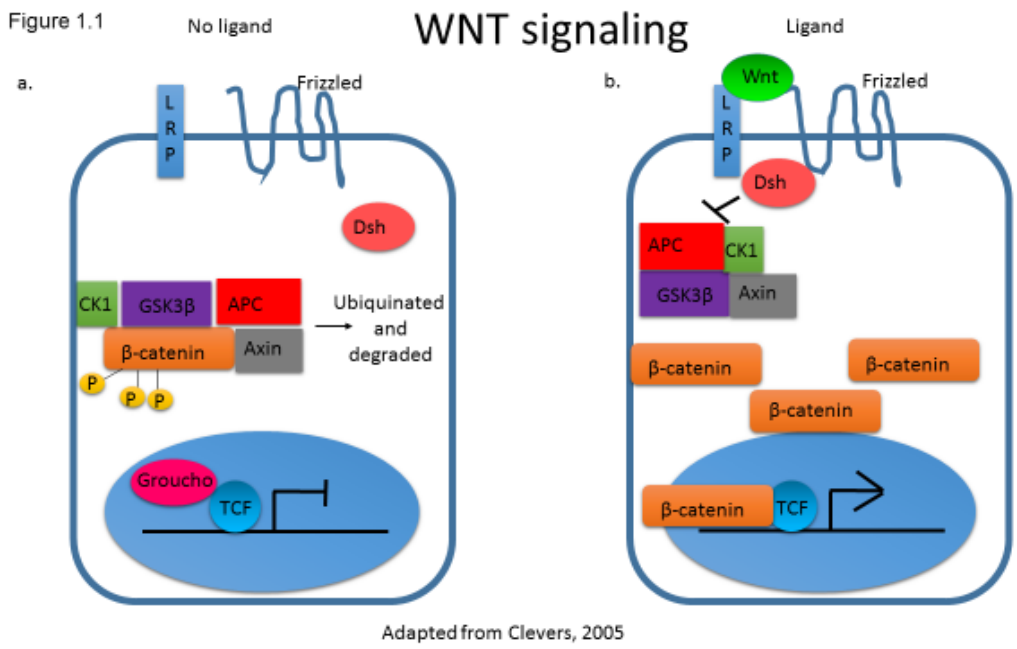


Figure 1.1. Diagram of canonical Wnt signaling pathway (adapted from [18]) a. Under circumstances where no Wnt ligand is present, β-catenin is targeted by the destruction complex, which consists of CK1, APC, Axin, and GSK-3 β. GSK-3 β phosphorylates β-catenin, leading to its ubiquitination and proteasomal degradation by the β-TrCP/SKP pathway. Thus, β-catenin does not translocate into the nucleus and Wnt signaling is not activated. b. Wnt binds to the co-receptors LRP5/6 and Frizzled, leading to inactivation of Dishveled and the ablation of the destruction complex. As a result, β-catenin is and translocated into the nucleus where it binds to its partners, such as TCF-1/LEF-and activate Wnt signaling.

As seen in Fig 1.1a, in the absence of Wnt proteins, basal levels of β-catenin are low due to the action of “destruction complex” composed of the tumor suppressor adenomatous polyposis coli (APC), GSK-3β, and Axin[19]. APC is the scaffold, by which “destruction complex” forms [20]. GSK-3β, then, phosphorylates β-catenin at four N-terminal serine and threonine residues [21, 22]. This phosphorylation event results in recognition by β-TrCP, and thus ubiquitinates β-catenin, labeling β-catenin to be degraded by the proteasome [11, 13, 16, 23].

Wnt proteins that initiate canonical Wnt signaling are a family of cysteine-rich glycoproteins [13, 24, 25]. These Wnt proteins serve as the ligands for the Frizzled (Fz) family of serpentine receptors [26-29] . Together with Fz, low-density lipoprotein receptor-related proteins (LRP-5 and -6)[30-33] bind to Wnt proteins to activate the Wnt cascade that results in inactivation of glycogen synthase kinase 3β (GSK3β) by a cytoplasmic protein known as Dishevelled, Dvl (Fig 1b) [34-38]. This results in the accumulation of β-catenin in the cytoplasm, and subsequent translocation into the nucleus, where it binds to members of the TCF/LEF family of transcription factors thereby activating Wnt target genes [11, 13, 16, 39-41].

Emerging evidence has shown that canonical Wnt signaling is important in the regulation of thymopoiesis and T cell differentiation, however, its role in naïve T cells is still unknown. Therefore by employing a conditional knockout of the *Apc* gene controlled by CD4-cre, we can ectopically activate Wnt signaling in later stages of T cell development, allowing us to further elucidate the role of Wnt in naïve T cells. Activation of Wnt initiates the upregulation of many downstream proteins that are regulators of the cell cycle, such as *c-Myc [42, 43]*. Interestingly, although *c-Myc* is known as an oncogene overexpressed in many cancers, *c-Myc,* in T cells, has be implicated in apoptosis via Fas/FasL interaction, death ligand tumor necrosis factor, and tumor necrosis factor related apoptosis-inducing ligand [44-46]. In this study, using a conditional knockout of *Apc*, we investigated the role of Wnt signaling and the effects of the upregulation of the known Wnt target gene, cMyc, in naïve T cells.

1.2 APC: Adenomatous Polyposis Coli

APC, a gene located on chromosome 5 in humans, was first identified as a tumor suppressor responsible for the onset of a rare autosomal dominant form of colorectal cancer known as familial adenomatous polyposis (FAP)[47-52]. It is somatically mutated in 80% of colorectal cancer and has been identified as the earliest known genetic alteration in colorectal cancers [53-55]. The mutation cluster region (MCR), found within the *APC* gene, is where the majority of colorectal cancer mutations are found, which results in a nonfunctional truncated APC protein lacking the carboxyl terminus [53, 56]. Though *Apc* has many other functions other than controlling Wnt signaling, many of the functions of *Apc,* in the hematopoiesis system, is β-catenin dependent [57].

Besides regulating β-catenin levels, APC also controls cell migration, apoptosis, and chromosomal segregation during mitosis [58]. APC regulates cell migration through its interactions with microtubules, EB1, and IQGAP1 [58-67]. APC has also been associated in chromosome instability (CIN). Colorectal adenomas with mutated APC display aneuploidy [68-70]. Embryonic stem (ES) cells from APC/min mice, but not wild-type ES cells, are more susceptible to chromosomal aberrations and aneuploidy, implicating APC in chromosomal segregation [71]. The role of APC in regulating apoptosis is still unclear and is likely to be tissue-dependent. APC can promote apoptosis by regulating Wnt target genes such as caspases or histone deacetylase 2 (HDAC2) [58, 72-74].

1.3 T cell development, maintenance and effector function

1.3.1 Thymocyte development

T lymphocytes originate from hematopoietic stem cells and progenitor cells that migrate from the bone marrow to the thymus to generate a population of immature thymocytes [75-78]. These immature thymocytes do not express CD4, CD8, nor CD3, therefore are designated as double negative (DN) thymocytes. DN thymocytes can further subcategorized by their expression of CD25 and CD44: DN1 ($CD44^{+}CD25^{-}$), DN2 ($CD44^{+}CD25^{+}$), DN3 ($CD44^{-}CD25^{+}$), and DN4 ($CD44^{-}CD25^{-}$) [75, 79-81]. Once a TCR β chain is successfully assembled [82], DN thymocytes will transition to double positive (DP) thymocytes. DP thymocytes undergo positive selection and negative selection and finally mature into SP CD4 or CD8 thymocytes [82-90]. Although, it has been shown that SP undergoes further maturation, the ultimate cellular or molecular factors involved in the final stage thymic maturation before thymic egress is largely unknown.

Once functionally mature, thymocytes exit the thymus to the peripheral lymphoid organs: the spleen and lymph nodes. This process ensures the maintenance of the peripheral T cell pool. However, the pathways that control thymic egress has not been fully defined. One possible transcriptional regulator of thymic egress is Krüppel-like factor 2 or KLF2, which licenses functionally mature thymocytes ($CD24^{lo}Qa\text{-}2^{+}$) to emigrate out of the thymus by regulating expression levels of sphingosine-1-phosphate (S1P) receptor S1P1, CD62L, and β7 integrin [91-94]. CD69 also regulates thymic emigration by negatively regulating the expression of S1P1 [95, 96].

Upon reaching the peripheral lymphoid organs, naïve T cells stay largely quiescent until they encounter their specific antigen and initiate immune response, as diagrammed in Fig. 1.2.

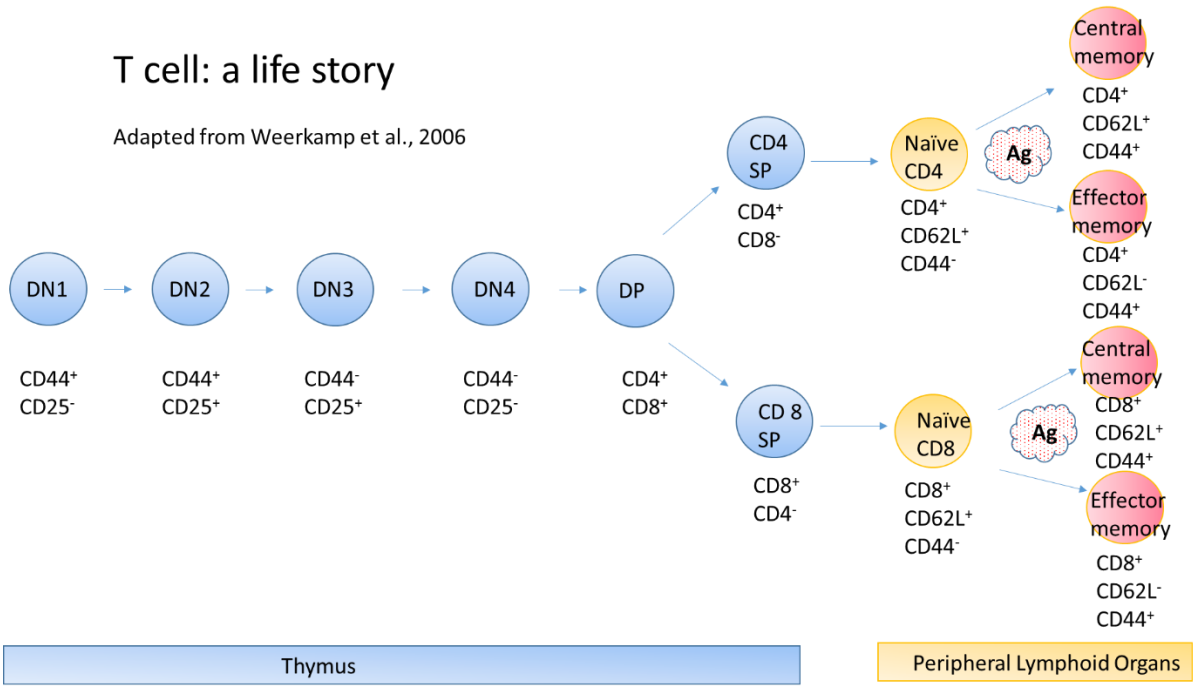


Figure 1.2. T cell: a life story. T cell development begins in the thymus as DN cells that do not express CD4 or CD8. These DN cells are subcategorized by their expression of CD44 and CD25 into DN1-4. After a successful β-chain rearrangement, DN cell transition in DP cells that express both CD4 and CD8. DP, then, transition into SP cells that express either CD4 or CD8. Once functionally mature, SP thymocytes exit out of the thymus entering the peripheral lymphoid organs as naïve T cells with high expression of an adhesion molecule, CD62L. Naïve T cells are held in a quiescent stage until an encounter with its cognate antigen, activating the cells to differentiate further into either central memory cells or effector memory cells that express high levels of CD44. Central memory cells can be distinguished from effector memory cells by their expression of CD62L. Adapted from [2].

1.3.2 Naïve T cell maintenance

In the periphery, naïve T cells are held in quiescence until they interact with their cognate antigen/MHC complex presented by an antigen presenting cell (APC). This interaction causes the naïve T cell to become activated and differentiate into an effector T cells. Quiescence is not a passive progress, rather, naïve T cell quiescence is enforced by several candidate pathways including mammalian target of rapamycin

(mTOR) [97, 98], forkhead box family members [99-101], and Schlafen-2 (Slfn2^{eka}) [102]. Genetic manipulations of these pathways lead to a loss of peripheral T cells and an increase of spontaneous activation and differentiation.

T cells deficient in the Fox family members have diminished peripheral T cell numbers, suggesting their importance in naïve T cell maintenance. Foxo1 has been shown to be important in expressing IL-7R. IL-7 signaling is essential for naïve T cell survival [100]. Foxp1 deletion leads to increased IL-7R(α) expression, which then sensitizes T cells to IL-7-driven proliferation and differentiation [99]. However, it is still unknown how these Fox members together interact synergistically to coordinate the expression IL-7R(α). Collectively, the Fox family members allow for naïve T cells to finely tune the response to IL-7 signaling.

Mice with T-lineage-specific deletion of tuberous sclerosis complex 1 (*Tsc1*), which leads to constitutive mTOR activation, also have a reduction of peripheral T cells that is associated with increased apoptosis; a phenotype that could be rescued through forced Bcl-2 expression [98]. T cells that are deficient in *Tsc1* have increased expression of positive regulators of the cell cycle, suggesting that mTOR inhibition restrains naïve T cell proliferation [97, 98]. These studies have provided very convincing evidence supporting the role of mTOR in the maintenance of naïve T cell quiescence, but it is still unclear what stimulates TSC1 to inhibit mTOR signaling so that naïve T cell quiescence can be held. In this study, we studied the functions of the gene *Apc*, a possible upstream regulator of mTOR signaling, in the maintenance of naïve T cells. Previously, it has been shown that APC can enhance the activity of GSK3β, which in turn, phosphorylates TSC2, thus inhibiting mTOR activation [103]. It is also known that β-

catenin becomes stabilized and translocates into the nucleus rapidly after TCR signaling though a phosphatidylinositol 3- kinase (PI3K) and phospholipase C (PCK) dependent manner [104]. By removing a known regulator of β-catenin via conditional deletion of *Apc*, we can examine the ectopic activation of Wnt and its effects on the regulation of mTOR activation and resulting naïve T cell maintenance, thereby connecting TCR signaling to TSC stimulation.

1.3.3 Effector Cell differentiation

After activation, $CD4^+$ T cells will expand and differentiate into distinct effector subsets: T helper (Th) cells, which are defined by their cytokines and transcription factors, as diagramed in Fig. 1.3. These subsets include: Th1, Th2, Th17, and T helper cells [105, 106]. Th1 cells are delineated by their production of interferon-Ɣ (IFN-Ɣ) and the expression of the transcription factor, T-bet. Th1 cell cytokines activate macrophages and defend against intracellular bacteria and some viruses [105, 106]. GATA-3 expressing Th2 cells produce Il-4, Il-13, and Il-5. Th2 cells activate eosinophils, aid B cells in antibody production, and mount an immune response against helmiths [105, 107, 108]. The transcription factor, FoxP3, is expressed by T regulatory (Treg) cells that produce immunosuppressive cytokines, such as TGFβ and Il-10[105, 107]. RAR-related orphan receptor gamma (RORγ) expressing Th17 cells produce Il-17A, IL-17F, and IL-22. Th17 cells are important in defense against some extracellular bacteria and fungi, and have been implicated with the pathogenesis of many autoimmune diseases [105, 109, 110]. Though the master transcription factors for each of these effector CD4 T cells have been well defined, when considering the complexity of inflammation, the system-

level cellular responses composed of multiple signaling pathways and how each of these interact to influence effector T cell differentiation is still unclear.

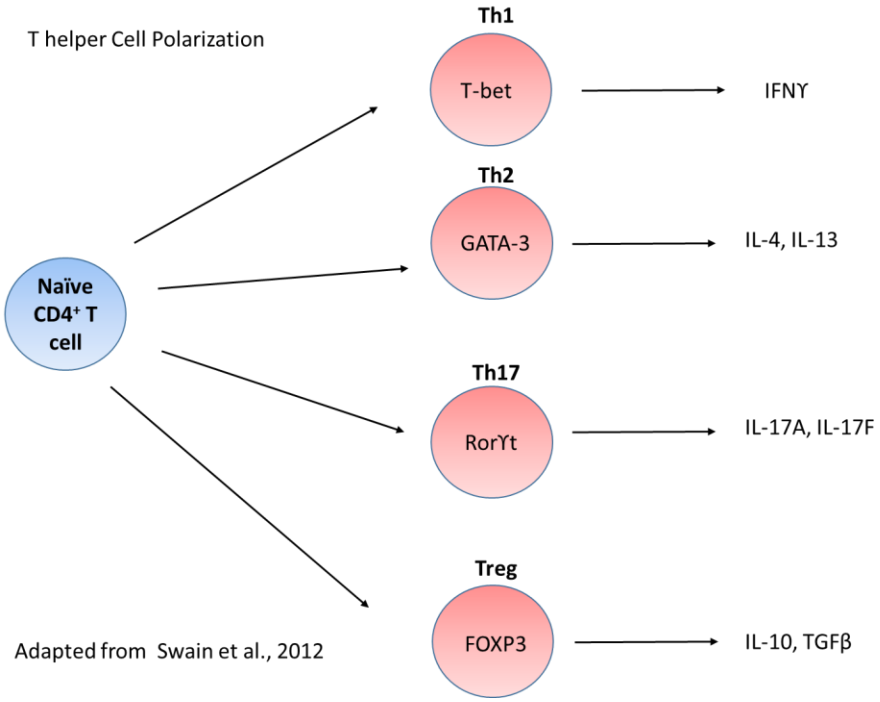


Figure 1.3 T helper cell differentiation. Naïve CD4 T cells will expand and differentiate into different T helper subsets: Th1, Th2, Th17, and Tregs. Th1 express the transcription factor T-bet and will produce IFNY. Th2 are defined by expression of GATA-3 and produce IL-4 and IL-13. Th17 express the transcription factor RORYt and produce the cytokines IL-17A and IL-17F. Finally, T regulatory cells express FOXP3 and produce IL-10 and TGF-β. Adapted [1].

1.4 Role of Wnt signaling in T cell biology

1.4.1 Wnt in thymocyte development

Many aspects of T cell biology: development, activation, and differentiation, are regulated by Wnt signaling. The importance of Wnt signaling in T cell development was

first illustrated with studies using either TCF1 or LEF1 deficient mice. Two different knockout mice for TCF1 were developed: deletion of exon 5 or exon 7 [111]. A deletion of exon 5 resulted in a truncated TCF protein that was still functional, therefore only caused a minor decrease in thymic cellularity. With an exon 7 deletion of TCF1, TCF-1 no longer has DNA binding activities, consequently nullifying TCF-1 transcriptional activity [111]. With an exon 7 deletion of TCF-1, thymocyte development was severely impacted: thymic cellularity was drastically reduced resulting from an age-dependent blockage at the immature SP (ISP) to DP transition. In addition to the developmental blockade at the ISP to DP transition, TCF1 exon 7 deletion caused a reduction of DN2 thru DN4 cells due to decreased cell proliferation [111]. Surprisingly, LEF1 deficiency does not affect T cell development. A deletion of both LEF1 and TCF1, using an exon 5 deletion of TCF1, resulted in a complete blockage in thymocyte development at the ISP stage of development, suggesting a redundant role for LEF1 and TCF1 in thymocyte development [112].

Despite these results demonstrating the importance of TCF-1/LEF-1 in thymocyte development, the dependence of canonical Wnt signaling mediating these defects is disputed. Conditionally deleting β-catenin using a T cell specific promoter, Lck-cre, resulted in a partial developmental blockages at the β-selection checkpoint of thymocyte development [113]. However, this β-catenin conditional deletion was not as striking as the TCF-1 exon 7 deletion, suggesting that some of the phenotypes seen in TCF-1 exon 7 deficient thymocytes may be the result of β-catenin independent functions. To add to the debate over the role of canonical Wnt signaling in thymocyte development, mice with a conditional deletion of β-catenin in the hematopoietic stem cell compartment did

not result in any phenotype in lymphopoeisis [12]. To exclude the possibility that β-catenin's homologue, ϒ-catenin, was compensating for the loss of β-catenin, double knockouts of both β-catenin and ϒ-catenin were made. However, no phenotype was observed, leading the authors to conclude that Wnt signaling is dispensable for hematopoiesis and lymphopoeisis [114, 115]. Furthermore, in a mouse model that overexpressed ICAT, a molecule that inhibits interactions between TCF-1 and β-catenin, was unable to recapitulate the extreme phenotype as seen TCF-1 exon 7 deleted thymocytes. ICAT overexpression, when expressed early in thymocyte development, did cause a developmental blockage at the DP stage, but when introduced later, did not inhibit SP thymocyte development [116]. Though TCF-1 has been shown to play a role in regulating thymocyte development, it remains to be demonstrated whether this role is dependent on canonical Wnt signaling.

In studies where gain-of-function mutations were made, the effects of ectopic Wnt activation has also created inconsistent conclusions. Loss of APC (Wnt activation) caused a developmental blockage at the DN3 to DN4 transition [117]. Likewise, knockin non-degradable β-catenin (deletion of exon 3) also affected thymopoiesis, where DP thymocyte development continued in the absence of pre-TCR signaling [118]. However, another group showed that stabilized β-catenin did not affect thymocyte development in young mice (6-8 weeks old). Instead, an overexpression of β-catenin accelerated thymic involution, as 3 month old mice thymic cellularity were drastically reduced [119]. With these conflicting studies, additional investigations are necessary to further delineate the role of Wnt in thymocyte development. In our study, we clarified the role of Wnt in thymocyte development by ectopically activating Wnt signaling via a conditional deletion

of APC through both Lck-cre and CD4-cre. Neither Lck-cre nor CD4-cre resulted in any thymocyte development blockade, but Lck-cre accelerated thymic involution as seen in a previous study. Thus, we demonstrated that Wnt activation is not sufficient to affect thymocyte development.

1.4.2 Wnt in T cell differentiation

Studies investigating the role of Wnt on T regulatory cell (Treg) functions have conflicting results. Enhancing Wnt signaling by a retroviral transduced β-catenin in $CD4^+CD25^+$ T cells improved Treg suppression of a model of inflammatory bowel disease that is induced by naïve T cell transfer. Stabilized β-catenin upregulated expression of Bcl-XL [120] thereby improving Treg survival; implying an immunosuppressive role for Wnt activation. Conversely, Tregs with an exon 3 deletion of β-catenin (constitutively active Wnt) could not suppress inflammatory bowel disease [121]. Another study found that inhibition of Wnt via a deletion of TCF-1 yielded Tregs with improved suppression of inflammation, though this result may be due to canonical Wnt-independent functions of TCF-1 [122]. Together, these studies have shown that Wnt's role in Treg function is still quite unclear.

The roles of canonical Wnt signaling in effector/memory T cell responses are also controversial. Activation of Wnt signaling, through in vitro addition of GSK-3β inhibitors, favors the production of memory CD8 T cells rather than the terminally differentiated effector T cells [123]. However, this effect was not observed by another study which showed that addition of GSK3β inhibitors did not affect CD8 T cell differentiation nor did increased Wnt signaling generate CD8 memory stem cells. Most importantly, deletion of

β-catenin did not perturb CD8 memory stem cells after priming [124]. The opposing data in these two reports remains to be reconciled.

The role of Wnt in the maintenance of memory CD8 T cells has also been studied via exon 7 deletion of TCF-1. Studies have shown that TCF-1, though a β-catenin-dependent regulation of eomes, increases the expression of IL-2βR, therefore maintaining CD8 T cell responsiveness to IL-15 [125]. In a conflicting study, Prlic et al. demonstrated that Wnt is dispensable for T effector cell function as mice with β-catenin-deficient T cells mounted a normal immune response to bacteria, as evidenced by comparable bacterial clearance, expression of CD62L and KLRG1, and no observed increase in memory CD8 T cells [126]. Perhaps this discrepancy in results is caused by the use of TCF-1 deletion rather than β-catenin deletions, thus the phenotypes seen in TCF-1 deficiency may not be the cause of Wnt activation, but its Wnt-independent roles.

1.5 Lymphopenia and autoimmune disease

The number of T cells in the peripheral lymphoid organs are relatively constant in an adult animal. T cell homeostasis is achieved by different mechanisms at different stages of life. In healthy young adults, the size of the peripheral T cell pool is dependent on seeding via thymopoiesis. As animals age, the thymus atrophies, thus shifts the maintenance of the peripheral T cell pool from the thymus to a process called homeostatic proliferation (HP). HP also occurs during the perinatal period when the number of T cells produced by the thymus is low (lymphopenic) compared with the space in the peripheral lymphoid organs [127].

HP occurs when T cells rapidly proliferate in response to lymphopenia. HP is distinctly different from the normal expansion of T cells due to antigen interactions [128]. During HP, T cells are allowed to proliferate in response to low affinity antigens and self-antigens. More importantly, this response is not toward a single foreign antigen, but is a polyclonal response. This expansion of T cells also allows low-affinity T cells that normally would not have been activated to acquire effector traits: expressing CD44, Ly6c and producing IFNγ [129-133]. During HP, T cells are allowed to expand in response to self-antigen, thus skewing the TCR repertoire toward self-reactivity [134-137]. This HP-driven self-reactivity is observed in H-2M mice. H-2M mice express CLIP peptide both in the thymus and in the periphery. In a non-lymphopenic host, $CD4^+$ T cells will be positively selected for while in the thymus, therefore peripheral T cells will not proliferate in the presence of the CLIP peptide. In contrast, lymphopenic H-2M mice have $CD4^+$ T cells expanding due to recognition of the CLIP peptide. In the context of lymphopenia, T cell proliferation has the ability to proliferate to self-antigen, thus skewing the TCR repertoire toward autoimmune disease [138].

The link between lymphopenia and autoimmune disease has been demonstrated through mouse models of inflammatory bowel disease. RAG-1 deficient mice, which lack both B and T cells, will develop inflammatory bowel disease with the transfer of small numbers of naïve $CD4^+$ T cells [139, 140]. More importantly, disease severity is correlated to the ability of these T cells to HP *in vivo* [139, 141]. Artificially inducing lymphopenia by neonatal thymectomies can also induce autoimmunity, however, it should be noted that severity and incidence of disease is dependent on the strain of mouse [142]. Thus, lymphopenia provides an ideal condition for the development of

autoimmune disease. However, as seen in the neonatal thymectomies, lymphopenia is not always sufficient in inducing autoimmunity.

Many human autoimmune diseases are associated with lymphopenia including: Sjogren's disease, rheumatoid arthritis, systemic lupus erythematosus (SLE), and Crohn's disease [134, 143-147]. Thymectomized myasthenia gravis patients that present with mild lymphopenia are predisposed to the development of SLE [148]. Rheumatoid arthritis patients, who are also lymphopenic, present with decreased TCR diversity. This has been attributed to HP because HP selects for T cell clones that have high affinity to self, thus skewing T cell repertoire toward self [149-151]. In addition to T cell repertoire skewing, T cells from RA patients acquire effector characteristics, defined by lymphopenia-driven HP [152-154]. Diabetic patients also present with aberrant T cell homeostasis. Many at-risk and newly diagnosed patients have increased percentages of activated T cells, but reduced numbers of peripheral T cells [155-157]. Murine studies have also shown a link between lymphopenia and autoimmunity. For instance, nonobese diabetic (NOD) mice were observed to have slightly lower numbers of T cells. The resulting population of T cells are activated and will eventually destroy β-cells causing diabetes. However, infusing the NOD mice with more T cells, the development of autoimmune diabetes can be delayed. Lymphopenia was not the only factor in in the development of diabetes. The investigators bred the NOD to a different background, Idd3.NOD mice, but did not develop autoimmune diabetes, though these mice still were lymphopenia. In addition to lymphopenia, NOD mice produced more IL-21 allowing for increased HP. This demonstrates that lymphopenia is not sufficient to generate autoimmunity, but a cofactor to autoimmunity [158].

Cell extrinsic factors, such as regulatory T cells, have also been hypothesized to play a role in suppressing lymphopenia-induced autoimmune disease. The addition of $CD4^+CD25^+$ Tregs can alleviate autoimmunity in thymectomized mice [142]. Still, depletion of Tregs in lymphopenic hosts is not always sufficient in inducing autoimmune disease [159]. When both naïve and Tregs are transferred into a lymphopenic host, the transferred Tregs do little to inhibit the proliferation of naïve T cells. It is possible that Tregs are not involved in the inhibition of HP in response to lymphopenia, but instead, are crucial in the suppression of the immune response in a much later stage of disease [160, 161].

Together, these studies have shown that lymphopenia-induced HP is a co-factor in autoimmune disease. However, the intrinsic T cell programs that are involved in the process are still unclear. Mutations in several genes lead to lymphopenia, such as deletions of *Tsc1* [97, 98] and *Klf2 [91, 92],* but were insufficient to cause autoimmune diseases. Components of Wnt signaling have been associated with autoimmune disease. SNPs in the TCF-4 promoter region are associated with increased incidence of ileal Crohn's disease due to impaired paneth cell production of α-defensins [162, 163]. The co-receptor for Wnt signaling, LPR6, has also been shown to be functionally mutated in early onset ileal Crohn's disease patients, damaging the regenerative potential of the intestinal crypts [164]. TCF7 SNPs result in β-cell dysfunction causing an increased risk in type II diabetes [165]. And finally, increased Wnt activation in the synovial fluid of rheumatoid arthritis patients is associated with increased severity of disease [166, 167]. Many of these studies are either related to dysfunction of tissue differentiation/remodeling, but Wnt activation T cells as a contributing factor for

autoimmunity has not been studied. Our study has implicated ectopic Wnt activation in autoimmune disease through a single T cell specific deletion that is sufficient to cause lymphopenia-driven autoimmune disease.

Chapter 2

On the Role of APC in early T cell development

2.1 Abstract

Wnt signaling has been found to regulate T lymphocyte development. It has been demonstrated that Wnt signaling plays a central role in early T cell development from studies where exon 7 deletion of TCF-1 inhibited thymocyte production. However, other studies dispute whether this developmental defect is a reflection of canonical Wnt signaling or of TCF-1-Wnt independent signaling. In this study, we further elucidated the role of Wnt signaling in thymocyte development by deleting a negative regulator of Wnt, Adenomatous polyposis coli (APC) using two lines of Cre-driver with minor differences in the time of *Apc* gene deletion within the DN3 stage of thymocyte development. Ectopic activation of Wnt signaling was observed in DN4 thymocytes. While *Apc* deletion ectopically activated Wnt signaling by increasing nuclear β-catenin and increased expression of Wnt target genes, such as *cMyc* and *Cd44* in mature thymocytes, the cellularity and major subset distribution among the thymocytes were not

affected. Our data demonstrated that ectopic activation of Wnt signaling starting at DN4 does not interfere with thymocyte development.

2.2 Introduction

Wnt signaling is an evolutionarily conserved pathway that is involved in cell differentiation, cell polarity, and maintenance of stem cell populations. Wnt signaling is initiated with the binding of Wnt proteins, cysteine-rich glycoproteins, to two receptors: Frizzled (FZ) and low-density lipoprotein receptor-related proteins (LRP) [3, 32, 33, 168]. As a result, Dishevelled, Dvl, inactives glycogen synthase kinase 3β (GSK3β). This allows for the accumulation and subsequent translocation of β-catenin into the nucleus, where it binds to members of the TCF/LEF family of transcription factors thereby activating Wnt target genes [11, 13, 16, 39-41]. In the absence of Wnt proteins, levels of β-catenin are regulated by the "destruction complex" composed of APC, GSK-3β, and Axin [19]. Identification of these key components provides us with ample opportunities to genetically manipulate the strength of Wnt signaling.

T lymphocyte development is among the best characterized among mammal tissues. From the bone marrow, progenitor cells migrate to the thymus to generate a population of immature thymocytes [75-78]. These immature thymocytes are designated as double negative (DN) thymocytes, because they do not express CD4 and CD8. DN (DN 1-4) thymocytes can further subdivided by their expression of CD25 and CD44 [75, 79-81]. DN thymocytes, mature into double positive (DP) thymocytes after a successful TCRβ chain has been assembled. DP thymocytes undergo positive selection and negative selection and finally develop into SP CD4 or CD8 thymoyctes [82-90].

Studies using TCF-1- and LEF-1-deficient mice suggest that Wnt signaling plays a critical role in thymocyte development [111, 112, 169]. TCF-1 was found to be important in driving the proliferation of DN2, DN4, and ISP thymocytes [111]. Ioannidis et al. demonstrated that this blockage can be recapitulated by deleting the β-catenin binding domain in TCF-1 [170], thus tying the defects of TCF-1 deficient thymocytes to the lack of Wnt signaling [170]. In support of this notion, constitutively activating Wnt, by ectopically expressing a non-degradable β-catenin at the DN3 stage, results in lack of the CD3-TCR complex on DP thymocytes. Ectopic Wnt activation in DN3 thymocytes bypasses pre-TCR signaling, and allows for the differentiation of DP thymocytes without surface expression of TCR-CD3 [171]. Ioannidis et al. also showed that these DP cells were also more resistant to apoptosis through the expression of BCL-X_L [170]. The importance of Wnt signaling was further confirmed in fetal thymi of $Wnt1^{-/-}Wnt4^{-/-}$ mice, where the lack of Wnt signaling affected thymocyte proliferation at the DN and ISP stages of development [172].

Directly deleting β-catenin, via a CRE-LOXP system controlled by the *Lck* promoter, leads to a developmental blockage at the DN3 stage, specifically at the TCRβ chain selection checkpoint. However, a TCF-1 deficiency did not cause a similar phenotype. Furthermore, Gounari et al. reported that deletion of *Apc,* using *Lck-Cre,* disturbs thymocyte development [117]. Despite of these reports, several lines of evidence dispute a role for Wnt signaling in thymocyte development. For example, mice with a broad β-catenin deletion via Mx-cre had normal lymphopoiesis [12]. It was postulated that the difference between this study and the study using β-catenin, via a Cre-LoxP system controlled by the *Lck* promoter, was due to either the differences between the

Cre drivers or the possibility of γ-catenin compensating for the loss of β-catenin. However, deletion of both β-catenin via MX-Cre and γ-catenin null allele also had no effect on lymphopoiesis [114, 115]. Overall, these studies illustrate that the role of Wnt signaling and its regulators in thymocyte development are not fully understood. In this study, we further investigated the role of Wnt signaling in thymocyte development by using an *Apc* conditional allele that has *loxP* sites inserted into introns 13 and 14 of the endogenous *Apc* gene under the control of both CD4-Cre and Lck-Cre transgene. We found that APC is not essential for later stages of thymocyte development.

2.3 Materials and Methods

Mice

CD45.1 C57BL/6 mice were obtained from Charles River Laboratories, through a contract with the National Cancer Institute. Mice with homozygous knockin of the floxed *Apc* [173] and transgenic mice expressing the *Cre* recombinase, under the control of either the proximal *Lck* promoter [174] or CD4 promoter [175], were obtained from Jackson Laboratories (Maine, USA). All mice used in this study have been backcrossed to C57BL/6 background for at least 10 generations. These strains were maintained in our animal facilities under pathogen-free conditions. All experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committees of the University of Michigan and the Children's National Medical Center.

Flow cytometric analysis

Mice were sacrificed at 6-8 weeks of age. Thymus and spleen tissues were homogenized to generate a single-cell suspension. Cells were stained at 4°C for 20

minutes in phosphate-buffered saline with 2% FBS with the following antibodies from BD Bioscience (1:200): CD4 (RM4-5, 25-0042-82), CD8 (SK-1, 8066-0087-120), B220 (HIS24, 25-0460-82), CD44 (IM7, 12-0441-83), CD62L (MEL-14, 17-0621-83), CD24 (30-F1, 12-0241-83), CD69 (H1.2F3, 12-0691-83), CCR7 (4B12, 17-1971-82), beta 7 integrin (FIB205, 12-5867-42), β-catenin (15B8, 50-2567-42), δϒ TCR (UC7-13D5, 11-5811) and CD25 (PC61.5, 17-0251-82). Samples were analyzed by using a BD LSR II Flow Cytometer.

For the co-localization experiment, splenocytes were stained for CD3 (145-2C11, 12-0031) and permeabilized using the BD fix/perm kit™ (554714). β-catenin was stained overnight (1:100) (15B8, 50-2567-42). DRAQ5 (ebioscience, 65-0880-92) was added (1:10,000) right before analysis using an Amnis Imaging Flow Cytometry. Analysis was carried out using IDEAS® software. Calculations were made by following IDEAS® software's wizard.

Statistics

Data was analyzed using 2-tailed unpaired Student's t-tests. All statistics were performed using GraphPad Prism, version 5 (GraphPad Software, San Diego, CA). *P<0.05, **P<0.01, ***P<0.001.

2.4 Results

2.4.1 Deletion of exon 14 floxed *Apc* by CD4-Cre results in loss of APC function and β-catenin accumulation/translocation

In order to study role of *Apc* in T cells, we used CD4-Cre to delete *Apc* and used a more commonly known *Apc* conditional allele that has *loxP* sites inserted into introns 13 and 14 of the endogenous *Apc* gene (*Apc^{fl})[173]*. These mice were labeled as cKO, while their *Cre*$^-$ littermates were used as a control and labeled as Ctrl. Based on the known specificity of the Cre promoter, *Apc$^{fl/fl}$;CD4-Cre*$^+$ mice should have a frameshift mutation at codon 580 and a truncated APC polypeptide. Before assessing the role of *Apc* in thymocyte development, we needed to confirm *Apc* deletion among thymocytes. CD4-cre expression should begin the DN3 stage (CD4$^-$CD8$^-$CD25$^+$CD44$^-$) of thymocyte development, thus we FACS-sorted CD4$^-$CD8$^-$ (DN) thymocytes based on their expression of CD4, CD8, CD44, and CD25. Using polymerase chain reaction (PCR) that yielded differently sized products from floxed (*fl*) and deleted (*del*) *Apc* alleles, we determined the kinetics of *Apc* deletion in developing DN thymocytes. As expected, no deletion was observed in DN1-4 of the Ctrl mice. In the cKO thymocytes, nearly equal amounts of *fl* and *del* alleles were observed in DN3. By DN4, most products were derived from the *del* allele (Fig. 2.1a). Thus, the *Apc* deletion began at the DN3 stage and was largely completed at the CD4$^-$CD8$^-$CD25$^-$CD44$^-$ DN4 stage in the cKO mice.

Since the primary function of the Apc protein is to mediate the destruction of β-catenin, we used intracellular accumulation of the protein as the primary readout for functional *Apc* inactivation throughout thymocyte development. As shown in Fig. 2.1b, despite a nearly 50% deletion of the *Apc* gene, no increase of intracellular β-catenin was observed in the DN3 stage. Likewise, although the *Apc* deletion was nearly complete in the DN4 stage, only a small subset showed functional inactivation of the Apc protein. However, significant accumulation of β-catenin was observed from the bulk of

$CD4^+CD8^+$ (DP), $CD4^+CD8^-$ (CD4 SP), and $CD4^-CD8^+$ (CD8 SP) thymocytes. The delayed functional inactivation of the *Apc* protein in relation to the gene deletion is likely due to residual Apc protein and/or mRNA. Regardless, the robust activation of Wnt signaling from the DP stage allowed us to assess the function of ectopic activation of Wnt in T cell development and function starting at the DP stage.

To investigate whether β-catenin accumulation is adequate for its translocation into the nucleus, we stained for $CD3^+$ and β-$catenin^+$ splenocytes of both ctrl and cKO mice. Using Aminis Imaging flow cytometry, we evaluated the levels of β-catenin in the nucleus marked with DRAQ5. We first gated on the $CD3^+$ and β-$catenin^+$ populations. As seen in Fig 2.1c, much like cKO thymocytes, splenic cKO had as much as 80% accumulation of β-catenin. In order to determine whether this accumulation results in translocation of β-catenin into the nucleus, we gated on the population that were observed to have β-catenin localizing with the nuclear stain, DRAQ5. Fig 1c clearly shows in over 50% of the case localizes in the nucleus, thus demonstrating that without APC, β-catenin will stabilization and translocate into the nucleus thereby ectopically activating Wnt signaling. Through sorting of DN populations, we showed that APC is deleted, on the DNA level, starting at the DN3 stage. Intracellular staining showed that this deletion of APC does functionally result in accumulation of β-catenin, and via Aminis imaging, we concluded that this accumulation results in the translocation of β-catenin, which thereby ectopically activating Wnt signaling.

2.4.2 Deletion of exon 14 floxed *Apc* by CD4-Cre does not grossly disturb thymocyte development

One method to evaluate thymocyte development is to assess thymic cellularity, which gives a general indication of the production of thymocytes. As shown in Fig. 2a, Ctrl and cKO thymi had comparable cellularity. To more closely assess thymocyte development, we stained thymocytes for CD4, CD8, CD25, and CD44, allowing us to investigate the DN, DP, and SP populations. Distribution of CD4 and CD8 markers among thymocytes revealed no gross abnormality in thymocyte development as the % (Fig. 2b) and numbers (Fig. 2.2c) of DP, CD4 SP, and CD8 SP thymocytes were similar. As expected from the largely normal β–catenin levels in cKO DN thymocytes, the frequency (Fig. 2d) and number (Fig. 2.2e) of double negative cells ($CD4^-CD8^-$, DN) were also comparable throughout stages 1-4. Thus, the deletion of *Apc* that began at the DN3 stage did not affect thymocyte development. However, we did observe an increase in apoptosis of *Apc*- deficient thymocytes (Fig 2.3a), although apoptosis was more pronounced in peripheral T cells (Fig 2.3b).

The differences observed between our results and those in a previous study [117] was not due to a subtle difference between the Lck-Cre used in the previous study and the CD4-Cre used here. In our hands, both CD4-Cre and Lck-cre had largely normal thymocyte development (Fig 2.4). However, the modest but statistically significant reduction in cellularity and decrease in SP thymocytes in Lck-Cre mice suggests that even minor differences in timing of gene deletion can affect the outcomes.

One potential phenotypic difference between Lck-Cre and CD4-Cre is in the expression of CD44 a known Wnt target gene. In the periphery, both CD8 and CD4 T cells from Lck-Cre and CD4-Cre upregulated CD44 at much higher levels than in the Ctrl mice.

However, thymocytes show a different pattern, where SP CD4 and CD8 thymocytes in CD4-Cre mice express much higher levels of CD44, while Lck-Cre levels of CD44, though higher, appears much more like Ctrl mice. In addition, we observed a loss of thymic cellularity and an apparent increase of DN thymocytes in older CD4-Cre-induced cKO mice that became moribund (Fig. 2.4c and 2.4d). This raised the intriguing possibility that abnormal thymocyte development may be secondary effect of overall immune activation in the mice, as reported by Martin et al. [176]. Overall, our data demonstrates that Wnt signaling starting at DN4 did not directly disturb normal T cell development in healthy young mice. This normal T cell development allowed us to study the function of Wnt signaling after T cell maturation.

Though thymcyocyte development looked relatively normal at 6-8 weeks of age, when the Lck-cre are aged, the thymic mass begins to decrease drastically (Fig 2.5). This is indicative of thymic involution, which has also been previously reported in mice with stabilized β-catenin, also under the control of Lck-cre [119].

Since $\gamma\delta$ T cells development divert from that of $\alpha\beta$ T cells prior to DN3, one would not expect significant effect of CD4-Cre-induced Apc deletion on the fate of $\gamma\delta$ T cells. As shown in Fig. 6, the numbers of gd T cells in the thymus and spleen were comparable between ctrl and cKO mice.

2.5 Discussion

In this study, we have demonstrated that *Apc* is not essential in later stages of thymocyte development. Ablation of *Apc* beginning from the late DN3 to DN4 stage

perturbs neither frequencies nor numbers of thymocyte subsets (DN, DP, CD4 SP, nor CD8 SP). We showed that the lack of developmental blockages were not due to an inefficient deletion of *A*pc, as seen by the efficient deletion at the DNA level of thymocytes at the DN4 stage. Furthermore, this deletion of *Apc* does effectively active Wnt signaling as see by the accumulation of β-catenin and nuclear translocation of β-catenin into the nucleus.

A previous report showed a dramatic developmental arrest of thymocytes at the DN4 stage by an *Apc* deletion [117]. This phenotype was not recapitulated when the interaction between β-catenin and Apc was inactivated by deletion of the Apc-binding domain on β-catenin [171]. The reason for the delayed activation of Wnt signaling is unknown, as both the Cre-driver and the Apc^{fl} differed in these studies. The previous study found β-catenin increased in most of the cells from the DN3 stage and on, while our study observed an increase in only a small fraction of DN4 and all of the DP and SP thymocytes. The delayed functional inactivation of *Apc* in our model resulted in normal thymocyte development. Since the CD4-Cre is activated later than the Lck-Cre in DN populations [177], we crossed the Apc^{fl} allele with the Lck-Cre to rule out this possibility. We also found largely normal thymocyte development and severe lymphopenia when the Lck-Cre was used. We did not, however, assess the deletion efficiency or deletion timing of the Lck-cre mice. It is possible that in our hands, Lck-cre did not efficiently delete *Apc* as the previous study or the deletion occurred later than what is expected in the literature.

In our hands, a deletion controlled by Lck-cre appears to have caused accelerated thymic atrophy, a phenotype also similar to a study using an exon 3 deletion of β-catenin (nondegradable form of β-catenin), though the role of Wnt signaling in thymic involution is still undefined [119]. Although subtle differences exist between the two: Lck-Cre- and CD4-Cre-induced cKO, these differences are not sufficient to account for the dramatic discrepancy between our study and the earlier work [117]. One explanation could be due to the use of different *Apc*^{fl} alleles rather than different Cre-drivers. Finally, since activation of T-cells in the periphery can cause loss of thymocytes [176], the possibility that activation of T cells in the periphery may account for a part of the previously described phenotype [117] remains to be excluded.

Figure 2.1

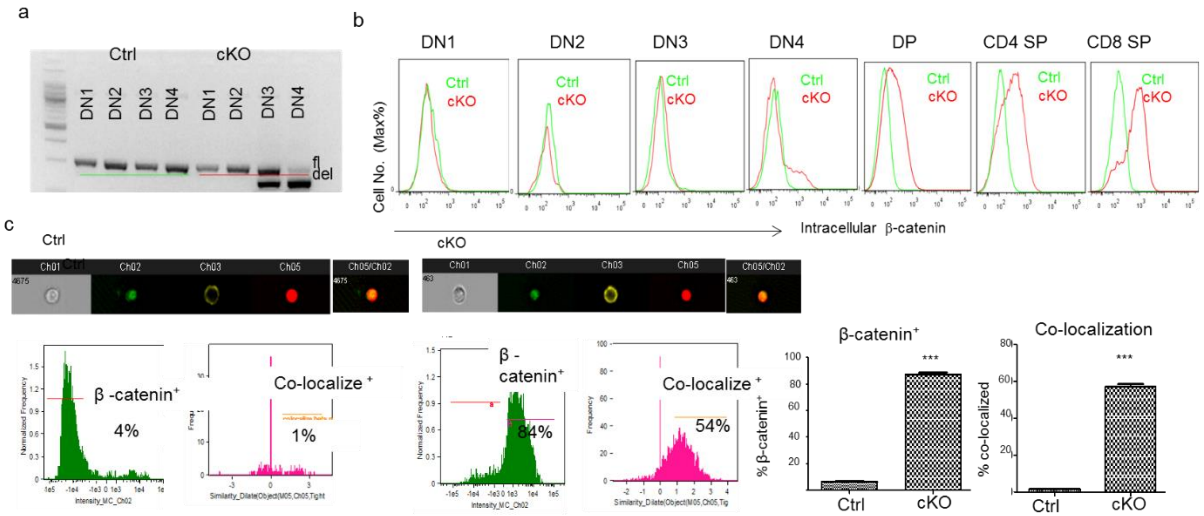

a
Ctrl
cKO
DN1
DN2
DN3
DN4
DN1
DN2
DN3
DN4
f
del
b
DN1
DN2
DN3
DN4
DP
CD4 SP
CD8 SP
Ctrl
cKO
Cell No. (Max%)
Intracellular β-catenin
c
Ctrl
cKO
Ch01
Ch02
Ch03
Ch05
Ch05/Ch02
β -catenin+
4%
Co-localize +
1%
β -catenin+
84%
Co-localize +
54%
β-catenin+
% β-catenin+
Co-localization
% co-localized
***
***
Ctrl
cKO
Ctrl
cKO

Fig 2.1. Deletion of exon 14 floxed *Apc* by CD4-Cre results in loss of APC function and β-catenin accumulation/translocation.

a. Deletion efficiency of cKO. 6-8 week old DN thymocytes were sorted using CD44 and CD25 expression. Using PCR, deletion was detected. Wt allele: 314 bp, deletion: 258 bp. Experiment was repeated twice. b. Expression of intracellular β-catenin in 6-8 week old ctrl or cKO. Stained for CD4, CD8, CD25, and CD44. Gated on DN population. n=9, per group. c. Splenocytes were stained for CD3, β-catenin, and DRAQ-5. Cells were gated on $CD3^+$ cells. Images were taken using Amis imaging flow cytometry and analysis was done using IDEAS software.

Figure 2.2

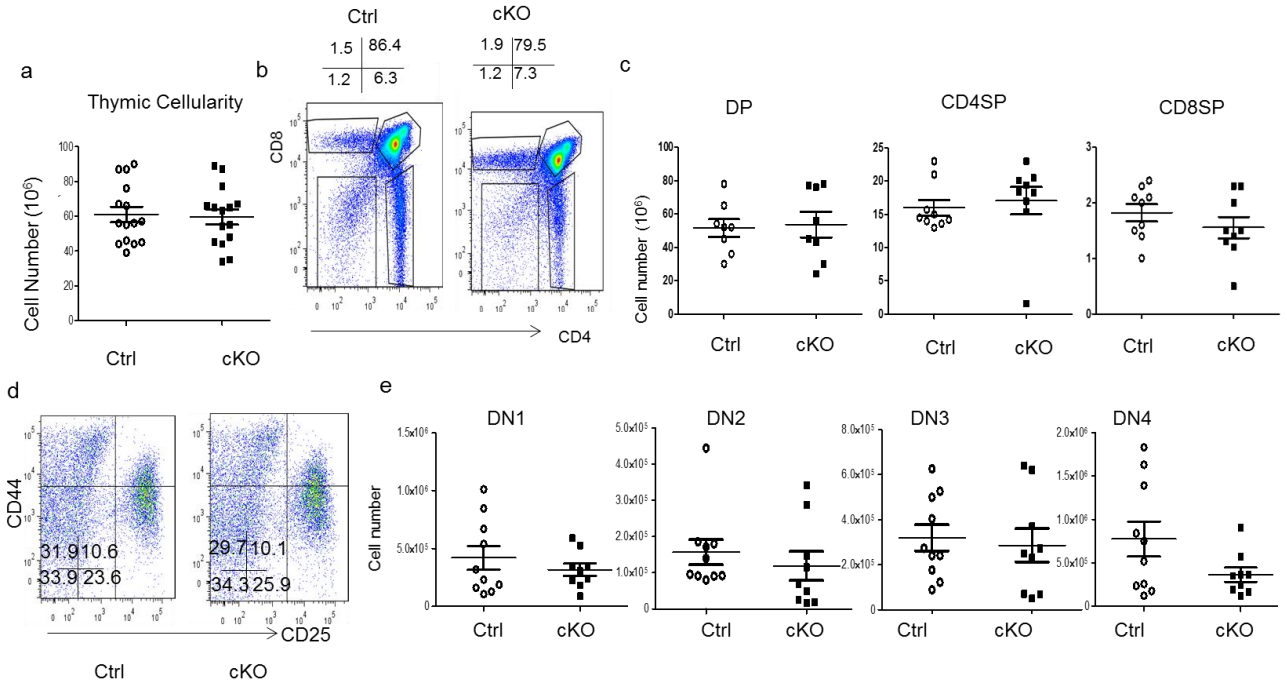


Fig. 2.2. Normal thymocyte develop in the cKO mice. a. Thymic cellularity of 6-8 week old Cre$^-$ controls (open circles) and APC cKO (closed circles) mice, n=15. b. Representative profiles of thymi from ctrl or cKO. c. Absolute numbers of DP, SP CD8 and CD4 thymi. n=9. d. Representative profiles of DN populations of control or APC cKO. e. Absolute number of DN populations from 6-8 week old ctrl or cKO mice. n=8.

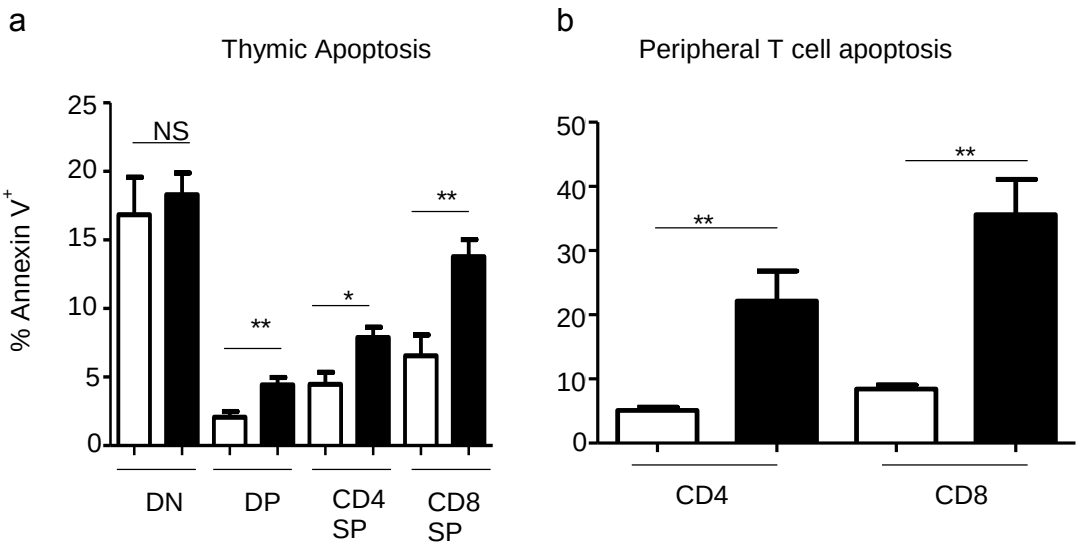


Figure 2.3. Increased apoptosis in cKO T cells in the thymus (a) and spleen (b). Ctrl (open bars) and cKO (filled bars) n=3 per group

Figure 2.4

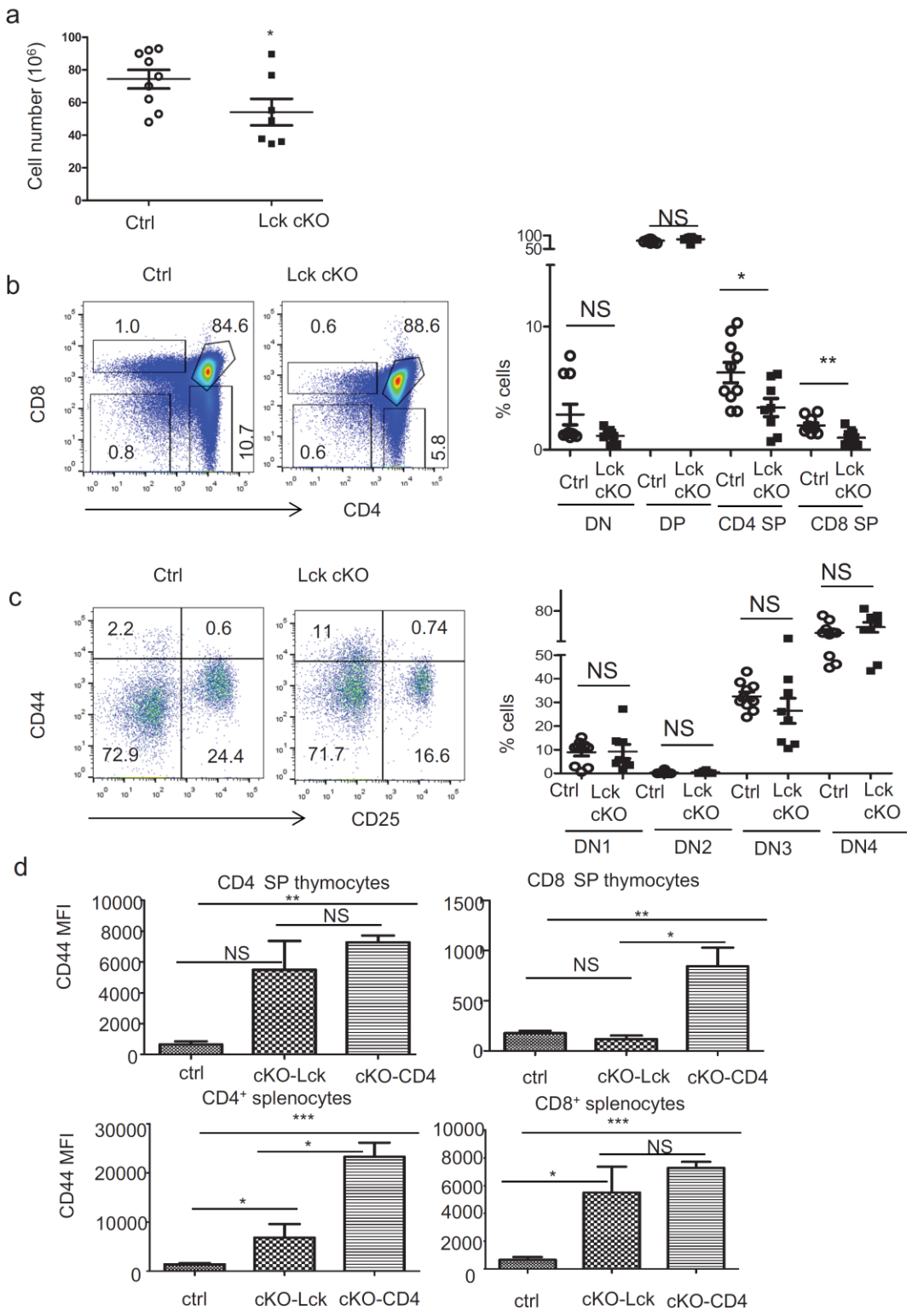

a
Cell number (10^6)
Ctrl
Lck cKO
b
Ctrl
Lck cKO
CD8
CD4
1.0
84.6
0.8
10.7
0.6
88.6
0.6
5.8
% cells
NS
DN
DP
CD4 SP
CD8 SP
c
CD44
CD25
2.2
0.6
72.9
24.4
11
0.74
71.7
16.6
DN1
DN2
DN3
DN4
d
CD4 SP thymocytes
CD8 SP thymocytes
CD44 MFI
ctrl
cKO-Lck
cKO-CD4
CD4+ splenocytes
CD8+ splenocytes

Fig. 2.4. Subtle thymocyte developmental defects caused by Lck-Cre-driven deletion of the Apc exon 14 in 6-8 week old mice. a. Thymic cellularity of *Apc*$^{fl/fl}$*;Lck-Cre*$^{-}$ (Ctrl) and the *Apc*$^{fl/fl}$*;Lck-Cre*$^{+}$ (Lck-cKO) mice. n=8. b. Thymocyte development based on distribution of CD4 and CD8 markers. Representative profiles are shown on the left, while summary data are presented on the right. n=9. c. Thymocyte development based on distribution of CD44 and CD25 markers among the CD4^{-}CD8^{-} thymocytes. Representative profiles are shown on the left, while summarized data is presented on the right. n=9. Note that the overall levels of CD44 are elevated among the DN4 subsets in the Lck cKO thymocytes. This is to be expected, as CD44 is a hallmark of Wnt signaling target gene. d. Summarized MFI expression, from one experiment, of CD44 in SP thymocytes and splenic T cells in Lck-cKO and CD-cKO. n=3. This experiment was repeated twice.

Figure 2.5

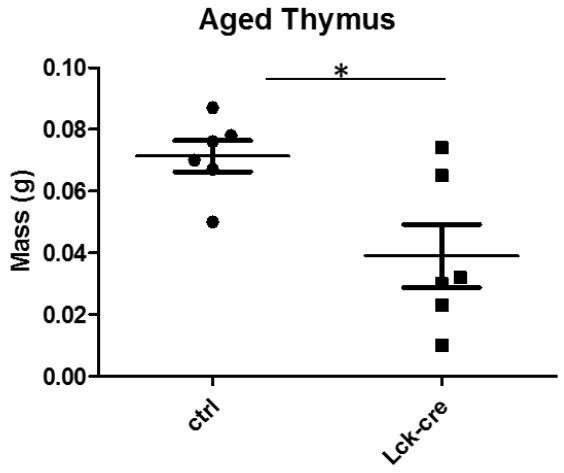


Figure 2.5

Thymic cellularities of 4-6 month old control (circles) or Lck-cre (squares). Lck-cre or ctrl thymi were weighed at 4-6 months of age.

Figure 2.6

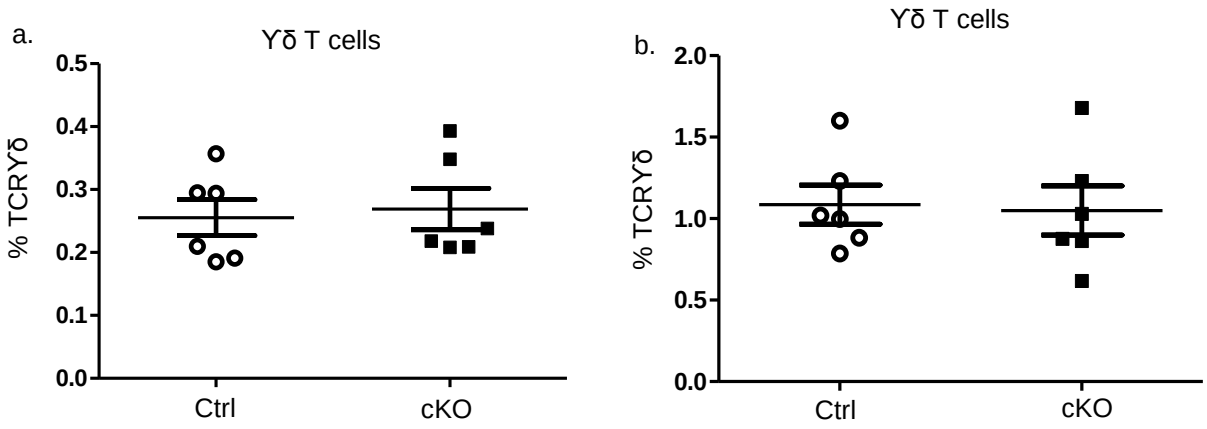


Figure 2.6. *Apc* deletion by *CD4-Cre* does not cause alteration in γδ T cells lineages. Ctrl (open circles) and cKO (filled squares) thymocytes (a) and splenocytes (b) were stained with anti-TCRβ and anti-TCRγδ complex mAbs. The data is shown as the mean and SEM of % γδ T cells, summarized from two independent experiments involving 6 mice at 6-8 weeks of ages in each group.

Chapter 3

APC regulates naïve T cell survival via c-Myc-dependent mechanisms

3.1 Abstract

The role of Wnt in thymocyte development and antigen-driven T cell differentiation and maintenance of memory populations has been extensively studied. However, very little is known about the role Wnt plays in maintenance of naïve T cells in the absence of antigens. Using mice with T-lineage-specific deletion of the *Apc* gene starting at the DN3 stage, we observed a massive loss of naïve T cells with concurrent with the up-regulation of cMyc expression and activation of the mTOR pathway. To address the role of cMyc activation in the loss of naïve cells, we created mice with a T-lineage-specific deletion of both Apc and cMyc. We found that heterozygous and homozygous deletion of cMyc attenuated the loss of naïve Apc-deficient T cells. However, cMyc deletion did not fully restore naïve T cell cellularity, raising the possibility that other pathways, such as Wnt-stimulated mTOR activation may also contribute to the loss of naïve T cells.

3.2 Introduction

Unless stimulated by their cognate antigen/MHC complex or homeostatic cues, naïve T cells proliferate at an exceptionally slow rate [178-180]. Under normal physiological

conditions, naïve T cell survival is dependent on both extrinsic factors and intrinsic programing.

Extrinsic factors that control naïve T cell survival include MHC molecules, adhesion molecules, and cytokines. During thymic development, through a process of positive selection, thymocytes that are able to recognize self-peptide-MHC complexes are allowed to mature into naïve T cells [181]. The ability to recognize self-ligands extends to peripheral naïve T cells. Naïve T cells recognize of their specific MHC and self-antigen for survival [178]. Cytokines also play a role in the maintenance of naïve T cells. In vitro culturing of naïve T cells with IL-4, IL-6, thymic stromal lymphopoietin, and IL-7 rescued these T cells from apoptosis [182-184]. In vivo studies suggest Il-7 is the most dominant cytokine involved in naïve T cell survival. Blocking activity of IL-7 through monoclonal antibodies or deletions of IL-7R limited the survival and expansion of naïve T cells [182, 185, 186]. Signaling through IL-7R allows for survival via the regulation of B cell lymphoma 2 (BCL-2) family member proteins, including BCL-2 and MCL1 [187, 188].

mTOR, FOXO family transcription factors, the transcriptional repressor Growth factor independent 1 (GFI1), KLF2, and Mst1 participate in cell intrinsic regulation of naïve T cell survival. Some control the responsiveness of naïve T cells to IL-7 signaling. Transcriptional regulation of IL-7R requires GFI1, in CD8 T cells, while FOXO (FOXO1 and FOXO3) transcription factors are utilized by both CD8 and CD4 T cells [100, 101, 189]. A different Fox factor, FOXP1, promotes the survival of naïve CD8 T cells through regulation of IL-7 signaling [99]. A deficiency of Schlafen-2 resulted in increased T cell apoptosis due to engagement of MHC/peptide complex or HP [102]. Recently our group

and others have shown that a deletion of a negative regulator of mTOR- tuberous sclerosis complex 1 (Tsc1) gene caused loss of naïve T cell quiescence and survival [97, 98]. However, it is still unclear what stimulates TSC1 to inhibit mTOR signaling. APC, being an upstream regulator of mTOR signaling, is a potential candidate as a regulator of naïve T cell survival. In this study, we investigated the role of *Apc* in naïve T cells. We showed in Chapter 2 that an *Apc* deletion does not affect thymocyte development, giving us a rare opportunity to examine the role of *Apc* in naïve T cells. Here, we show that *Apc* is an important cell intrinsic regulator of naïve T cell survival, in part via inhibition of a Wnt target gene, c-Myc.

3.3 Materials and Methods

Mice

CD45.1 C57BL/6 mice were obtained from Charles River Laboratories, through a contract with the National Cancer Institute. Mice with homozygous knockin of the floxed *Apc* [173] and transgenic mice expressing the *Cre* recombinase, under the control of either the proximal *Lck* promoter [174] or CD4 promoter [175], were obtained from Jackson Laboratories (Maine, USA). Mice with floxed *cMyc* locus [190] were kindly provided by Dr. De Alboran. All mice used in this study have been backcrossed to C57BL/6 background for at least 10 generations. These strains were maintained in our animal facilities under pathogen-free conditions. All experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committees of the University of Michigan and the Children's National Medical Center.

Adoptive transfer of T cells

Mature CD24^{-}thymocytes were isolated by Dynal negative selection using biotinylated anti-CD24 mAb M1/69 (Ebioscience, 13-0242-85). Cells were labeled with 10 μM CFSE and injected intravenously to CD45.1 recipients (1x10^{6}/mouse).

Western blot analysis

Tissues were lysed in a lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% NP-40) supplemented with a protease inhibitor cocktail (Pierce, 78437). Cell lysates were separated by SDS-PAGE and transferred to polyvinylidene fluoride membranes. The membranes were incubated with anti-c-Myc (Abcam, AU016757, Ab39688,1:2000) and anti-actin (Abcam ACTN05, Ab1801, 1:5000). Either anti-rabbit or anti-mouse IgG horseradish peroxidase-linked antibody (Santa Cruz, 1:3000, SC-2054 and SC-2005 respectively) was used as a secondary antibody. A chemiluminescence kit (GE healthcare, RPN2133) was used to visualize blots.

Flow cytometry

Mice were sacrificed at 6-8 weeks of age. Thymus and spleen tissues were homogenized to generate a single-cell suspension. Cells were stained at 4°C for 20 minutes in phosphate-buffered saline with 2% FBS with the following antibodies from BD Bioscience (1:200): CD4 (RM4-5, 25-0042-82), CD8 (SK-1, 8066-0087-120), B220 (HIS24, 25-0460-82), CD44 (IM7, 12-0441-83), CD62L (MEL-14, 17-0621-83), CD24 (30-F1, 12-0241-83), CD69 (H1.2F3, 12-0691-83), CCR7 (4B12, 17-1971-82), beta 7 integrin (FIB205, 12-5867-42), β-catenin (15B8, 50-2567-42), δϒ TCR (UC7-13D5, 11-

5811) and CD25 (PC61.5, 17-0251-82). Samples were analyzed by using a BD LSR II Flow Cytometer.

Intrathymic injections

Five week old mice were anesthetized with isoflurane. A midline incision was used to expose the ribs. CFSE (10 μl of a 1 mM solution, Invitrogen, C1157) was injected into each lobe of the thymus. Mice were sacrificed at either 6 or 24 hours. The thymus, spleen, and lymph nodes were collected and analyzed by flow cytometry.

Adoptive transfer of T cells

Mature $CD24^-$thymocytes were isolated by Dynal negative selection using biotinylated anti-CD24 mAb M1/69 (Ebioscience, 13-0242-85). Cells were labeled with 10 μM CFSE and injected intravenously to CD45.1 recipients ($1x10^6$/mouse).

Irradiation chimera

$CD45.1^+$ B6 mice were lethally irradiated (11 Gy delivered in 2 installments, 4 hours apart, as reported[191]) and reconstituted with of $2.5x10^6$ $CD45.1^+$ bone marrow cells in conjunction with equal numbers of either cKO or Ctrl bone marrow cells. Reconstitution was confirmed at 8 weeks from peripheral blood staining of CD45.1 and CD45.2. Bone marrow chimeras were analyzed for T cell numbers and phenotypes at 10 weeks post-transplantation.

Statistics

Data was analyzed using 2-tailed unpaired Student's t-tests. All statistics were performed using GraphPad Prism, version 5 (GraphPad Software, San Diego, CA). *P<0.05, **P<0.01, ***P<0.001.

3.4 Results

3.4.1 *Apc* deficiency causes loss of naïve T cells.

In the previous chapter, we demonstrated that an *Apc* deletion did not affect later stages of thymocyte development. Surprisingly, we observed a marked reduction of T cell numbers in the periphery. As seen in Fig 3.1a, the frequencies of $CD4^+$ or $CD8^+$ T cells in the cKO mice were reduced by 10-fold or more in comparison to the Ctrl mice. In addition to the loss of $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells, approximately 1/3 of the T cells in the cKO mice lost expression of CD4 and CD8 (Fig. 3.1b). This loss of CD4 and CD8, however, is not the result of a lineage switch to γδ T cells, as cKO mice have similar numbers of $\gamma\delta$ T cells in the thymus and the spleen (Fig. 3.1c and 3.1d). The ontogeny of these cells remains to be determined. Moreover, in both $CD4^+$ (Fig. 3.2a) and $CD8^+$ (Fig. 3.2b) T cell compartments, a profound loss of naïve T cells was observed in the spleen. While more than 60% of the T cells in Ctrl mice exhibited the naïve phenotype, $CD44^{lo}CD62L^{hi}$, while only approximately 5% or less of the cKO T cells did. In fact, most of the cKO T cells displayed markers associated with effector memory T cells ($CD44^{hi}CD62L^{lo}$), although significant increases in the central memory

compartment were also observed. In addition, expression of CD69 was also elevated in the cKO T cells (Fig. 3.2c).

There are three explanations for the reduction of peripheral T cells: (1) a developmental blockage during thymic development, (2) a defect of thymic emigration into the periphery, or (3) survival defect of peripheral T cells. As seen from Chapter 2, a deletion of *Apc* did not affect thymic development, therefore, a thymic developmental blockage could not be an explanation for the loss of peripheral T cells. To evaluate whether a defective thymic output was responsible for the loss of peripheral T cells, we injected CFSE intrathymically and tracked recent thymic emigrants in the periphery. To avoid potential artifacts associated with peripheral labeling, we monitored the labeling of the thymus and excluded any samples that had less than 3% labeling of either CD4 SP or CD8 SP thymocytes. Moreover, since the efficacy of CFSE labeling of thymocytes is varies between mice, we normalized the % of $CFSE^+$ T cells in the lymph node by dividing them with the % $CFSE^+$ of either CD4 or CD8 SP thymocytes. The overall thymic output in cKO and ctrl were comparable, as cKO produced as many recent thymic emigrants as the ctrls (Fig 3.3). Thus, the reduction of peripheral T cells in cKOs is the not the result of aberrant thymic emigration.

The data in Fig. 3 raised the possibility that the lack of naïve T cells is due to poor survival in the periphery. To test this hypothesis, we isolated $CD24^-$ mature thymocytes from both Ctrl and cKO mice (both are $CD45.2^+$) and transferred these cells into congenic hosts that are $CD45.1^+$, so that we can follow their persistence in the peripheral blood and lymphoid organs. As shown in Fig. 4a upper panel, similar

numbers of CFSE$^+$ Ctrl and cKO T cells were observed in the spleens of the congenic hosts at 6 hours after transfer. By 24 hours, the numbers of cKO T cells in the spleen were less than 1/3 of their Ctrl counterparts. The frequencies of cKO T cells in the pooled lymph nodes were reduced by more than 10-fold (Fig. 3.4b). The rapid loss of adoptive transferred cells demonstrate a survival defect in cKO T cells.

To further demonstrate that this disappearance of cKO was due to apoptosis, we evaluated cell death via 7-AAD and Annexin V staining at 6 hours post-transplant, when we could detect similar percentages of CD45.2$^+$ in both Ctrl and cKO. As seen in Fig 3.4c, there was a higher % of 7-AAD$^+$ and/or Annexin V$^+$ T cells in the cKO mice (Fig. 3.4c). Taken together, data in Fig. 4 demonstrates that while T cell emigration from the thymus was unaffected by the deletion of the *Apc* gene, increased apoptosis lead to reduced accumulation of Apc-deficient T cells after their emigration from the thymus.

3.4.2 Naïve Cell death in *Apc* deficient T cells is cell intrinsic

To determine whether the loss of peripheral T cells in the cKO mice was cell intrinsic, we created bone marrow chimeras using cKO or Ctrl donor-type (CD45.2) cells mixed with an equal number of recipient-type (CD45.1) bone marrow cells. This allowed for both cKO and Ctrl thymocytes to develop into naïve T cells in the same environment (same MHC/antigen interactions and cytokines). After 10 weeks, we harvested the thymi and spleens to investigate the reconstitution of CD45.2$^+$ donor cells. As shown in Fig. 3.5a, the percentage of CD45.2$^+$ cells in the thymus was unaffected by the deletion of *Apc.* Based on the distribution of CD4 and CD8 markers, the development of both Ctrl and cKO thymocytes was grossly normal, as all major subsets were present among

CD45.2^{+} thymocytes (Fig. 3.5b). However, the percentage of SP thymocytes were reduced by approximately 2-fold (Fig. 3.5b), perhaps due to a subtle survival disadvantage of the cKO thymocytes, as described in Chapter 2. In the context of a competitive reconstitution, the apoptosis of cKO thymocytes is further amplified. To further elucidate whether a deletion of *Apc* can affect thymic egress, we assessed the percentages of mature SP thymocytes, using the markers: Qa-2 and CD24. Among SP thymocytes, the percentages of Qa-2^{hi} and CD24^{lo} thymocytes were comparable between cKO and Ctrl groups (Fig. 3.5c). Therefore a deficiency of *Apc* does not cause preferential accumulation of mature thymocytes.

In sharp contrast to a roughly normal thymocyte development, severe cKO T-cell loss was observed in the spleens of chimera mice. Thus, while the frequency of CD45.2 donor leukocytes was unaffected by *Apc* deletion (Fig. 3.6a, b), more than a 20-fold reduction was observed in the frequency of T cells within the CD45.2 splenocytes (Fig. 3.6c, d). In addition to the loss of T cell cellularity, the remaining T cells exhibited a marked difference in cell surface markers. Even though the majority of the Ctrl T cells displayed CD44^{lo}CD62L^{hi} markers of naïve T cells, most of the cKO T cells had either central or effector memory markers (Fig. 3.6e, f). Loss of naïve T cell markers and acquisition of memory/effector T cell markers in cKO T cells were reminiscent of what was observed in the Ctrl and cKO mice. On the other hand, cellularity of the recipient-type T cells was not affected in a trans-fashion by the genotype of the donor-type cells in the recipients (Fig. 3.7a and 3.7b). Since the cKO T cells have developed and emigrated into a normal environment, both the loss of cellularity and the acquisition of activation markers following *Apc* deletion is cell-intrinsic.

3.4.3 cMyc over-expression contributes to apoptosis in mature T cells

cMyc is a β-catenin target gene that causes T-cell apoptosis through promotion of Fas/FasL interaction, and increased expression of death ligand tumor necrosis factor, and tumor necrosis factor related apoptosis-inducing ligand [44-46]. As the first step to investigate its potential contribution to T cell death in cKO mice, we evaluated cMyc upregulation in $CD24^-$ thymocytes from Ctrl and cKO mice by quantitative PCR. As shown Fig 3.8a, the *cMyc* transcript was doubled in the thymocytes of cKO mice. However, cMyc protein was not obviously upregulated among the mature thymocytes. In contrast, peripheral cKO T cells had drastically higher levels of cMyc than Ctrl T cells (Fig 3.8b). We therefore generated the $cMyc^{fl/+}Apc^{fl/fl};CD4\text{-}Cre^+$ ($Myc^{+/-}cKO$) mice to diminish the impact of the transcriptional activation of cMyc by eptopic Wnt signaling. Using PCR to detect the deletion of *cMyc* on the DNA level, we isolated peripheral T cells from either ctrl cre(-) or $Apc^{-/-}cMyc^{+/-}$. As shown in Fig 3.8c, only $Apc^{-/-}cMyc^{+/-}$ splenic T cells efficiently deleted one allele of *cMyc,* while cntrl splenic T cells still retained both wildtype alleles. Heterozygous deletion of *cMyc* resulted in a substantial reduction of the cMyc protein (Fig. 3.8b) and partially restored the numbers of cKO T cells in the periphery, as an approximately 3-fold increase of both CD4 and CD8 T cells was also observed in $Myc^{+/-}$ cKO mice in comparison to $cMyc^{+/+}$cKO mice (Fig. 3.9d, e). By reducing the transcriptional levels of *cMyc,* we are thereby able to observe an increase of naïve T cells in the periphery by decreasing naïve cell death. To further demonstrate that upregulation of *cMyc* caused apoptosis of $Apc^{-/-}$ T cells, we adoptively transferred either $Apc^{-/-}$ or $Apc^{-/-}cMyc^{+/-}$ mature thymocytes into WT mice and followed the survival of the T cells in the host at 6 hours post-injection. As shown in Fig. 3.9a,

Apc$^{-/-}$*cMyc*$^{+/-}$ T cells exhibited reduced apoptosis and therefore increased survival in the WT hosts. This data demonstrates that the poor survival of *Apc*$^{-/-}$ T cells can be rescued by down-regulation of *cMyc* expression. In addition to an overall increase in T cell numbers, the % of naïve CD4 and CD8 T cells increased by 3-fold or more, while that of naïve CD8 T cells increased by 50% (Fig. 3.9b, c). A corresponding reduction of central and effector memory T cells was also observed in CD4 T cells, although the accumulation of effector memory CD8 T cells was less affected by an *cMyc* deletion (Fig. 3.9 b,c).

Our data in Fig. 3.8b demonstrated that heterozygous deletion of *cMyc* failed to reduce cMyc protein levels in cKO mice to WT levels. To determine whether the partial effect of a heterozygous cMyc deletion was due to an insufficient reduction in cMyc protein, we generated mice with a homozygously floxed *cMyc* locus. To validate that deleting both alleles of *cMyc* only affected peripheral T cell functions, but not thymocyte development, we analyzed thymocyte development by flow cytometry. Deletion of *cMyc* had no effect on thymocyte development (Fig. 3.10a) and T cell cellularity in the periphery (Fig. 3.10b), although a reduction of CD44 levels was observed on both CD4 and CD8 T cells (Fig. 3.10c). Combinational deletion of both Apc and cMyc did not affect thymocyte development (Fig. 3.10d). However, a homozygous deletion of *cMyc* resulted in a further rescue of T cell survival defects in the cKO mice, although it did not completely restore T cell cellularity and survival defects (Fig. 3.10, f).

3.4.4 mTOR activation contributes to apoptosis in mature T cells

Recently, it has been shown that APC directs mTOR signaling via GSK3β's negative regulation of TSC2 [103]. Because mTOR activation results in loss of quiescence in naïve T cells [97], we hypothesized that the loss of APC and resulting loss of naïve T cells may also be the result of activation of mTOR signaling. To test this hypothesis, we detected a well-known downstream target of mTOR signaling: phosphorylated S6 [192]. Using intracellular staining, we found that cKO $CD4^+$ and $CD8^+$ splenocytes had increased levels of phosphorylated S6, indicative of higher mTOR signaling (Fig 3.11a). To confirm this increase in peripheral T cells, we isolated T cells from the spleen and lymph nodes from cKO and ctrl mice via Miltenyi biotec's pan T cell isolation kit. From western blot analysis, we observed significantly higher levels of phosphorylated S6 in cKO than in Ctrl T cells (Fig 3.11b). This data demonstrates that the loss of APC results in the activation of mTOR signaling as seen by the increase of phosphorylated S6 expression.

It is known that constitutive activation of mTOR signaling results in the activation of p53, thus sensitizing cells to apoptosis [193]. It is possible that activation of mTOR may be also contributing to the increased rate of apoptosis through p53. To detect p53 levels, we isolated peripheral T cells from Ctrl and cKO and blotted for p53. Though western blot analysis, cKO have higher levels of p53, thus the loss of APC allows increased mTOR signaling, which then may cause the activation of p53. (Fig 3.11c).

To establish whether mTOR activation is involved in cKO naïve T cell death, we used a well-known inhibitor of mTOR, rapamycin, to rescue cell death *in vitro*. Ctrl or cKO thymocytes were treated with varying concentrations of rapamycin *in vitro* for an

overnight culture, then assayed for cell death using Annexin V and 7-ADD staining. As seen in figure 3.11d, inhibition of mTOR via treatment of rapamycin does decrease apoptosis at very low concentrations for CD4 SP thymocytes, but not for CD8 SP thymocytes.

We also tested mTOR inhibition *in vivo (*4 mg/kg, every other day), however, rather than rescuing naïve T cell death, at least 60% of the cKO rapamycin-treated mice had died, while ctrl vehicle/rapamycin treated or cKO vehicle treated all survived (Fig 3.12a). Rapamycin-treated cKO mice exhibited massive weight loss by day 3 of treatment if rapamycin was given daily (Fig 3.12b). Since rapamycin treatment cause rapid and severe health deterioration, we hypothesized that this was consist with a cytokine storm. Using serum for ctrl or cKO mice treated with rapamycin or vehicle over a 3 day period, we measured pro-inflammatory cytokines levels using a cytokine bead array assay. cKO rapamycin treated mice producde more pro-inflammatory cytokines, particularly TNFα (Fig 3.12c). Intracellular staining confirmed that the cells that produced the pro-inflammatory cytokine, TNFα, were T cells (Fig 3.12d).

3.5 Discussion

Though many studies have shown the importance of extrinsic factors, such as MHC interactions and cytokines, very little is known about the intrinsic cell programs that are involved in naïve T cell survival. Due to the heavy emphasis on Wnt signaling in either thymocyte development or T effector cell differentiation, little has been studied on the role of Wnt in naïve T cells. In this study, we report a possible role of Wnt signaling in naïve T cells. Thus, Wnt signaling joins other transcription factors (FOXO1, FOXO3,

FOXP1, KLF2) known to promote naïve T cell survival. However, while these transcription factors were shown to control the expression of IL-7R [99-101], we have not observed any effect of Wnt signaling on IL-7R levels.

We demonstrated that this loss of *Apc* and resulting in ectopic activation of Wnt leads to the upregulation of *cMyc*, which is known to play a role in activation cell death via through Fas/FasL interaction, death ligand tumor necrosis factor, and tumor necrosis factor related apoptosis-inducing ligand [44-46]. Though the deletion of *cMyc* in cKO mice does attenuate cell death and CD44 expression, naïve T cell numbers are not entirely rescued, suggesting that there are other pathways involved in loss of naïve T cells. To test whether the apoptosis in cKO T cells were associated with cMyc's regulation of death ligand expression (extrinsic apoptosis pathway) or the intrinsic apoptosis pathway, we detected the levels of either caspase 8 (implicating the extrinsic apoptosis pathway) or caspase 9 (implicating the intrinsic apoptosis pathway). We observed an upregualtion of both caspases (data not shown), suggesting that both pathways are involved in the observed cell death in cKO T cells. Due to the involvement of both p53 and caspase 9, it is worth exploring the role of *Apc* in regulating the intrinsic apoptosis pathway in naïve T cells.

Previous reports have demonstrated that Wnt activates mTOR through inactivation of GSK3β, which is critical for Tsc function. Here we showed that *Apc* deletion resulted in increased mTOR activation. Since our group and others have also shown that mTOR signaling regulates naive T cell quiescence [78, 174], we attempted to decrease mTOR activation via rapamycin treatment. We show that rapamycin rescues CD4 SP

thymocyte cell death via inhibition of mTOR signaling, however this is not observed in CD8 SP thymocytes. This may be due to the fact that CD8 T cells are more sensitive to mTOR activation as seen in TSC1 cKO T cells as seen in the deletion of TSC1 and the resulting mTOR activating significantly affected CD8 T cells more than CD4 T cells [78, 174].

We also have attempted to rescue T cells by rapamycin. Surprisingly, rapamycin caused severe weight loss, increased production of pro-inflammatory cytokines specifically in the cKO mice. Although rapamycin is known for its uses as an immunsuppressive, but it also has been shown to have pro-inflammatory effects: kidney-transplant recipients developing post-transplantation glomerulonephritis and proteinuria after rapamycin treatment [194, 195] and transplant patients developing anemia and increased pro-inflammatory cytokines post-rapamycin treatment[196]. It has been previously reported that treatment of monocytes with rapamycin, in conjunction with LPS or bacteria, promotes pro-inflammatory cytokine production dependent on the transcription factor NF-κB [197]. Here we found that loss of *Apc* resulted in a moderate increase of TNFα production in T cells, which is massively elevated by treatment by rapamycin. This observation is paradoxical as mTOR activation was generally associated with increased production of inflammatory cytokines. How a deletion of Apc alters the function of mTOR is of great interest and deserves further investigation. Weichhart et al. demonstrated that it is the TSC2-mTOR pathway that mediates the effects of rapamycin to produce pro-inflammatory cytokine. In light of this observation, we suggest that inactivation of Apc resulted in inactivation of Tsc2, which need to be balanced by high mTORC1 activity to prevent cytokine storm.

It would be also interesting to dissect the role of Wnt and mTOR in the control of the cell cycle in cKO T cells, for both pathways have been implicated in regulation of the cell cycle. APC's other regulatory roles, independent of Wnt signaling, are also worth examining. For instances, APC's role in chromosomal stability may also be a contributing factor in the loss of peripheral T cells, which may explain the upregulation of p53 due to DNA damage. cMyc is also known to be important in p53-dependent apoptosis, where *cMyc* deficient cells are do not undergo apoptosis due to DNA damage due to the inability to upregulate p53 [198]. Further investigates are need to know whether our *cMyc*$^{-/-}$ cko T cells can undergo p53-dependent apoptosis. Our study has shown that *Apc* is a crucial regulator in naïve T cell survival by controlling levels of *cMyc* and mTOR activation.

Figure 3.1

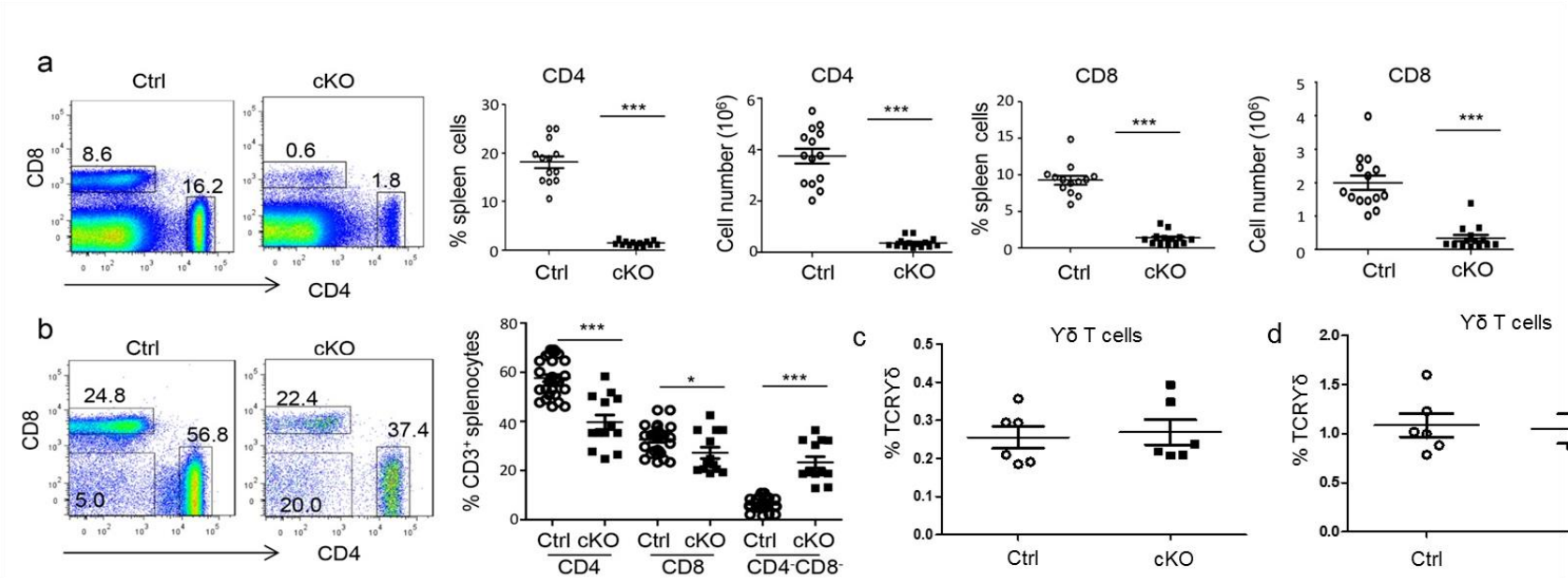

a
Ctrl
cKO
CD8
CD4
8.6
16.2
0.6
1.8
CD4
% spleen cells
Cell number (10⁶)
CD8
***
Ctrl
cKO
b
24.8
56.8
5.0
22.4
37.4
20.0
% CD3⁺ splenocytes
*
CD4
CD8
CD4⁻CD8⁻
c
Yδ T cells
% TCRYδ
d

Fig. 3.1. T-lineage-specific activation of Wnt signaling causes T cell lymphopenia a. Wnt signaling causes massive reductions in both CD4 and CD8 T cells. Representative profile depicting the frequencies of CD4 and CD8 T cells in 6-8 week old WT and cKO spleens are shown on the left, while the summary is presented on the right (n=13). b. Wnt signaling causes loss of CD4 and CD8 markers in T cells. Representative profiles for CD4 and CD8 markers among $CD3^+$ spleen cells are shown on the left, while the summary is shown on the right (n=11). c, d. *Apc* deletion by *CD4-Cre* does not cause alteration in γδ T cells lineages. Ctrl (open circles) and cKO (filled squares) thymocytes (a) and splenocytes (b) were stained with anti-TCRβ and anti-TCRγδ complex mAbs. The data is shown as the mean and SEM of % γδ T cells, summarized from two independent experiments involving 6 mice at 6-8 weeks of ages in each group.

Figure 3.2

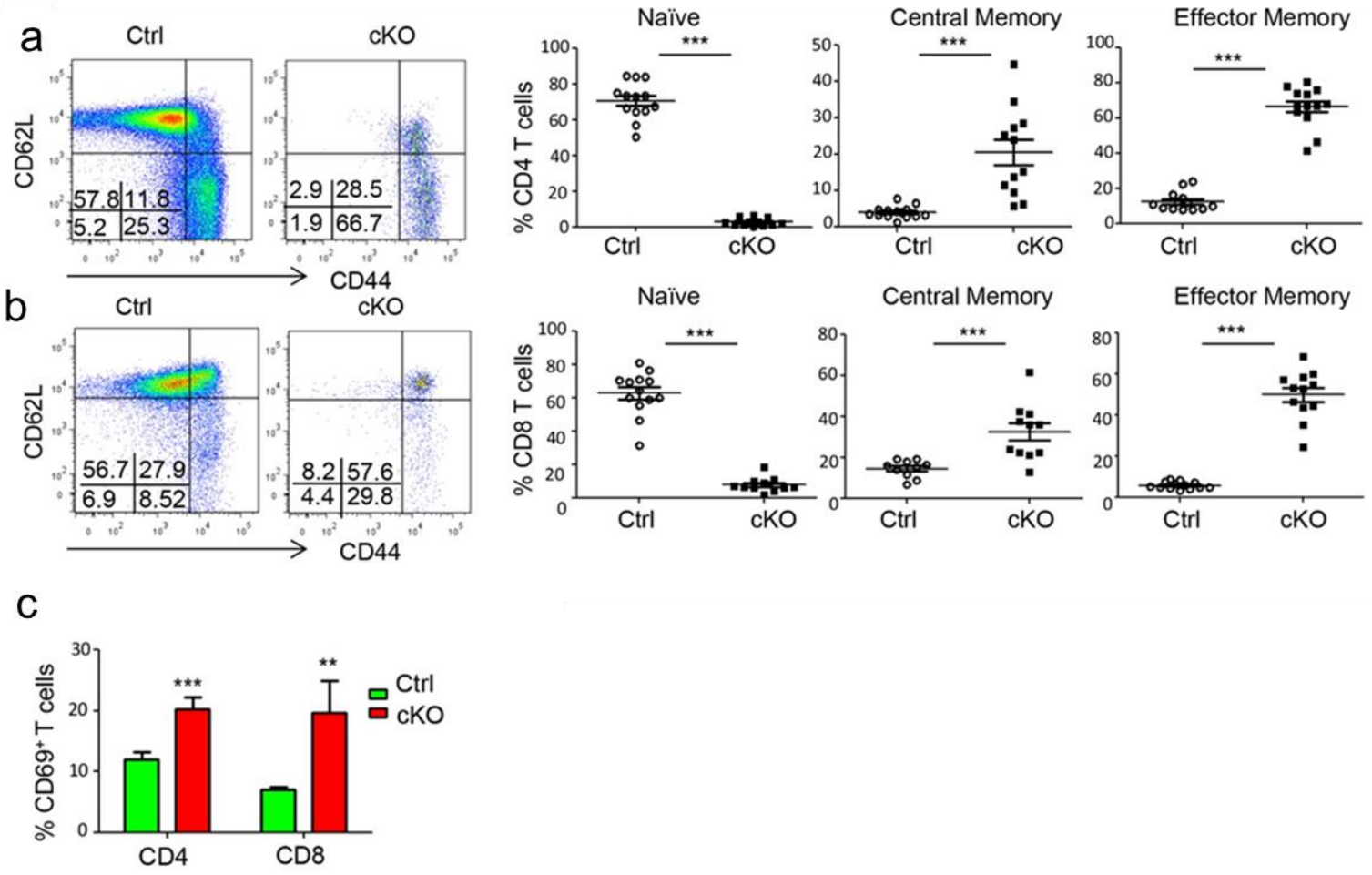
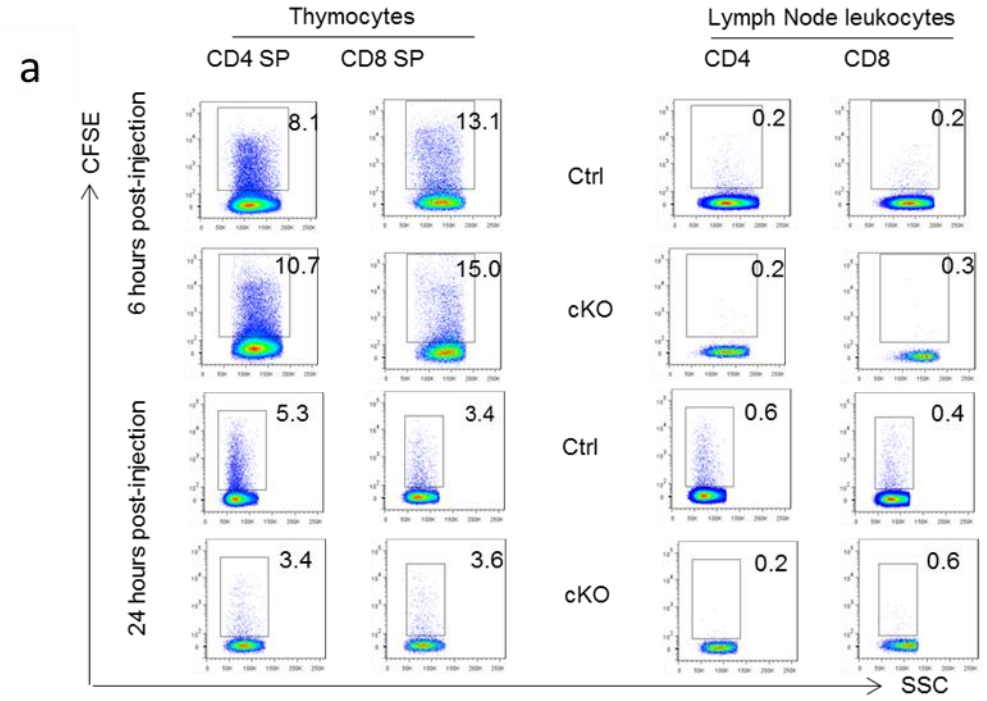

a
Ctrl
cKO
CD62L
CD44
57.8
11.8
5.2
25.3
2.9
28.5
1.9
66.7
b
Ctrl
cKO
CD62L
CD44
56.7
27.9
6.9
8.52
8.2
57.6
4.4
29.8
Naïve
Central Memory
Effector Memory
% CD4 T cells
% CD8 T cells
***
Ctrl
cKO
c
% CD69+ T cells
**
Ctrl
cKO
CD4
CD8

Fig. 3.2. Wnt signaling causes loss of naïve CD4 (a) and CD8 (b) T cells in the spleen. Left panels show representative profiles depicting the distribution of CD44 and CD62L markers, while the right panels show the summary of the frequencies of naïve ($CD62L^{hi}$ $CD44^{lo}$), central memory ($CD62L^{hi}$ $CD44^{hi}$), or effector memory ($CD44^{hi}CD62L^{lo}$) cells (n=13). c. Increased CD69 expression among cKO T cells. Data presented are % of $CD69^{+}$ T cells among CD4 and CD8 T cells (n=9). Data are either representative or summary of those from at least three independent experiments.

Figure 3.3


a
Thymocytes
CD4 SP
CD8 SP
Lymph Node leukocytes
CD4
CD8
CFSE
6 hours post-injection
24 hours post-injection
8.1
13.1
0.2
0.2
Ctrl
10.7
15.0
0.2
0.3
cKO
5.3
3.4
0.6
0.4
Ctrl
3.4
3.6
0.2
0.6
cKO
SSC

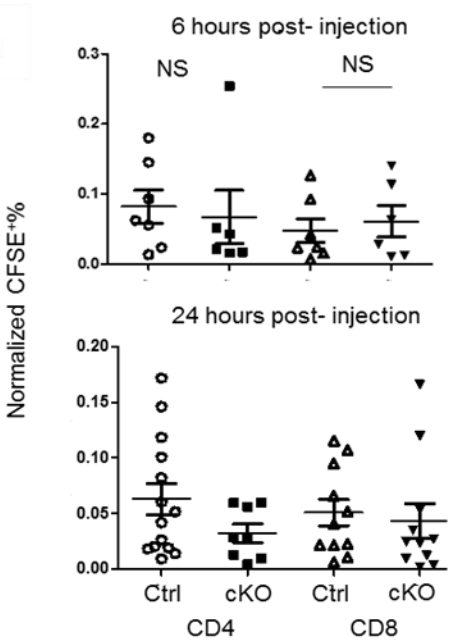

b
6 hours post- injection
NS
NS
24 hours post- injection
Normalized CFSE+%
0.3
0.2
0.1
0.0
0.20
0.15
0.10
0.05
0.00
Ctrl
cKO
Ctrl
cKO
CD4
CD8

Figure 3.3.

Thymic output is not affected by an *Apc* deficiency in the T cell linenage. Thymocytes were labeled by intrathymic injections of CSFE. After 6 or 24 hours, Ctrl and cKO thymi and lymph nodes were harvested to analyze the frequency of $CFSE^+$ CD4 or CD8 T cells. FACS profiles depicting the $CFSE^+$ cells among gated CD4 or CD8 T cells in the thymus or lymph nodes are presented in a, while the summary from one experiment involving 6-14 mice per group from 2-3 independent experiments are shown in d. Rare mice with <3% labeling of either CD4 or CD8 SP thymocytes were excluded from the analyses

Figure 3.4

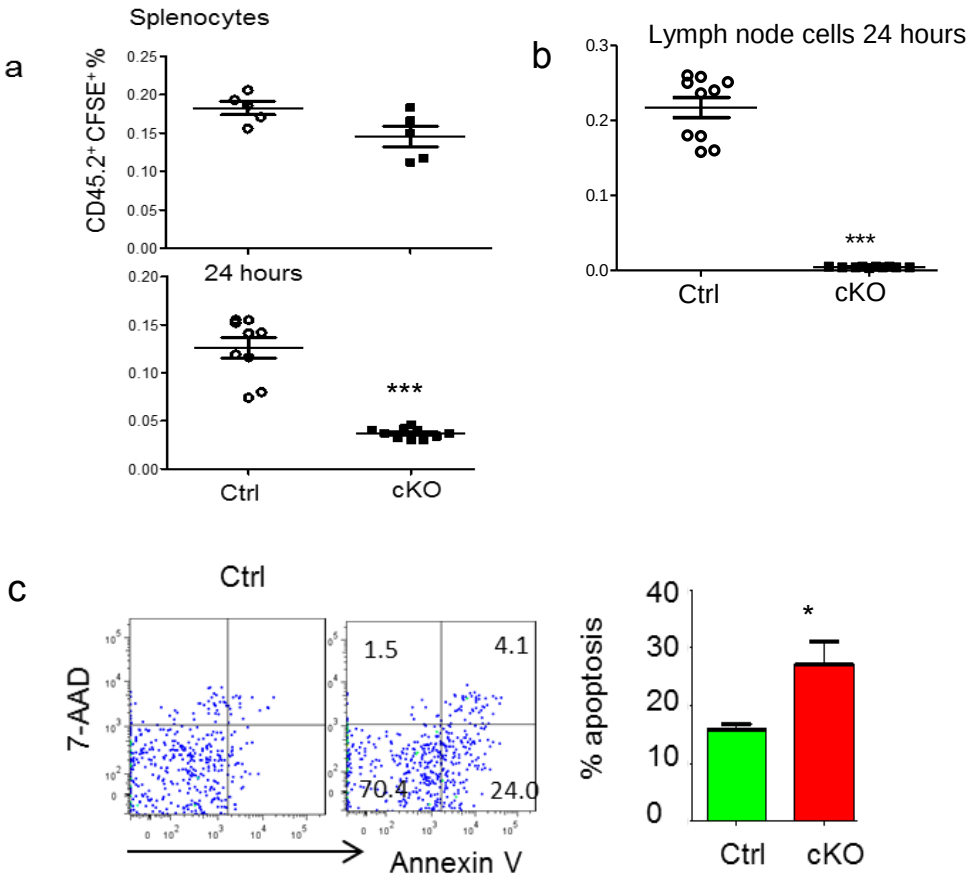

a
Splenocytes
CD45.2+CFSE+%
0.25
0.20
0.15
0.10
0.05
0.00
24 hours
0.20
0.15
0.10
0.05
0.00
***
Ctrl
cKO
b
Lymph node cells 24 hours
0.3
0.2
0.1
0.0
***
Ctrl
cKO
c
Ctrl
7-AAD
1.5
4.1
70.4
24.0
Annexin V
% apoptosis
40
30
20
10
0
*
Ctrl
cKO

Fig. 3.4. Rapid loss of mature cKO thymocytes in the periphery. $CD24^-$ mature T cells were isolated from Ctrl or cKO mice (CD45.2) and injected intravenously into congenic CD45.1 mice. The frequency of $CD45.2^+CFSE^+$ cells in the spleen (a) or lymph nodes (b) are summarized from three independent experiments. c. Increased apoptosis of mature cKO thymocytes at 6 hours after adoptive transfer. % apoptosis was detected via staining of 7-AAD and Annexin V. Representative FACS profiles are shown in the left, while summary data involving 3-4 mice per group were shown in the right. The data have been reproduced in two independent experiments.

Figure 3.5

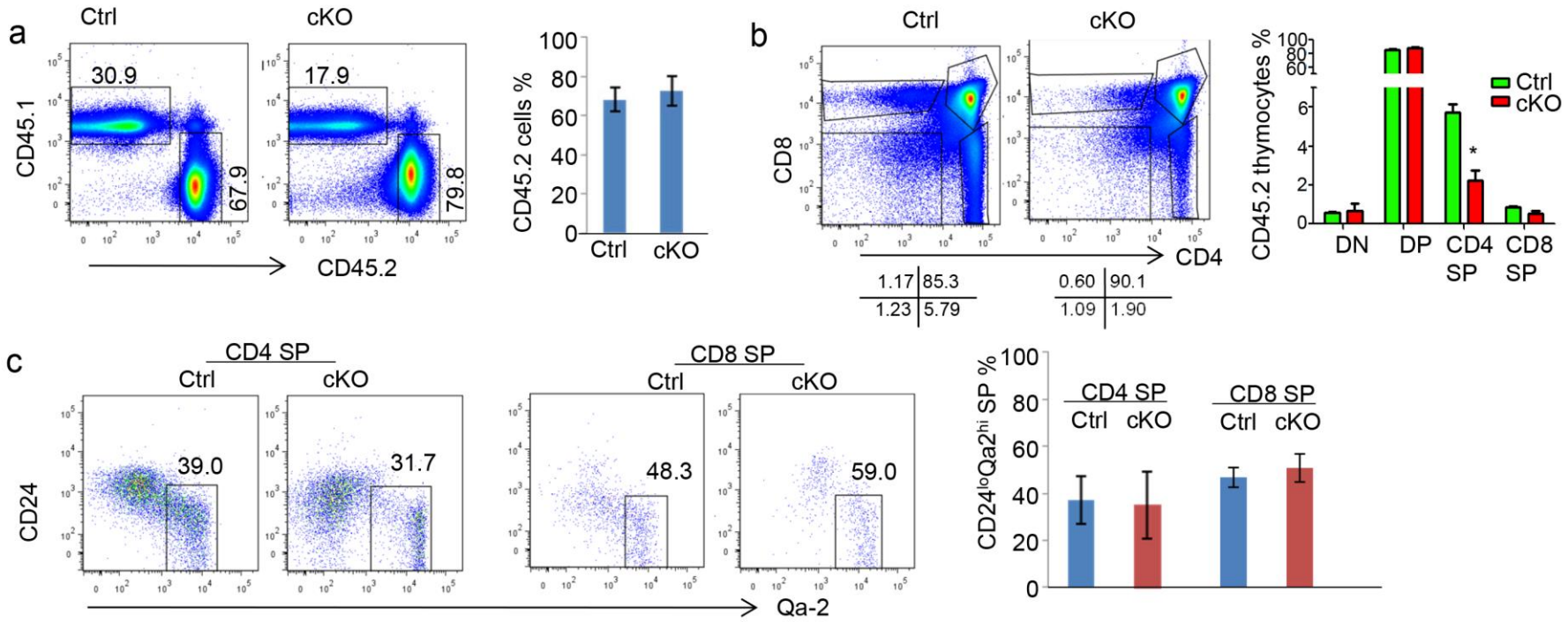

a
Ctrl
cKO
30.9
17.9
67.9
79.8
CD45.1
CD45.2
CD45.2 cells %
100
80
60
40
20
0
Ctrl
cKO
b
Ctrl
cKO
CD8
CD4
1.17
85.3
1.23
5.79
0.60
90.1
1.09
1.90
CD45.2 thymocytes %
100
80
60
6
4
2
0
Ctrl
cKO
*
DN
DP
CD4 SP
CD8 SP
c
CD4 SP
CD8 SP
Ctrl
cKO
Ctrl
cKO
CD24
39.0
31.7
48.3
59.0
Qa-2
CD24loQa2hi SP %
100
80
60
40
20
0
CD4 SP
CD8 SP
Ctrl
cKO
Ctrl
cKO

Fig. 3.5. Grossly normal development of Apc-deficient T cells in bone marrow chimera mice**.** Bone marrow chimeras were generated by injecting $2.5x10^6$ of either Ctrl or cKO bone marrow cells, in conjunction with an equal number of recipient CD45.1-type bone marrow cells into lethally irradiated mice. Mice were sacrificed 10 weeks after bone marrow transplantation. For all panels, representative profiles are shown on the left, while the summarized data from one experiment involving 4 mice per group are presented on the right. All data have been reproduced in two independent experiments. a. In the chimeric mouse thymus, *Apc* deletion does not affect thymocyte production. Data shown are frequencies of donor-derived T cells. b. Development of $CD45.2^+$ thymocytes was assessed based on the distribution of CD4 and CD8 markers. c. Normal thymocyte maturation was assessed based on CD24 and Qa-2 expression on donor-type CD4 SP and CD8 SP thymocytes.

Figure 3.6

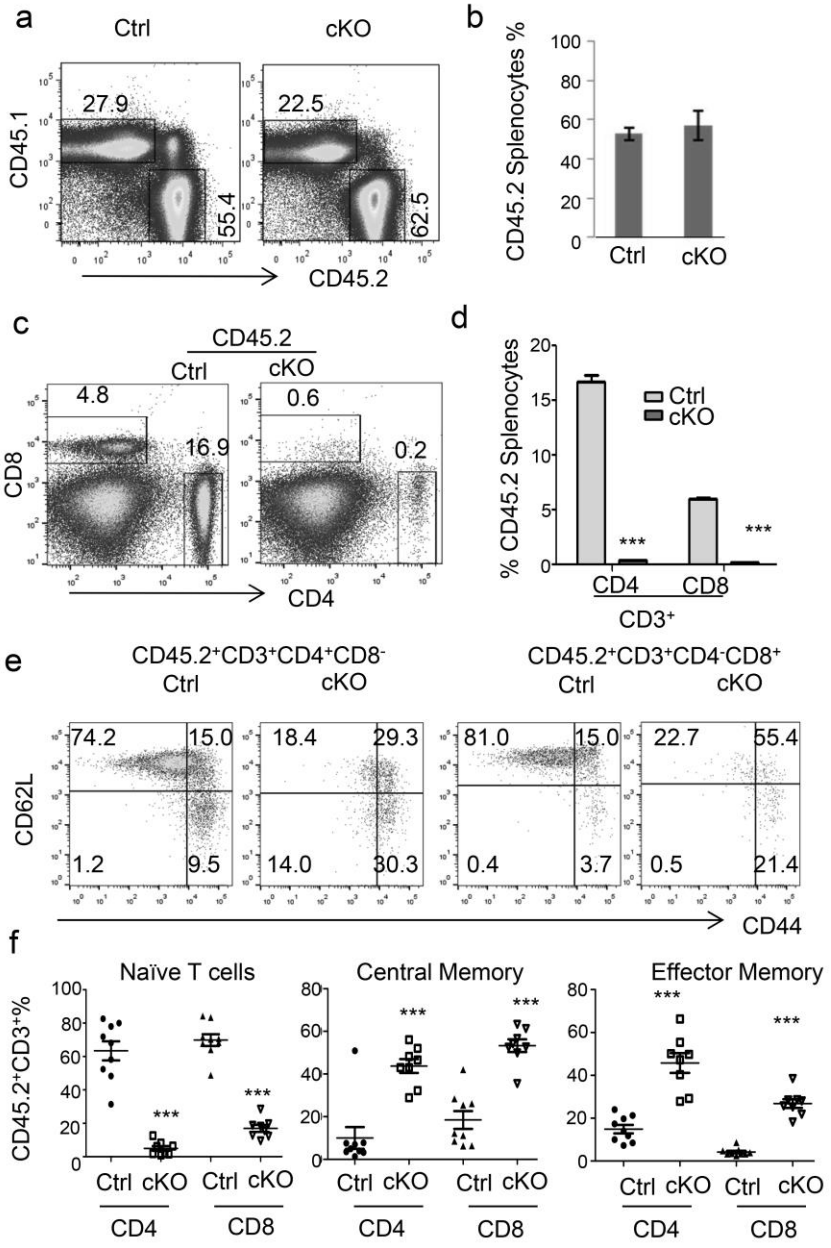

a
Ctrl
cKO
CD45.1
27.9
55.4
22.5
62.5
CD45.2
b
CD45.2 Splenocytes %
100
80
60
40
20
0
Ctrl
cKO
c
CD45.2
Ctrl
cKO
CD8
4.8
16.9
0.6
0.2
CD4
d
% CD45.2 Splenocytes
20
15
10
5
0
Ctrl
cKO
***
***
CD4
CD8
CD3+
e
CD45.2+CD3+CD4+CD8-
Ctrl
cKO
CD45.2+CD3+CD4-CD8+
Ctrl
cKO
CD62L
74.2
15.0
1.2
9.5
18.4
29.3
14.0
30.3
81.0
15.0
0.4
3.7
22.7
55.4
0.5
21.4
CD44
f
Naïve T cells
Central Memory
Effector Memory
CD45.2+CD3+%
100
80
60
40
20
0
***
***
***
***
***
***
Ctrl
cKO
Ctrl
cKO
CD4
CD8

Fig. 3.6. T-cell intrinsic function of Wnt signaling and T cell survival. a, b. Chimerism in the spleen was assessed based on the expression of congenic markers. c, d. Cell-intrinsic lymphopenia after lineage-specific *Apc* deletion was assessed based on 5-color flow cytometry using antibodies specific for CD45.1, CD45.2, CD3, CD4, and CD8. e, f. Spontaneous loss of naïve T cells is due to cell-intrinsic *Apc* deletion. The frequencies of naïve, central, and effector memory T cells were determined using CD45.2, CD44, CD62L, CD4 and CD8. Representative FACS profiles are shown in a, c and e, while summary data from two independent experiments involving 4 mice per group are shown in b, d and f.

Figure 3.7

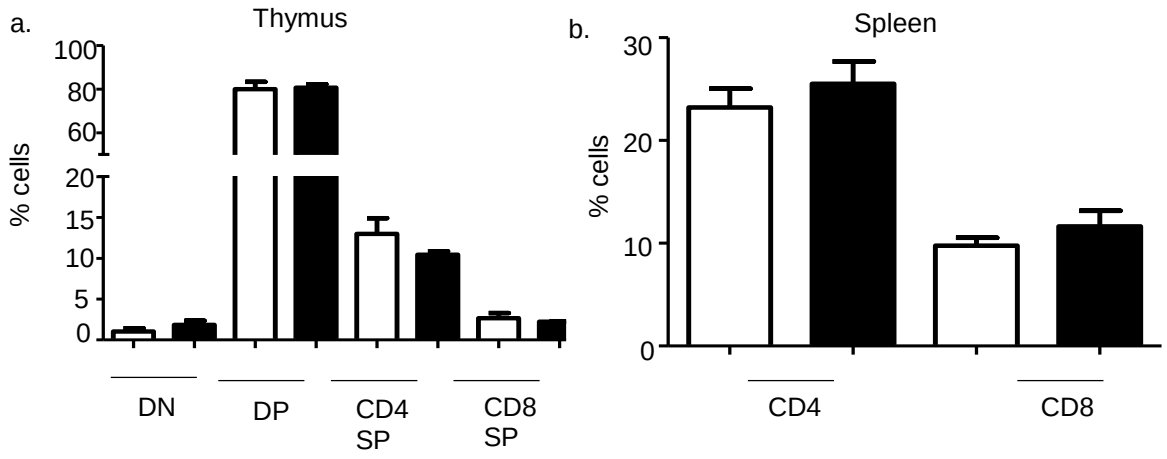


Figure 3.7 (a and b) In mixed bone marrow chimeras, *Apc* deletion by *CD4-Cre* in donor-type T cells does not affect the development and survival of recipient-type T cells. a. % WT CD45.1^{+} recipient-type cells developed in the presence of either Ctrl (open bars) or cKO (filled bars) donor-type (CD45.2^{+}) cells. b. As in c, except spleen cells were analyzed. The data is shown as the mean and SEM, summarized from 3 independent experiments, n=10.

Figure 3.8

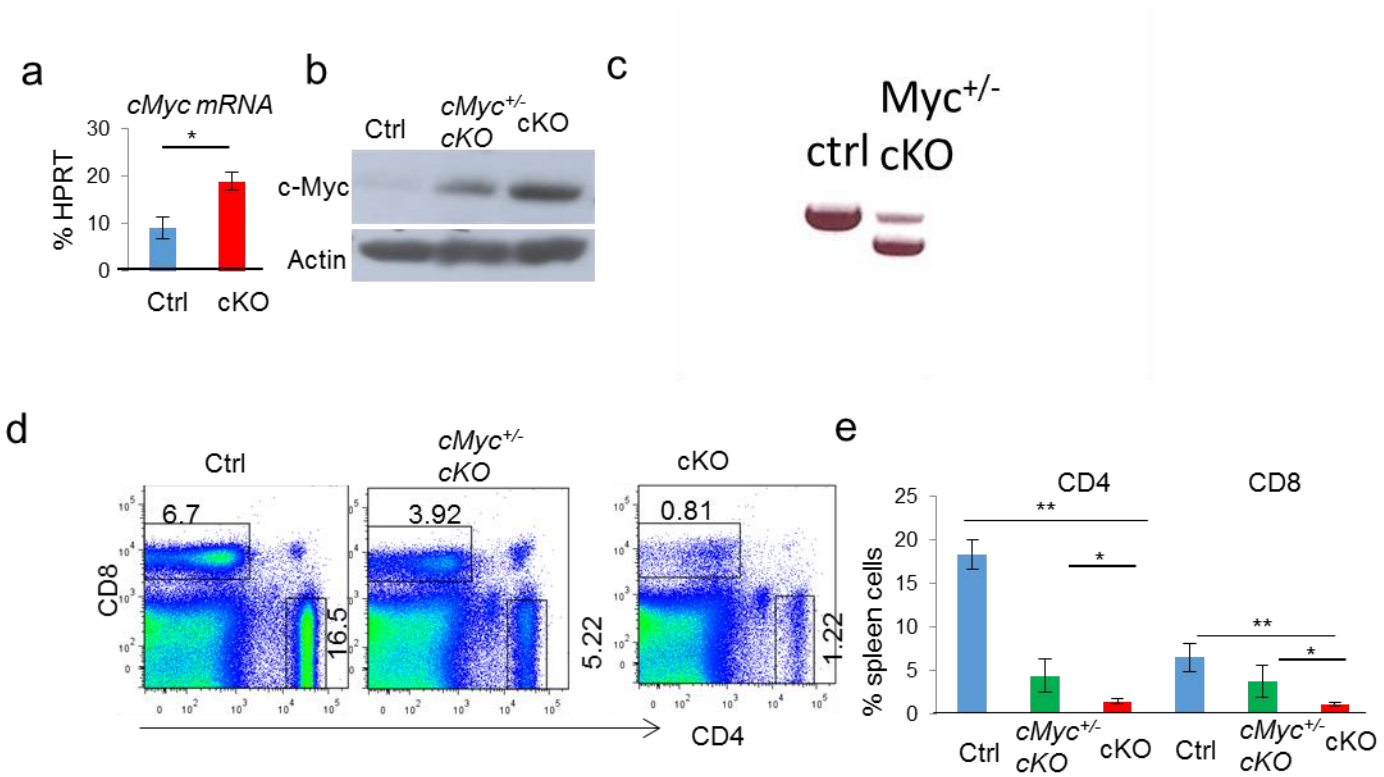

a
cMyc mRNA
% HPRT
30
20
10
0
*
Ctrl
cKO
b
Ctrl
cMyc+/- cKO
cKO
c-Myc
Actin
c
Myc+/-
ctrl
cKO
d
Ctrl
cMyc+/- cKO
cKO
6.7
3.92
0.81
CD8
16.5
5.22
1.22
CD4
e
CD4
CD8
% spleen cells
25
20
15
10
5
0
**
*
**
*
Ctrl
cMyc+/- cKO
cKO
Ctrl
cMyc+/- cKO
cKO

Fig. 3.8. Heterozygous deletion of cMyc partially attenuate loss of T cells caused by *Apc* deletion**.** a. *Apc* deletion increases *cMyc* mRNA in $CD24^-$ mature thymocytes as measured by quantitative PCR. Data shown are means+/- SEM (n=3). These data have been reproduced 3 times. b. Western blot analysis of c-Myc protein in pooled $CD24^-$ mature thymocytes from Ctrl (n=3), $cMyc^{+/-}cKO$ (n=6), and $cMyc^{+/+}cKO$ (n=9). These data have been reproduced twice. c. Representative profiles of CD4 and CD8 T cells in Ctrl, $Myc^{+/-}cKO$, or *cKO* spleens. d. Summary of the frequencies of CD4 or CD8 T cells in in Ctrl, $Myc^{+/-}$ *cKO*, or *cKO* spleens (n=9). e. detect of deletion of *cMyc* allele by PCR of splenic T cells from either control or $Myc^{+/-}$ *cKO* mice.

Figure 3.9

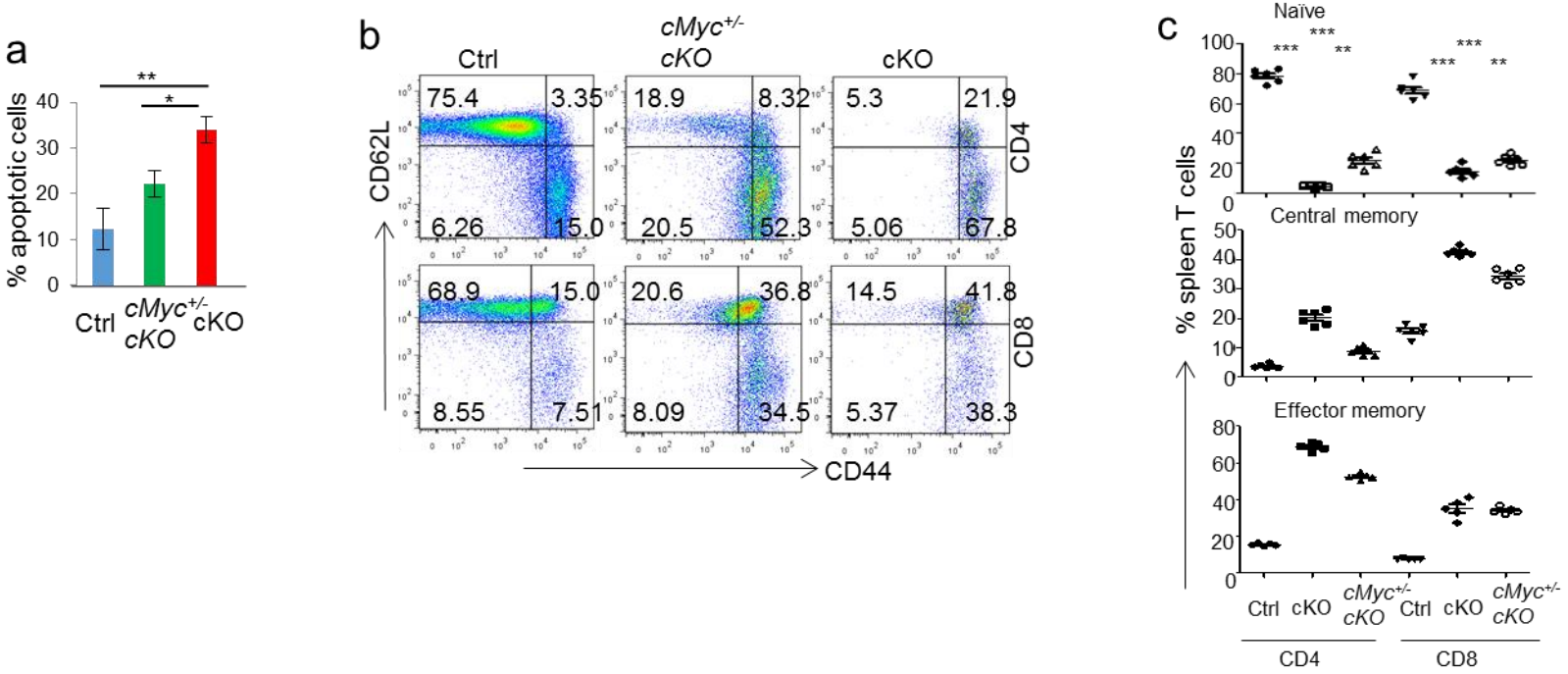

a
% apoptotic cells
Ctrl
cMyc+/- cKO
cKO
b
Ctrl
cMyc+/- cKO
cKO
CD62L
CD44
CD4
CD8
75.4
3.35
6.26
15.0
18.9
8.32
20.5
52.3
5.3
21.9
5.06
67.8
68.9
15.0
8.55
7.51
20.6
36.8
8.09
34.5
14.5
41.8
5.37
38.3
c
Naïve
Central memory
Effector memory
% spleen T cells
Ctrl
cKO
cMyc+/- cKO
CD4
CD8

Figure 3.9. Heterozygous deletion attenuates apoptosis and partially restore naïve T cells. a. Heterozygous deletion of *cMyc* in T cells attenuates apoptosis of $CD24^-$ mature thymocytes at 6 hours after adoptive transfer via intravenous route. Aliquots of $1x10^6$ CFSE-label thymocytes were injected into congenic CD45.1 recipients. Apoptosis was measured by staining with 7-AAD and Annexin V. Data shown are means and SEM (n=9). b. Representative profiles depicting the distribution of CD44 and CD62L markers on CD4 and CD8 T cells in Ctrl, $Myc^{+/-}$ *cKO*, or *cKO* spleens. c. Frequencies of naïve, central memory, or effector memory splenic T cells in 6-8 week old Ctrl, $cMyc^{+/-}$*cKO*, or *cKO* mice (n=9). Data from a-c are either representative or summary of those from three independent experiments.

Figure 3.10

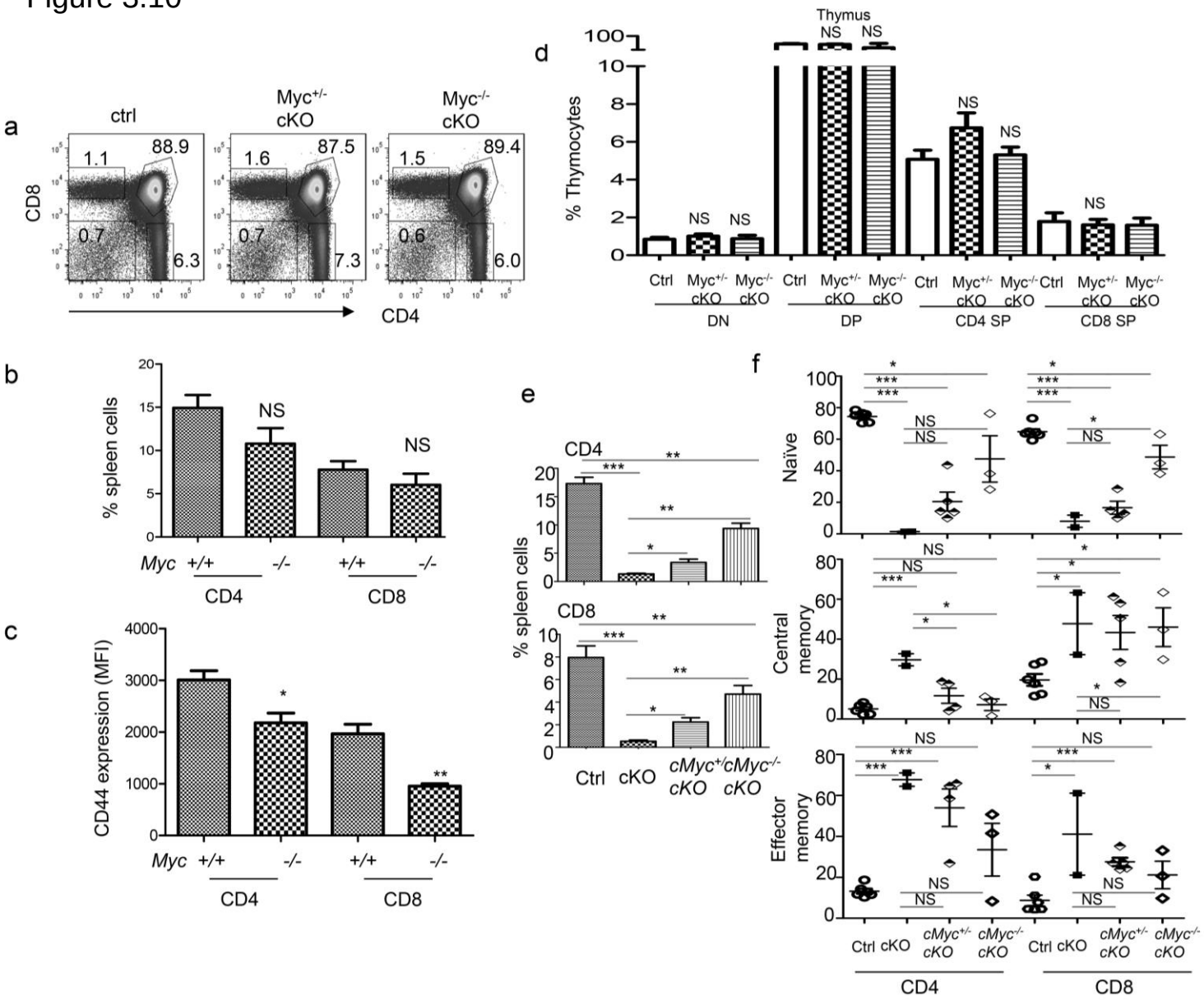

a
ctrl
Myc+/- cKO
Myc-/- cKO
CD8
CD4
1.1
88.9
0.7
6.3
1.6
87.5
0.7
7.3
1.5
89.4
0.6
6.0
b
% spleen cells
NS
NS
Myc +/+ -/- +/+ -/-
CD4
CD8
c
CD44 expression (MFI)
Myc +/+ -/- +/+ -/-
CD4
CD8
d
Thymus
% Thymocytes
NS
Ctrl Myc+/- cKO Myc-/- cKO
DN
DP
CD4 SP
CD8 SP
e
CD4
CD8
% spleen cells
Ctrl cKO cMyc+/- cKO cMyc-/- cKO
f
Naïve
Central memory
Effector memory
Ctrl cKO cMyc+/- cKO cMyc-/- cKO
CD4
CD8

Fig. 3.10. Homozygous deletion of *cMyc* substantially rescues lymphopenia and spontaneous activation of *Apc*-deficient T cells. a-c. *cMyc* deletion has minimal impact on thymocyte development and T lymphocyte cellularity but reduces CD44 expression in spleen T cells. a. Representative profiles depicting thymocyte development based on distribution of CD4 and CD8 markers. b. Deletion of cMyc does not significantly reduce T cell cellularity. Summary data showing the frequencies of CD4 and CD8 T cells, data shown are means and SEM (n=9). c. cMyc deletion reduces CD44 expression. Data shown are means and SEM (n=9) of mean fluorescence intensities, depicting CD44 expression among CD4 and CD8 T cells. d. Summary data on thymocytes subsets among 6-8 weeks old mice with or without deletion of Apc and genes. Data shown are means and SEM (n=9). e. Frequencies of CD4 or CD8 T cell from Ctrl (n=6), cKO (n=2), or, *cMyc*$^{+/-}$*cKO* (n=5), or *cMyc*$^{-/-}$*cKO* (n=3). f. Frequencies of naïve, central memory and effector memory T cells in the spleen from Ctrl (n=6), cKO (n=2), *Myc*$^{+/-}$ *cKO* (n=5), or *cMyc*$^{-/-}$*cKO* (n=3) mice.

Figure 3.11

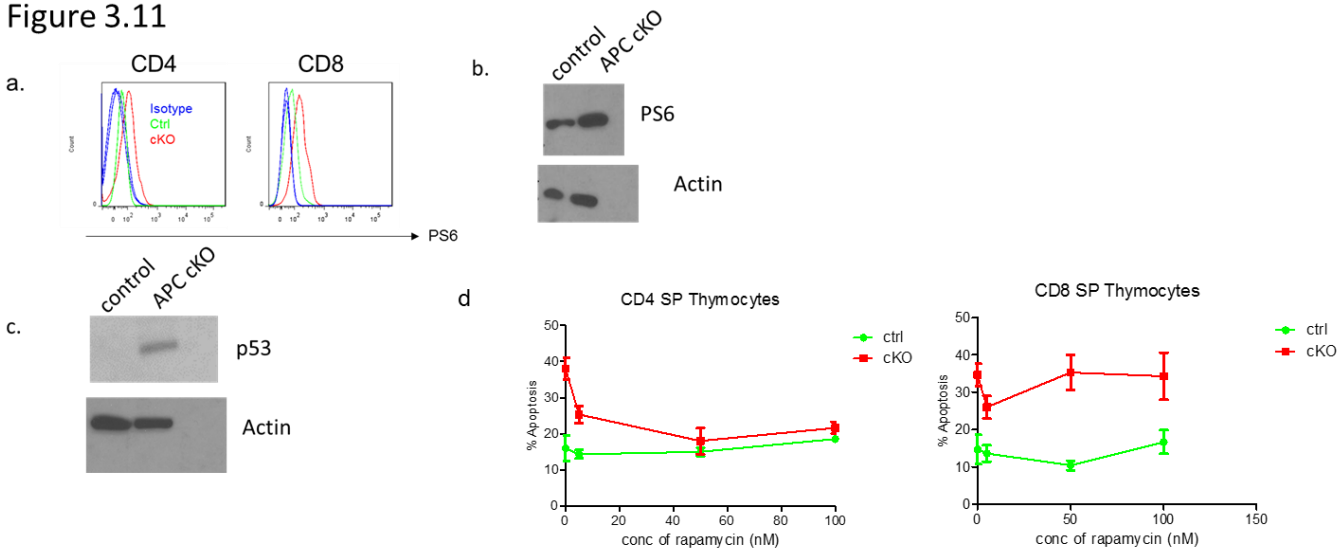


Fig. 3.11. Deletion of *Apc* results in hyperactivation of mTOR signaling a. Splenocytes were isolated from 6-8 week cntrl and cKO mice. Cells were for surface markers (CD3, CD4, and CD8). Cells were permeablized, then stained with anti- pS6 (1:30) or an isotype control. Similar data were obtained in 3 independent experiments, involving 9 mice per group in total. (b and c). Splenocytes were isolated from cntrl or cKO mice. T cells were isolated using Macs pan T cell kit through negative selection. T cells were lysed in RIPA buffer. Protein levels were analyzed using western blot analysis. These data have been repeated twice. d. thymocytes from ctrl or cKO mice were cultured overnight in RMPI with 10%FBS, pen/strip, and 50mM of 2-Me with varying concentrations of rapamaycin (0-5 nm). Apoptosis was detected via Annexin V and 7-AAD staining. This experiment was repeated twice with 3 mice per group in each experiment.

Figure 3.12

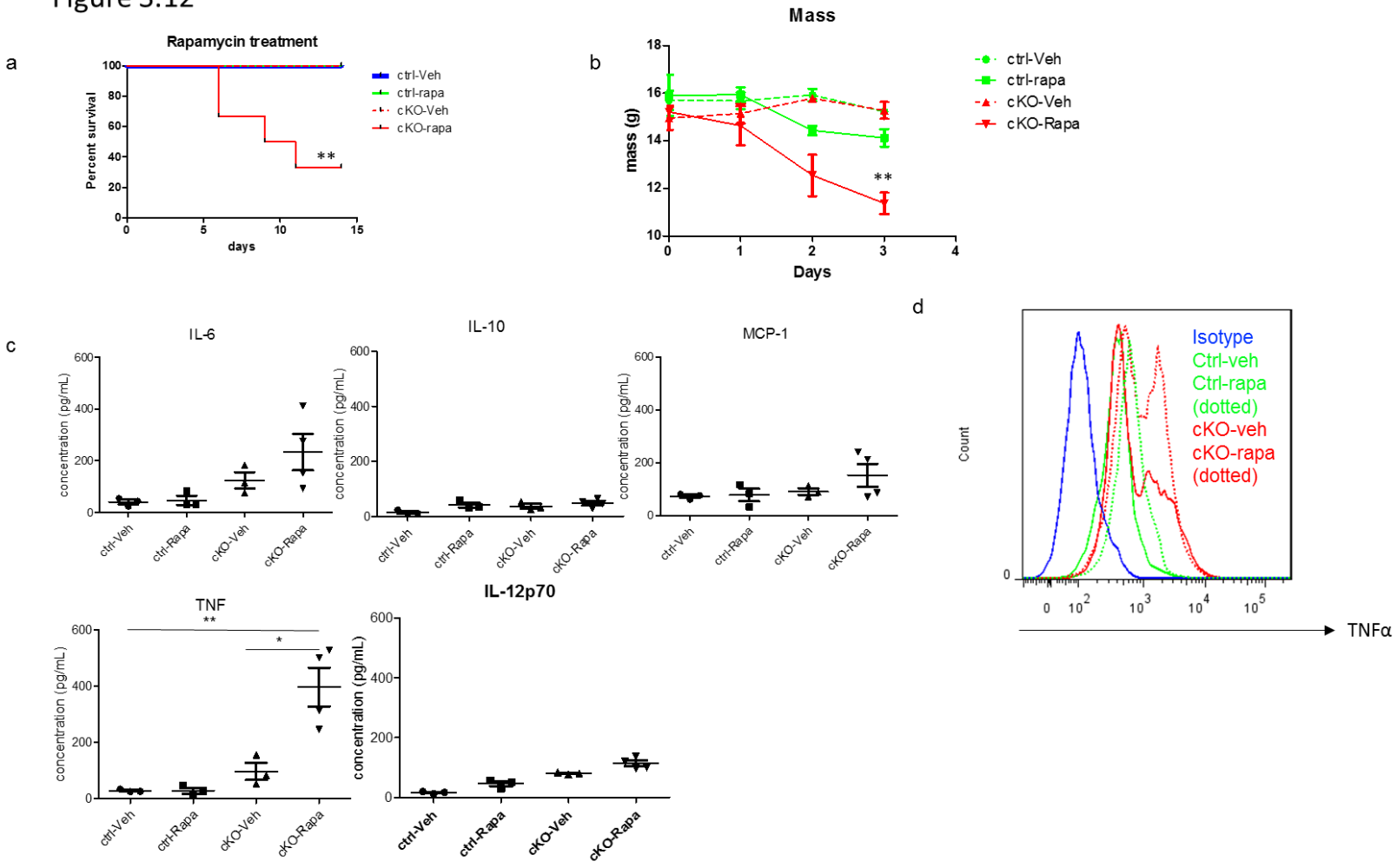

a
Rapamycin treatment
Percent survival
days
ctrl-Veh
ctrl-rapa
cKO-Veh
cKO-rapa
**
b
Mass
mass (g)
Days
ctrl-Veh
ctrl-rapa
cKO-Veh
cKO-Rapa
**
c
IL-6
IL-10
MCP-1
TNF
IL-12p70
concentration (pg/mL)
ctrl-Veh
ctrl-Rapa
cKO-Veh
cKO-Rapa
d
Count
Isotype
Ctrl-veh
Ctrl-rapa
(dotted)
cKO-veh
cKO-rapa
(dotted)
TNFα

Fig. 3.12. Treatment of rapamycin *in vivo* results in massive cytokine storm in mice with T cell specific *Apc* deficiency. a. ctrl or cKO mice were treated every other day for 2 weeks with 4 mg/kg of rapamycin or vehicle control (n=6) . Survival was recorded for 2 weeks. b. ctrl (n=3) or cKO mice (n=4) were treated with 4 mg/kg of rapamycin daily 3 times. Weight was recorded daily. c. Serum was collected on day 3 post-treatment and cytokine levels were determined using a pro-inflammatory bead array assay. d. Splenocytes were treated in vitro for three days with rapamycin. On day three, cells were treated with PMA (500 ng/mL) and ionomycin (500 ng/mL) to stimulate T cell cytokine production for 4 hours. Golgi blockers was added by hour 2. Cells were gated on $CD3^+$ splenic T cells and TNFα production was analyzed. (n=4).

Chapter 4

T-cell Intrinsic Deletion of the *Adenomatous Polyposis Coli* Gene Causes Homeostatic Proliferation and Inflammatory Bowel Disease

4.1 Abstract

Many autoimmune diseases have been linked to lymphopenia, a conditional associated with lower than normal lymophocytes. However, to our knowledge, none of the known single gene defects that cause lymphopenia are sufficient to cause autoimmune diseases. In addition, the intracellular programing involved in lymphopenia-driven homeostatic proliferation (HP) remains to be elucidated. Using a conditional knockout of adenomatous polyposis coli (*Apc)* under the control of CD4-cre, we showed that *Apc* deletion causes both T cell activation, lymphopenia and HP. Using this mouse model, we studied the association between lymphopenia-driven HP, and autoimmune diseases. We showed that APC deficiency and the resulting activation of Wnt signaling in T cells causes a fatal inflammatory bowel disease with Crohn's disease-like pathology. Development of autoimmune diseases requires lymphopenia. This data provide the first genetic evidence that a T-cell intrinsic single-gene defect is sufficient to cause lymphopenia, HP and autoimmune diseases.

4.2 Introduction

Many human autoimmune diseases, including type 1 diabetes [156-158, 199], rheumatoid arthritis [150-154, 200, 201], Sjogren's syndrome [161], systemic lupus erythematous [143, 145, 146], inflammatory bowel diseases (IBD) [140, 202, 203], and celiac disease [204, 205], have been associated with lymphopenia [206]. Transient lymphopenia caused by rubella infections is linked to autoimmune diabetes mellitus [207, 208], while Parvovirus B19 infection is to associated with rheumatoid arthritis [209, 210] and multiple sclerosis risk is linked to common childhood infections [211]. The link between autoimmune disease and lymphopenia has been demonstrated in various mouse models of autoimmune diseases including: IBD, gastritis, and diabetes. The mouse model of inflammatory bowel disease (IBD) involves HP by transferring T cells into a lymphopenic host. In addition, mice that were thymectomized at day 3 of age, and therefore lymphopenic, developed gastritis, caused by expansion of T cells that were specific to a self-antigen, H/K ATPase [212, 213]. Furthermore, nonobese diabetic (NOD) mice develop diabetes due to the destruction of β-cells by T lymphocytes. This T cell-mediated autoimmune disease was linked to the reduction of T cell numbers, as diabetes was alleviated by infusing NOD mice with syngeneic T cells.

Lymphopenia participates in the pathogenesis of autoimmune disease via a process known as T cell HP. HP occurs when T cell numbers decrease, and they homeostatically expand [130-132, 214]. HP is distinct from T cell expansion in response to a foreign antigen, as it allows for expansion of T cells that have a low affinity for self-antigens. These self-reactive T cells become activated and acquire features of memory T cells, including expression CD44 and Ly6c markers and producing IFNϒ, granzyme

B, and perforin following short-term PMA stimulation [129-133]. Therefore, HP allows for the proliferation and activation of possible self-reactive T cells creating an environment for the development of autoimmunity.

In addition to lymphopenia, It has also been suggested that gut associated microbes also are required for optimal HP. When $CD4^+$ T cells were transferred into SCID mice maintained under germ-free conditions, these T cells had reduced HP [215]. Other regulators of HP include a surface co-inhibitory molecules. BTLA deletion in T cells exacerbate HP [216], while deletion of CD24, in T cells, attenuates it [217]. While T regulatory cells do not suppress HP, co-transfer of T regulatory cells prevented inflammatory bowel diseases caused by $CD45RB^{hi}$ CD4 T cells [140, 142, 203, 218].

Though lymphopenia seems to be interconnected with autoimmunity, it alone is not sufficient to cause autoimmunity. The model of IBD and also of gastritis requires, not only lymphopenia, but the lack of T regulatory cells to cause disease. In addition, T cell-mediated diabetes in NOD mice requires a specific background of mouse that over-produces IL-21, a cytokine that aids in HP [137]. Therefore, while lymphopenia appears to play a role in the etiology of autoimmunity, the link between lymphopenia and autoimmune disease remains to be strengthened in at least two levels. First, although a number of genetic studies have indicated that lymphopenia can be caused by deletion of genes involved in T cell development [219-221], emigration [91, 222], and maintenance of quiescence [97, 223], to our knowledge, none of these mutations alone are sufficient to cause autoimmune disease. Second, although it is well established that T cells undergo homeostatic proliferation in response to lymphopenia and acquire memory phenotypes [131, 214], the lack of understanding of the intracellular program

for lymphopenia-driven HP makes it difficult to definitively assess how lymphopenia and its associated HP serve as drivers for autoimmune disease. In this context, although the cytokines IL-7, IL-15 [186, 224], and IL-21[158], the cell surface protein CD24 [217, 225], and the interaction of TCR with self-peptide-MHC [127, 130, 138, 226] all contribute to HP in response to lymphopenia for naïve T cells, the intracellular signaling pathway responsible for lymphopenia-driven HP is largely uncharacterized. From the previous chapters, we have shown that a deletion of *Apc* does not affect thymocyte development, but instead, causes T cell death in naïve T cells. The resulting lymphopenia allows us to further elucidate a link between HP and autoimmune disease. Here, we show that lymphopenia and activation of Wnt signaling by *Apc* deletion in T cells causes HP and inflammatory bowel disease (IBD). These data greatly strengthen the link between lymphopenia, HP, and autoimmune disease.

4.3 Materials and Methods

Mice

CD45.1 C57BL/6 mice were obtained from Charles River Laboratories through a contract with the National Cancer Institute. Mice with homozygous knockin of the Floxed *Apc [173]* or *Ctnnb1* [227] loci and transgenic mice expressing the *Cre* recombinase under the control of CD4 promoter [175] were obtained from Jackson Laboratories (Maine, USA). Mice with floxed cMyc locus [190] were kindly provided by Dr. De Alboran. All mice used in this study have been backcrossed to C57BL/6 background for at least 10 generations. These strains were maintained in our animal

facilities under pathogen-free conditions. All experiments were in accordance with guidelines of the Institutional Animal Care and Use Committee of University of Michigan and the Children's National Medical Center.

Flow cytometric analysis

Single cell suspensions were stained at 4°C for 20 minutes in phosphate-buffered saline with 2% FBS with the following antibodies (BD Bioscience, 1:200): CD4 (RM4-5, 25-0042-82), CD8 (SK-1, 8066-0087-120), B220 (HIS24, 25-0460-82), Samples were analyzed by using BD LSR II Flow Cytometer. Ki-67 (SolA15, 12-5698-82) staining was performed by fixing and permealbilizing cells at room temperature for 30 minutes using perm/fix buffer (BD Bioscience, 554714) and stained at 1:200 at 4°C overnight. For staining of intracellular cytokines, mononuclear cells were stimulated for 6 hours with PMA and ionomycin in the presence of golgi blocker (BD Bioscience, 554724) prior to staining using the same methods.

CFSE dilution experiments

T cells isolated from the *Ctnnb1$^{f/f}$;CD4-Cre^{-}* (WT) or *Ctnnb1$^{f/f}$; CD4-Cre^{+}* (*Ctnnb1$^{-/-}$*) mice were labeled with 10 μM CFSE (Invitrogen, C34554) and injected intravenously (10^6 cells/mouse) into 550 Rad-irradiated congenic mice as described [217, 225] . Five days later, spleen cells were harvested and analyzed for CFSE dilution among the CD45.2^{+}CD3^{+} T cells.

Irradiation chimera

Two types of bone marrow chimeras were created. First, CD45.1 mice that had received 6 Gy irradiation were reconstituted with 1x10^6 bone marrow cells from

Apc$^{f/f}$*;CD4-Cre*$^{-}$ (Ctrl) or *Apc*$^{f/f}$*;CD4-Cre*$^{+}$ (cKO) mice were analyzed for the presence of B220^{+} B cells and T cells at 6 weeks after transplantation. The mice were observed for survival using weight loss of greater than 20% as the endpoint. Second, CD45.1^{+} B6 mice were lethally irradiated (11 Gy delivered in 2 installment 4 hours apart, as reported [228]) and reconstituted with 2.5x10^{6} CD45.1^{+} bone marrow cells and an equal number of either cKO or Ctrl bone marrow were observed for the development of survival and the % of Ki67^{+} T cells in the PBL.

Statistics

Data was analyzed using 2-tailed unpaired Student's t-test. All statistics were performed using GraphPad Prizm, version 5 (GraphPad Software, San Diego, CA). *P<0.05, **P<0.01, ***P<0.001.

4.4 Results and Discussion

4.4.1 APC deficiency causes loss of naïve T cells that results in homeostatic proliferation and autoimmune disease.

In chapter 3, we demonstrated that a deletion of *Apc* resulted in naïve T cell death, resulting in lymphopenia and highly activated T cells. To determine whether *Apc* deficient T cells undergo HP in response to lymphopenia, we used Ki-67 expression as a marker to study cycling T cell *in vivo*. As shown in the left panel of Fig. 4.1a and summarized in Fig.4.1b, cKO T cells had 5-fold more Ki-67^{+} T cells than Ctrl T cells. To determine whether cKO T cell proliferation is the result of lymphopenia we created chimeras consisting of bone marrow from either ctrl or cKO (CD45.2^{+}) in conjunction with CD45.1^{+} WT bone marrow. We demonstrated in Chapter 3 and in Fig 4.2 that cKO

bone marrow is unable to fully reconstitute the peripheral T cell compartment, but the host will not be lymphopenic as the CD45.1$^+$ bone marrow is able to fill this loss. Thus, by creating bone marrow chimeras, we can determine whether the observed proliferation of cKO can be prevented by restoring T cell cellularity. Again using Ki-67 as a marker of proliferation, we compared the Ki-67 expression in cKO T cells in a lymphopenic host with those in a non-lymphopenic host. As shown in the right panel of Fig. 4.1a and summarized in Fig 4.1b, the percentage of proliferating cKO T cells was reduced in chimera mice due to filled T cell compartment comprised of CD45.1$^+$ T cells. Therefore, the proliferation of cKO T cells is considered homeostatic in nature.

Remarkably, the majority of the mice with T-lineage-specific deletion of *Apc* developed rectal prolapse, with blood in the feces, diarrhea, and weight loss (data not shown). Most of the cKO mice died within 100 days of age, while most Ctrl mice survived throughout the observation period of 300 days (Fig. 4.3a). Gross anatomical images revealed severe swelling in the colon (Fig. 4.3b).

We conducted a histological analysis of all internal organs and found pathological changes limited to both the small and large intestines, a pathology more similar to Crohn's disease than ulcerative colitis. As shown in Fig. 4.3c, leukocyte infiltration was observed in both the small intestines (top panels) and the colon (middle panels), with a severe loss of mucin production by the colon epithelial cells (bottom panels) and a significant thickening of the colonic muscular layer (middle panels). Together, the bias toward Th1 and Th17, the involvement of both small intestines and colon and,

histopathological features further support the notion that the disease resembles Crohn's disease.

To determine whether this pathogenesis is linked to cKO T cells, we investigated known cytokines that have been shown to play a role in driving the pathogenesis of inflammation in the intestines: IL-17A and IFN-γ. We observed significant elevation of IFN-γ–producing Th1 CD4 T cells and IL-17-producing Th17 cells in the spleen and mesenteric lymph nodes (Fig. 4.4a-d). The bias towards Th1 and Th17, the involvement of both the small intestines and colon, and the histopathological features, such as increased thickness of muscular layer and inflammation in smooth muscle layer, were highly suggestive of Crohn's disease [229].

Genome wide analysis studies (GWAS) have correlated many genes involved in Th17 differentiation: IL-12B, JAK2, IL23R, STAT3, CCR6, and TNFSF15, to susceptibility to Crohn's disease [202, 230, 231]. It is possible that the loss of *Apc* may result in a predisposition of cKO T cells to differentiate into disease-causing Th17 cells. To establish whether *Apc* can regulate Th17 polarization, we polarized mature $CD24^-$ CD4 SP thymocytes. Though the standardized protocol for T helper cell polarization requires the use of naïve CD4 T cells, unfortunately, peripheral cKO T cells are predominantly effector T cells; thus, we use mature $CD24^-$ CD4 SP thymocytes. After polarization, cKO thymocytes do have a tendency to differentiate IL-17A-producing Rorγt expressing Th17 cells (Fig 4.4e). cKO T cells have a higher predisposition to differentiate into Th17 cells.

To show that the autoimmune disease can be prevented by the addition of T cells to create a lymphoreplete environment is the gold standard of determine whether an

autoimmune disease is due to homeostatic proliferation. To address this issue, we generated bone marrow chimera consisting of both WT CD45.1$^+$ T cells and cKO T cells in order to fill the T cell compartment, thus preventing HP (Fig. 4.2). To determine whether the infusion of WT T cells in chimera mouse corrected the survival defects, we compared the survival of chimera mice with cKO mice. As shown in Fig. 4.3a, all of the chimera mice survived throughout an observation period of 300 days. Therefore, provision of WT T cells in the same host prevented both lymphoproliferation and IBD.

4.4.2 A critical role for Wnt signaling in T cell homeostatic proliferation

The link between *Apc* deletion and homeostatic proliferation (Fig. 4.1) suggested a critical role for Wnt signaling in homeostatic proliferation. To verify this, we crossed the floxed *Ctnnb1* locus into the cKO mice to generate *Apc$^{fl/fl}$Ctnnb1$^{fl/fl}$;CD4-Cre$^+$* mice. Unfortunately, the T cells developed in the *Apc$^{fl/fl}$Ctnnb1$^{fl/fl}$;CD4-Cre$^+$* mice retained *Apc* and/or *Ctnnb1* (data not shown), perhaps due to selective pressure against doubly deleted T cells.

To confirm the importance of Wnt signaling, one would have to use naïve CFSE labeled T cells from cKO, *Ctnnb1$^{-/-}$*, and Ctrl mice, observe their proliferation in a lymphopenic host. However, cKO mice do not have an abundance of naïve T cells. If we were to utilize total T cells, we would be comparing cKO effector T cells to naïve Ctrl T cells; two distinctly different populations with different proliferation potentials. On the other hand, a sufficient number of *Ctnnb1$^{-/-}$* T cells could be isolated from *Ctnnb1$^{fl/fl}$;CD4-Cre$^+$* mice (Fig. 4.5a). To determine whether β-catenin is required for homeostatic proliferation, we transferred CFSE-labeled *Ctnnb1$^{+/+}$* and *Ctnnb1$^{-/-}$* T cells into CD45.1 congenic mice

that were made lymphopenic by sublethal irradiation. Five days after adoptive transfer, the spleen cells were harvested to analyze the distribution of the CFSE intensities among the adoptively transferred CD45.2 T cells. As shown in Fig. 4.5b and c, while WT T cells underwent homeostatic proliferation, the β-catenin-deficient T cells barely divided. Therefore, β-catenin is required for homeostatic proliferation.

4.4.3 Myc is not required for homeostatic proliferation

Given the critical role for cMyc in the lymphopenia-induced by the *Apc* deletion, we tested if the increased Myc transcription is critical for homeostatic proliferation, using the Ki-67 expression in the lymphopenic host as an indication of expansion. *cMyc*$^{-/-}$ T cells do have a significantly reduced proliferation rate as seen in the reduced Ki-67 percentages in CD3^{+} T cells, as well as both CD4^{+} and CD8^{+} compartments (Fig 4.6). However, Heterozygous deletion of *cMyc* in cKO T cells did not appreciably reduce HP (Fig 4.6). Homozygous cMyc deletion attenuated the HP somewhat, although substantial HP remains. The modest impact by homozygous *cMyc* deletion was observed in CD8 but not CD4 T cells (Fig. 4.6). Therefore, the genetic requirement for lymphopenia and homeostatic proliferation can be distinguished by cMyc. Consistent with the role for homeostatic proliferation in the pathogenesis of inflammatory bowel disease, neither heterozygous nor homozygous cMyc deletion significantly abrogated the inflammatory bowel disease in the cKO mice (Fig. 4.7).

4.5 Discussion

Previous studies showed that single gene mutations that resulted in lymphopenia appeared to be insufficient to cause autoimmune disease. Thus, although deletion or shRNA silencing of the *Iddm1* locus (*GIMAP5*) causes defective thymocyte development and/or loss of naïve T cells in the periphery, mice with a targeted mutation of the gene do not show signs of autoimmune disease within their relatively short life-span [232-234]. Likewise, in human subjects with a hypomorphic mutation of the *RAG1* gene, which reduces lymphocyte output, none of the patients with lymphopenia develop autoimmune disease [235]. Furthermore, targeted mutations of the *Klf2* and *Tsc1* genes, which respectively cause defective thymic emigration [91] and survival of naïve cells [97, 223], result in lymphopenia without autoimmune disease. In addition, the development of diabetes, in NOD mice, requires lymphopenia, but also requires a specific background of mouse that over-produces IL-21, a cytokine that aids in HP [137].

Here, we report that T-lineage-specific deletion of *Apc* alone was sufficient to cause spontaneous and lethal autoimmune disease within 100 days of age in a C57BL/6 background. Deleting *Apc* and thereby activating Wnt signaling, causes the cKO T cells to be highly activated and die by apoptosis. The deficiency of *Apc* causes T helper cells to polarize to more pro-inflammatory Th17 and Th1 cell types. Previously, it has been shown that Tcf can bind to and activate the *RorγT* gene*,* which encodes the transcription factor for Th17 differentiation; through the loss of *Apc,* Wnt activation can drive Th17 development [236]. HP has also been implicated in the biased differentiation

of gut trophic Th17 cells [237]. It still remains to be determined if it is the activation of Wnt that directly drives Th17 differentiation or lymphopenia-driven HP that skewed T helper cell towards Th17.

Surprising, the autoimmune disease that manifests in *Apc*-deficient T cells is most similar to Crohn's disease, as seen by the histological analysis of the small and large intestines, loss of mucin-producing cells in the intestines, and pro-inflammatory cytokine production of IL-17A and IFNγ. Analysis of the other organs of the cKO mice revealed leukocyte infiltration and inflammation was limited to the small and large intestines. It is still unclear why the inflammation can only be observed in the intestines, but not any other organ, when the conditional knockout is in T cells, not an intestine specific cre-recombinase. It is possible that the loss *Apc* may induce the expression of integrins involved in migration to the intestines, therefore preferentially causing the migration of highly activated cKO T cells to the intestines, causing intestinal inflammation. Also, the majority of the T cell compartment of cKO mice are recent thymic emigrants, due to the high apoptotic rates of naïve T cells. Recent thymic emigrants are known to preferentially migrate to the intestines [238]; perhaps this innate property of recent thymic emigrant migration may therefore target the intestines for the development of autoimmune disease.

Not only does the loss of *Apc* result in a more pro-inflammatory T cell subsets, but also T cells that homeostatically proliferate more efficiently, thus increasing the risk of developing autoimmune disease. To verify HP as a driver of autoimmune disease in cKO mice, we created bone marrow chimeras to introduce cKO T cells in a

lymphoreplete environment. Though cKO T cells continue to be highly activated in the chimera mice, no autoimmune disease has been observed. We also demonstrated that Wnt signaling is required for efficient HP, by using β-catenin-deficient T cells. Interestingly, though *cMyc* was shown to play a large role in causing naïve T cell death in cKO mice and is also a known driver of proliferation, here we showed that cMyc does not control homeostatic proliferation. The underlying mechanism by which Wnt drives homeostatic proliferation still remains unclear. Through this study, we have shown a more compelling link between lymphopenia-driven HP and autoimmune disease by generating a single T-cell intrinsic gene defect that sufficiently drives autoimmune disease.

Previous studies have suggested a genetic and epigenetic association between Crohn's disease and components of Wnt signaling, including LRP6 [164] and *TCF4* [163, 239]. Most studies on Wnt signaling in IBD have focused on Wnt function in tissue remodeling partly because *TCF4* is expressed at high levels in the intestinal epithelial cells. Since the IBD described herein is initiated by *Apc* deletion in T cell lineages, our data suggest a direct role for Wnt signaling in T cells as an alternative interpretation of these genetic data.

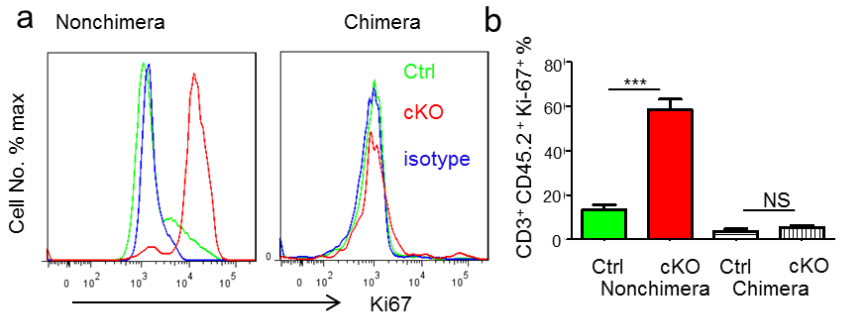


Figure 4.1

Fig 4.1 Enhanced HP APC cKO mice. a. Representative profiles of Ki-67 expression in $CD3^+$ T cells in peripheral blood of ctrl or cKO (left) or bone marrow chimera consisting of both WT and cKO T cells (right panel) . b. Summarized % of Ki-67 positive cells. n=4. These data have been reproduced in 3 independent experiments.

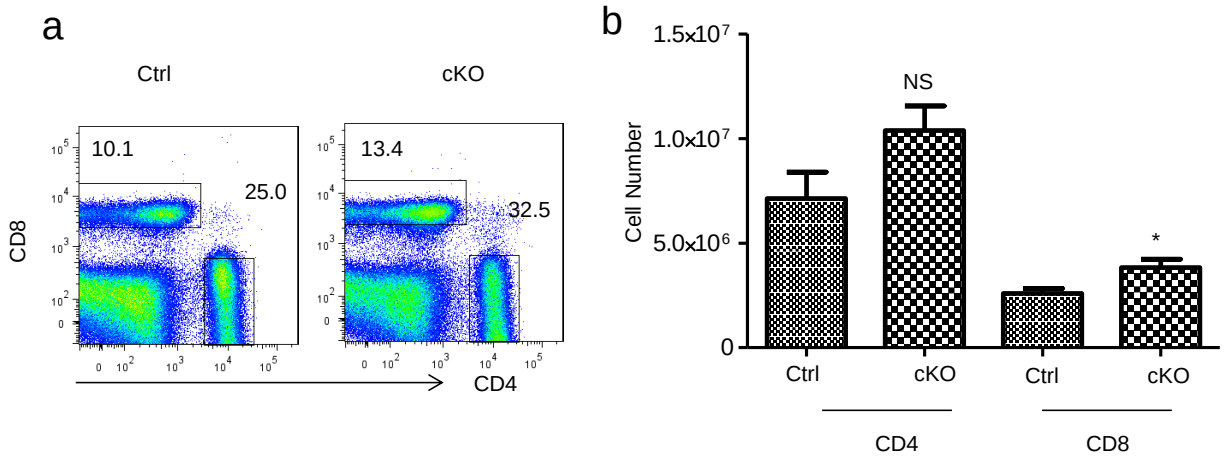


Fig. 4.2. Normal cellularity of recipient-type T cells in the chimera mice. Bone marrow from the CD45.2$^+$ cKO and Ctrl donors were mixed at a 1:1 ratio with CD45.1$^+$ recipient-type bone marrow cells (total bone marrow cell number: 5x10^6/mouse). The mixtures were injected into lethally irradiated CD45.1$^+$ hosts. Mice were sacrificed at 12 weeks post-transplantation to analyze reconstitution in the spleen. Representative profile of gated CD45.1$^+$ cells are shown in the left and on the right are absolute numbers of CD45.1$^+$ recipient-type splenic T cells in chimera mice consists of either Ctrl or cKO donot-type bone marrow cells. n=5.

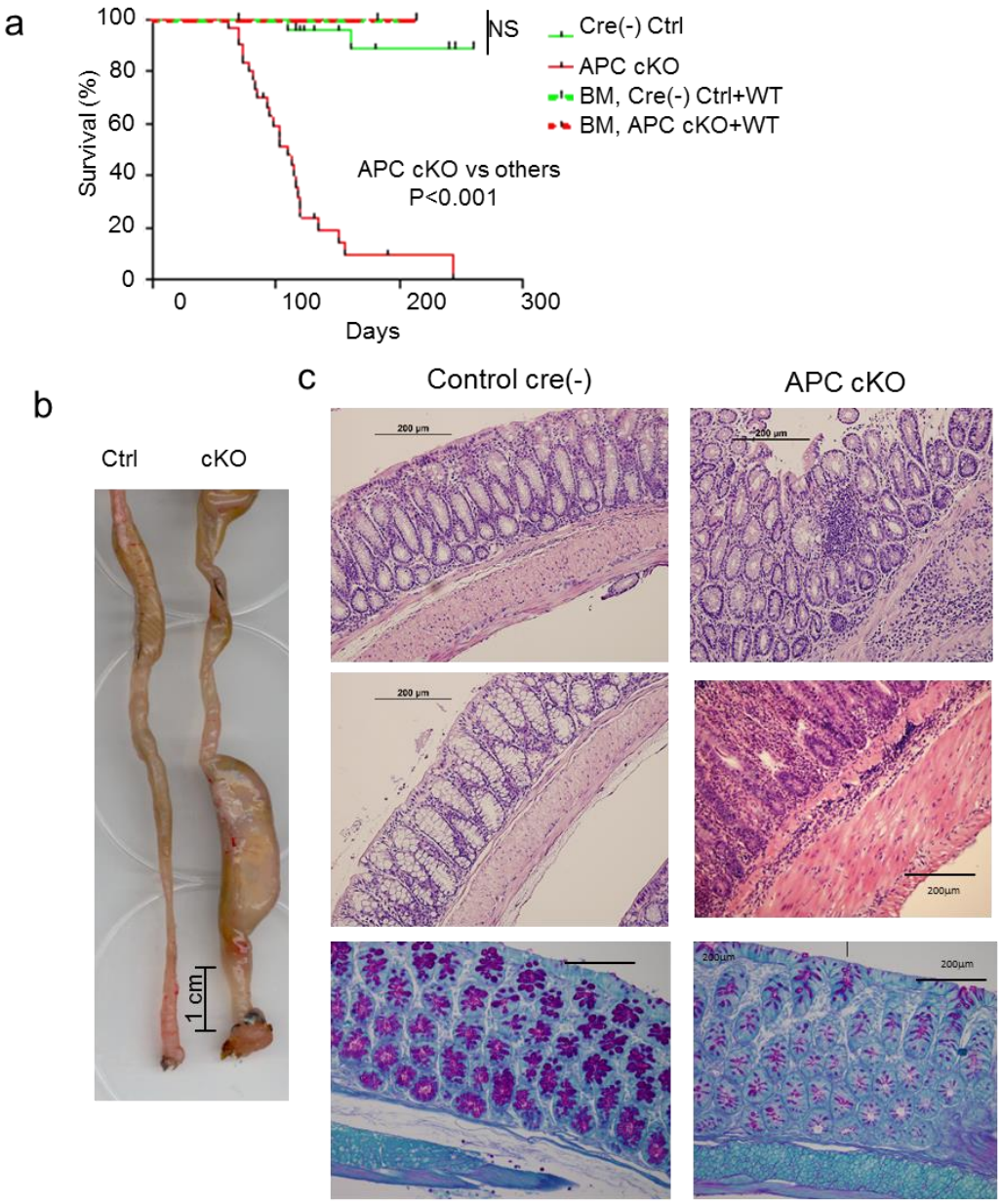

a
100
80
60
40
20
0
Survival (%)
0
100
200
300
Days
NS
Cre(-) Ctrl
APC cKO
BM, Cre(-) Ctrl+WT
BM, APC cKO+WT
APC cKO vs others
P<0.001
b
Ctrl
cKO
1 cm
c
Control cre(-)
APC cKO
200 µm
200µm

Figure 4.3

Figure 4.3. T-lineage-specific deletion of Apc causes IBD with Crohn's disease-like pathology: association with lymphopenia.a. Kaplan Meier survival curve of ctrl (green), lymphopenic cKO (red), ctrl bone marrow chimera (green dotted) or non-lymphopenic cKO bone marrow chimeras (red dotted) mice. Mice were determined moribund by drastic weight loss, rectal bleed, or bloody diarrhea. b. Gross anatomy images showing the swelling of the colon in cKO mice. c. representative H&E stains of intestines of ctrl or cKO intestines. Comparison of the histological features from the intestines of Ctrl and cKO mice. Note microcyst formation and inflammation in smooth muscle layer in the small intestine of cKO but not Ctrl (top) and inflammation to and expansion of the muscle layer in colon (middle panel) of cKO but not Ctrl mice and significant thickening of the colonic muscular layer (middle panels) and reduced production of mucin in the colon (bottom panels).

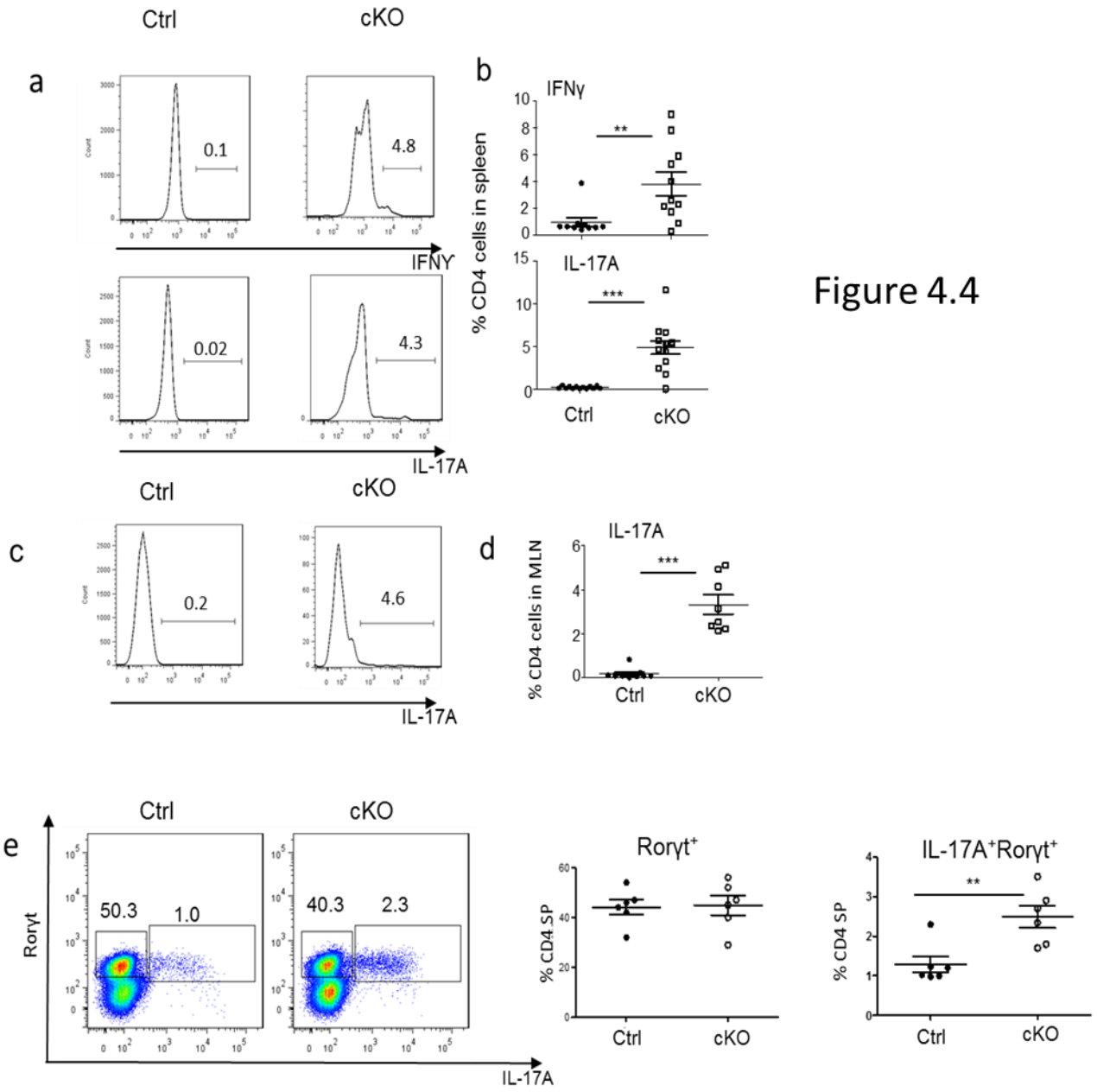

Ctrl
cKO
a
0.1
4.8
IFNY
0.02
4.3
IL-17A
b
IFNγ
% CD4 cells in spleen
**
IL-17A
***
Ctrl
cKO
c
0.2
4.6
IL-17A
d
IL-17A
% CD4 cells in MLN
***
Ctrl
cKO
e
Ctrl
cKO
Rorγt
50.3
1.0
40.3
2.3
IL-17A
Rorγt+
% CD4 SP
Ctrl
cKO
IL-17A+Rorγt+
**
% CD4 SP
Ctrl
cKO

Figure 4.4

Figure 4.4.

Apc deletion promote development of Th1 and Th17 cells

a-b. Apc deletion enhanced accumulation of Th1 and Th17 cells in the spleens, Splenocytes from WT and cKO mice were stimulated with PMA and ionomycin for 6 hours prior to intracellular staining with anti-IFNγ and IL-17A mAbs. Representative profiles were shown in a, while the summary data are shown in b. n=9. c-d. IL-17A production in MLN of ctrl or cKO; n=9. e. CD4 SP thymocytes were polarized in Th17 polarizing medium for 5 days and stained for Rorγt. Representative profiles are shown in the left while summary data involving 6 mice per group were shown in the right panels.

Figure 4.5

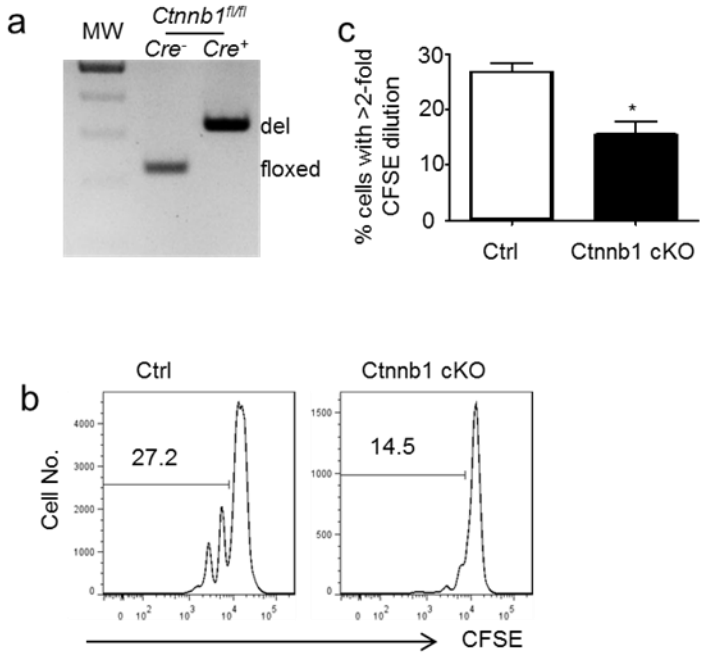


Figure 4.5. Role for β-catenin in HP of Apc-deficient T cells. a. Efficacy of *Ctnnb1* deletion in T cells. Genomic DNA was isolated from the purified splenic T cells from either *Cre*$^{-}$ or *Cre*$^{+}$ littermates of the *Ctnnb1*$^{fl/fl}$ mice. Genotype was performed with a set of three primers that give distinct sizes for the Floxed (fl) and deleted (del) allele. b and c. Defective homeostatic proliferation of *Ctnnb1*$^{-/-}$ T cells in a lymphopenic host. b. Representative profiles of WT and *Ctnnb1*$^{-/-}$ T cells. c. Summary of the data from b with 3 mice per group. These data have been reproduced twice.

Figure 4.6

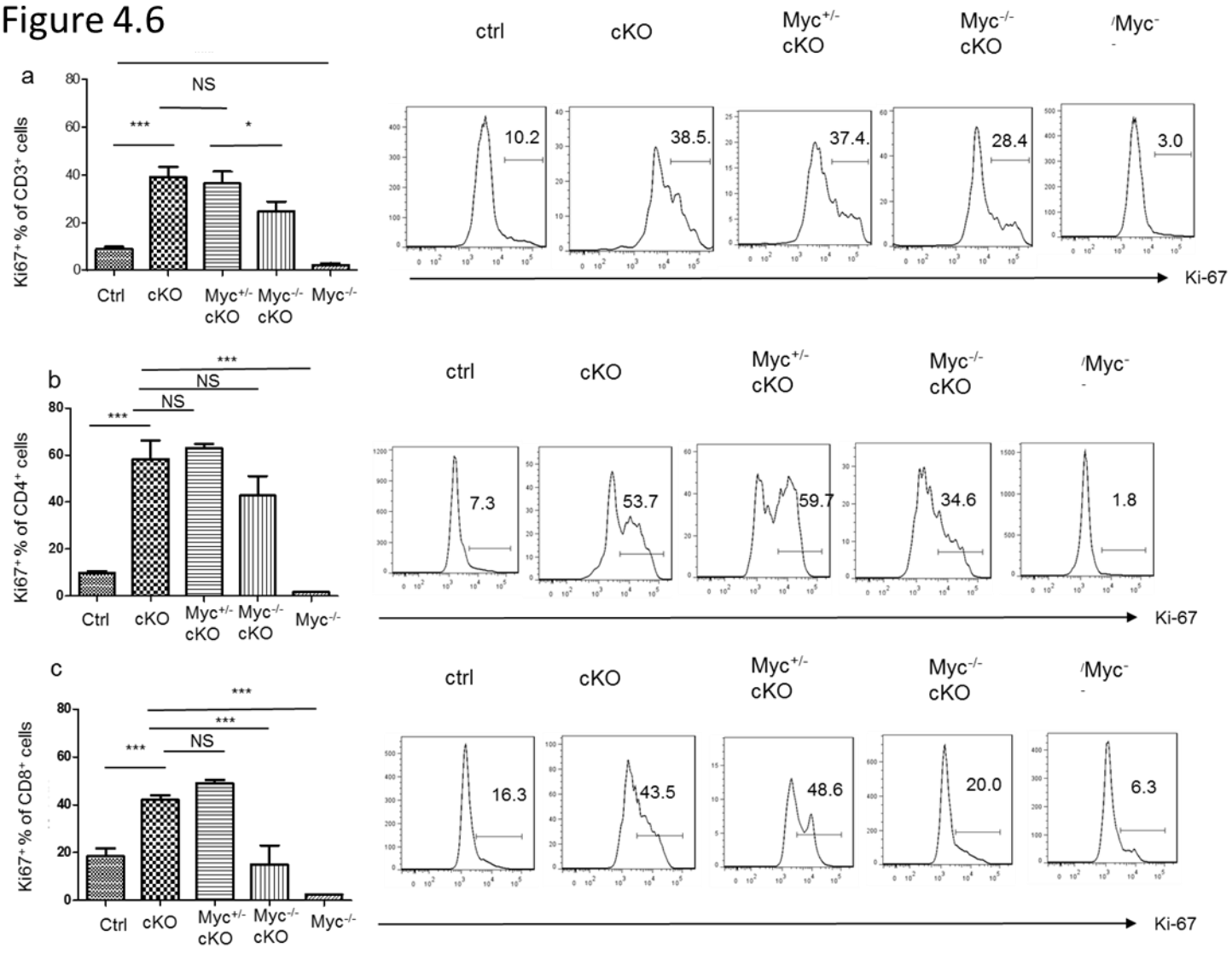

a
b
c
ctrl
cKO
Myc+/- cKO
Myc-/- cKO
-/Myc-
Ki67+ % of CD3+ cells
Ki67+ % of CD4+ cells
Ki67+ % of CD8+ cells
Ctrl
cKO
Myc+/- cKO
Myc-/- cKO
Myc-/-
NS
***
*
10.2
38.5.
37.4.
28.4
3.0
7.3
53.7
59.7
34.6
1.8
16.3
43.5
48.6
20.0
6.3
Ki-67

Fig 4.6. *cMyc* activation does not contribute to homeostatic proliferation of Apc-deficient T cells. (a-c) Peripheral blood from ctrl (n=9), cKO (n=9), $Myc^{+/-}$ cKO (n=9), or $Myc^{+/-}$ cKO (n=6) were collected from 6-8 week old mice. Red blood cells were lysed. Cells were stained for surface markers: CD3, CD4, and CD8, then permealbilized and stained for Ki-67. Levels of Ki-67 on $CD3^+$ T cells (a) $CD3^+$ $CD4^+$ T cells (b) and $CD3^+CD8^+$ T cells (c) Representative profiles are shown in the left and summary data are presented in the right panels.

Figure 4.7

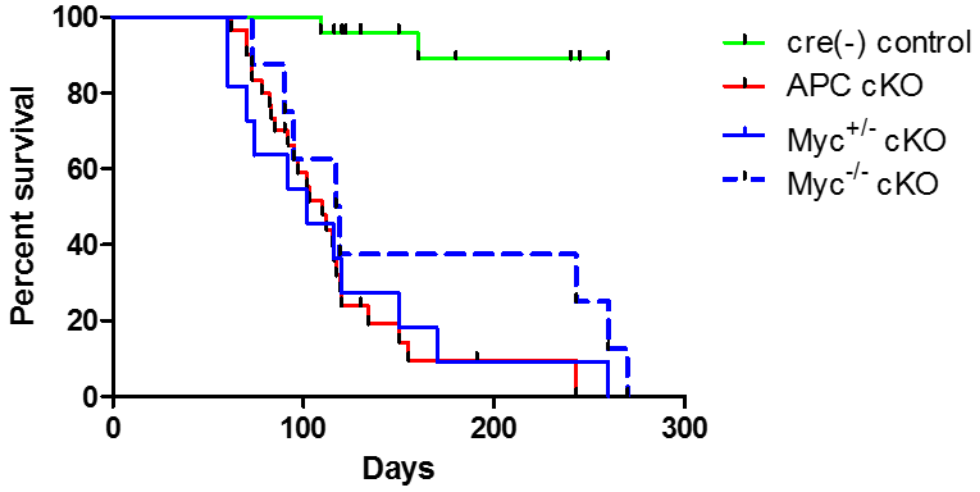


Figure 4.7 cMyc deletion is insufficient to prevent autoimmune disease in cKO mice. Survival curve of ctrl, cKO, *Myc*$^{+/-}$ cKO, and *Myc*$^{-/-}$ cKO. Mice were sacrificed when determined moribund by: greater than 20% weight loss and bloody diarrhea.

Chapter 5

Concluding Remarks

Wnt signaling regulates many aspects of T cell development, from early thymocyte development to peripheral T cell differentiation and maintenance. However, conflicting studies using β-catenin deficient thymocytes challenges the exact functional role Wnt plays in thymocyte development. In chapter 2, we sought to readdress the role of Wnt in thymocyte development by deleting the regulator of Wnt, *Apc*. We show that without *Apc*, thymocyte development occurs fairly normally. We did detect increased SP thymocyte death, but it was not significantly sufficient to alter SP percentages. Any aberrations to thymocyte development could only be seen in the context of a competitive reconstitution, as seen in chapter 3.

This modest difference is a stark contrast to a previous study using a deletion of *Apc,* but with Lck-cre. To verify that the differences observed were not the result of differently timed cre promoters, we crossed our *Apc* deletion to Lck-cre. Unlike the previous study which saw a large developmental blockage at the DN3 to DN4 transition, we only saw a small decrease of SP thymocytes and age-dependent thymic atrophy. This difference may be due to the differences in loxP sites, where the resulting protein in our study was slightly larger than what was previously use. It is also possible that in our Lck-cre

system, *Apc* was not deleted as efficiently or as early as the previous study, for we have yet to test the deletion efficient or timing of our Lck-cre system.

As previously described, deletion of *Apc,* under the control of Lck-cre, enhanced thymic involution [119]. It is possible that β-catenin accumulation enhances thymic involution by mimicking the effects of chronic TCR signaling, though the involvement of Wnt signaling is still unknown. Thymic involution, in humans, occurs due to aging. It would be of interest to determine whether aged mice have an accumulation of β-catenin in the DN compartments, mimicking the accumulation of β-catenin via Lck-cre controlled deletion of *Apc*.

Because *Apc* also is involved in many Wnt-independent signaling pathways, it is important to determine whether it is the activation of Wnt that affects thymocyte development. Using an exon 3 deletion of β-catenin, where the β-catenin is constitutively active, we can verify if Wnt activation will be a phenocopy of our cKO T cells. It is possible that many of the phenotypes seen in cKO mice is the result of Wnt-independent roles of *Apc.* Future investigations should focus on the roles of *Apc* that are Wnt-independent, such the role of *Apc* in mitosis (chromosomal segregation and cytokinesis), cell migration, DNA repair, and cell cycle control. Further studies are needed to determine whether the observed phenotypes are all Wnt-dependent.

The rather normal thymocyte development in cKO mice gave us a rare opportunity to study the role of *Apc* in naïve T cells. In chapter 3 we reported that *Apc* deficiency causes a massive loss of peripheral naïve T cells. Though Wnt signaling has been extensively studied in thymocyte development and mature effector T cells, the role of

Wnt signaling in naïve T cells has not yet been recognized. Preliminary evidence have shown that Wnt signaling regulation is vital to naïve T cells. Resting human T cells has higher levels of both LEF and TCF-1, however, both isoforms expressed are inhibitory isoforms [240]. In our study, the ectopic activation of Wnt caused the overexpression of *cMyc,* and T cell loss can be alleviated by reducing *cMyc* expression. Though the reduction of *cMyc* in cKO mice does attenuate cell death, naïve T cell numbers are not entirely rescued, suggesting that there are other pathways involved in loss of naïve T cells.

Therefore, we investigated whether the loss of *Apc* could result in increased mTOR signaling. We detected higher mTOR signaling in cKO T cells, though it still remains to be determined the extent mTOR signaling may play in cKO T cell apoptosis. Perhaps most surprising, treatment of rapamycin of cKO mice resulted in a cytokine storm that caused the eventual death in over 60% of the cKO mice. Rapamycin, a known inhibitor of mTOR, is known as an immunosuppressant, used in prevention of transplantation rejection by enhancing Treg expansion by inhibiting the expression of other T effector cell types [241-243], diminishing the co-stimulatory abilities of dendritic cells [244, 245], and stimulating the release of anti-inflammatory cytokines [197, 246]. However, when treated in conjunction with LPS or other TLR agonists, the presence of rapamycin incites the production of pro-inflammatory cytokines in dendritic cells [197]. This can also be extended into T cells: perhaps, in pro-inflammatory conditions (highly activated IFNγ and IL17A-producting cKO T cells), mTOR acts as a negative regulator of the production of pro-inflammatory cytokines, TNFα.

Future investigations are needed to determine the mechanism by which rapamycin treatment induces this production of TNFα in cKO mice. Firstly, it is important to determine whether it is the specific inhibition of mTOR that causes pro-inflammatory cytokine release. Rapamycin is a pharmaceutical inhibitor that could also affect other cellular pathways, therefore, it is important to determine whether rapamycin is not causing off-target effects. We need to treat both control and cKO mice with other inhibitors of mTOR (torin1/2, MK-866, or RAD001) to verify the inhibition of mTOR signaling is the cause of the cytokine storm in cKO mice. Secondly, we need to confirm whether the cause of the cytokine storm is cell intrinsic or cell extrinsic (Tregs or innate APCs). Though it is well-established that rapamycin promotes the expansion of Tregs [241], it is conceivable that inhibition of mTOR signaling in cKO T cells may be affecting Treg suppression by affect Treg survival or function (inhibition of IL-10). Therefore, it could be a dysfunction of Tregs that allows for the uninhibited production of pro-inflammatory cytokines. Previously, it has been reported that under conditions of inflammation, inhibition of mTOR promotes IL-12 and prevents the production of IL-10 in monocytes. Possibly, the treatment of rapamycin affects monocytes, which then in turn further activates cKO T cells. Finally, it is also conceivable that the cytokine storm is a cell intrinsic effect that causes cKO T cells to become even more activated. Rapamycin treatment of LPS treated mice caused increased STAT3 signaling [197]. Perhaps increased STAT3 signaling in T cells could be driving the polarization to pathogenic Th17 cells in our *Apc*-deficient, rapamycin-treated T cells. Another possible pathway that could be the cause of pro-inflammatory cytokines could be the result of a downstream target of mTOR, hypoxia inducible transcription factor (HIF-1α) [247, 248].

It has been shown that HIF-1α deficient T cells causes increased production of pro-inflammatory cytokines in the context of an *in vivo* model of bacterial sepsis [249]. By inhibiting mTOR signaling, HIF-1α may also be reduced, thus resulting in increased pro-inflammatory cytokine production. Therefore, further studies are necessary to determine if the inhibition of mTOR is the cause of the cytokine storm and whether this production of pro-inflammatory is cell intrinsic or cell extrinsic programming.

Due to the fact that deletion of cMyc did not fully rescue naïve T cell apoptosis and the observation that both the intrinsic and extrinsic pathways of apoptosis are involved, we sought to look for other pathways involved in naïve T cell apoptosis. Another possible mechanism involved in the survival defect of the naïve cKO T cells is the p53 pathway. p53 upregulation is the result of cellular stress, including: genotoxicity [250-254], cytokines [250, 255], hypoxia [256, 257], activated oncogenes [258], growth factors [259-261], and metabolic changes [193, 262]. We determined that p53 is upregulated in cKO T cells. It is important to define the mechanism by which p53 is upregulated. A deletion of *Apc* may cause chromosomal instability, thus causing the upregulation of p53 [58, 71]. It is also possible that the deletion of *Apc* causes the accumulation of β-catenin, causing oncogene-induced p53 expression. It has been previously shown that β-catenin accumulation can activate ARF, which in turn, will cause p53 expression [263]. In addition, constitutive mTOR activation has been shown to activate p53 [193, 262]. It will be of interest to determine whether in vitro rapamycin treated cKO T cells will affect p53 expression. cMyc has also been implicated as a requirement in p53-induced apoptosis due to DNA damage [198]. It still remains to be determined whether cKO cMyc$^{-/-}$ T cell apoptosis involves p53.

To improve our investigation of the role of *Apc* in naïve T cells, we need to verify that these subtle aberrations in bone marrow chimera SP thymocyte development did not affect the phenotypes we observed in naïve T cells. In this study, we focused on the role of *Apc* in Wnt, but *Apc* plays many other roles, including chromosomal stability. By deleting *Apc* at a much earlier time point, we could be causing genomic instability that may also be contributing to cell death in naïve T cell. Changing to a different cre, distal lck-cre, may also help elucidate the role of *Apc* in naïve T cells. Distal Lck-cre deletion begins much later than CD4-cre. CD4-cre is already active during the neonatal lymphopenic period, while dLck-cre occurs slowly in the peripheral T cells. By altering the cre promoter, we will be able to better examine the role of *Apc,* specifically in naïve T cells.

Finally, in chapter 4, we investigated the role of *Apc* lymphopenia-driven homeostatic proliferation and autoimmune disease. We showed that the loss of *Apc* and the resulting lymphopenia instigated a Crohn's disease-like autoimmune disease. This study is the first to show a single gene deletion having the ability to sufficiently initiate autoimmune disease in a context of lymphopenia due to the highly activated nature of cKO T cells, but also the hyperproliferation capabilities of activated Wnt T cells. Factors that can affect optimal homeostatic proliferation include the influence of extrinsic factors (cytokines, commensal bacteria, and T regulator cells) and also intrinsic factors (TCR stimulation and co-stimulator molecules); further studies are necessary to determine the extent each of these factors may contribute to the development of autoimmune disease seen in cKO mice. Wnt signaling may be important for T regulatory cell function and/or survival [120-122]. It is possible that cKO T regulatory cells may be unable to suppress

the homeostatic proliferation of cKO T cells, allowing for the development of disease, rather than an enhanced ability to homeostatically proliferate. Using naïve T cell transfer to *Rag*1$^{-/-}$ mice, we plan to clarify the function of T regulatory cells in causing autoimmune disease in cKO mice. Commensal bacteria are another factor that can influence homeostatic proliferation because these bacteria provide inflammatory signals and antigens that are required for the development of autoimmunity in the context of lymphopenic environments. Antibiotic treatment of recipient Rag1$^{-/-}$ before transfer of naïve T cells will verify the involvement of commensal bacteria in driving homeostatic proliferation-driven lymphopenia in cKO mice. Finally, to delineate the degree TCR stimulation plays a role in the hyperproliferation of cKO T cells, using OT-I cKO, we could determine the proliferation to either a weak or strong TCR stimulation. Also breeding cKO mice to an OT-1 background would allow us to investigate whether this hyperproliferation requires TCR/MHC interaction to proliferative. Detection of CD5 on cKO T cells may also determine the levels of TCR stimulation. Mechanistically, these studies will assist our understanding of Wnt signaling post-TCR stimulation. TCR stimulation is often followed by the accumulation/stabilization of β-catenin [104], however the physiological significance is still unclear. Perhaps, cKO T cells are hyperproliferative because of the higher levels of β-catenin causing these T cells to have hypersensitive TCRs.

The etiology of Crohn's disease is still unknown, however, recent human studies have shown a link between the incidence of Crohn's disease and a reduction of diversity of the patients' microbiota [264, 265]. It is still unknown what causes this shift in microbiota, whether it is an ineffective immune system that cannot clear an infection or if

it is the host's immune cells that drives the colonization of more pathogenic bacteria. It would be of interest to determine whether if the cytokine milieu produced by *Apc* deficient T cells is sufficiently alters the microbiota, which then instigates the development of the Crohn's like disease in cKO mice. We have shown the *Apc* deficient T cells produce more IFNγ and IL-17A, which could possibly affect the homeostasis of commensal bacteria, allowing for the colonization of more pathogenic bacteria leading to a severe pro-inflammatory response driving the development of autoimmunity. It is also possible that *Apc* deficient T cells are ineffective at clearing infections, which then allow for the colonization of pathogenic bacteria. Either hypothesizes provide an interesting insight in how the microbiota and T cells may both play a role in the development of Crohn's disease.

This study has shown that *Apc* is not essential in later stages of thymocyte development nor thymic emigration. Instead we found that regulation of *Apc* is essential in the maintenance of naïve T cells. Our insight into the intracellular programs, which regulates the maintenance of a peripheral pool of incredibility diverse repertoire of naïve T cells, plays an essential role in our understanding of how the adaptive immune system responses to an assortment of foreign antigens. By retaining these naïve T cells in a quiescence state, these intrinsic programs allow for the suppression of the development of autoimmune disease by restraining the acquisition effector traits. Previously, the mTOR pathway has been shown to play a vital role in holding naïve T cell quiescence [97, 98]. Rather, the survival of naïve T cells require *Apc* to regulate Wnt signaling in order to maintain both low levels of cMyc and mTOR signaling. By deleting *Apc*, we also show that Wnt signaling is necessary for an optimal homeostatic proliferation.

Constitutively activating Wnt results in a highly inflammatory cKO T cell that leads the development of a Crohn's disease-like autoimmune disease.

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