THE ROLE OF NDFIP1 IN T CELL TOLERANCE

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ABSTRACT

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Without the ability to suppress its responses, the immune system, instead of being advantageous to the individual, would elicit deleterious consequences. Thus, mechanisms to contain the activity of immune cells are necessary to prevent immune responses against non-pathogenic antigens and the severe immunopathology that would otherwise result. In T cells, these mechanisms are termed T cell tolerance. T cell tolerance is defined as the collection of T cell intrinsic and extrinsic processes that suppress T cell responses to these non-pathogenic antigens. A breach in T cell tolerance could have fatal results, as evidenced in several autoimmune syndromes. Many of these mechanisms of tolerance occur during T cell development (central T cell tolerance), whereas, others exist in mature T cells (peripheral T cell tolerance). This thesis is focused on the involvement of the adaptor protein Ndfip1 in mechanisms of peripheral T cell tolerance.

Ndfip1 is an adaptor for E3 ubiquitin ligases of the Nedd4 family. In Th2 cells, Ndfip1 is necessary for the degradation of JunB by the Nedd4 family E3 ligase Itch. Consequently, Ndfip1 limits IL-4 expression in Th2 cells. Here we present

evidence that Ndfip1 also limits IL-4 expression during the differentiation of inducible Tregs (iTregs), promoting their suppressive program. Thus, by promoting iTreg differentiation, Ndfip1 enforces the proper balance between effector and tolerogenic T cell responses. Ndfip1 also promotes T cell tolerance by regulating T cell activation. Ndfip1 constrains T cell activation by limiting IL-2 mRNA expression. Ndfip1 is part of a negative regulatory system in which factors that induce IL-2 expression downstream of T cell receptor engagement also induce the expression of Ndfip1 to limit the extent of IL-2 production and, thus, control T cell activation. By regulating these two processes, Ndfip1 favors peripheral T cell tolerance and prevents the development of a T cell mediated fatal inflammatory disease.

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CHAPTER 1

Introduction

DYSFUNCTIONAL IMMUNE RESPONSES AND DISEASE

While the purpose of the immune system is to protect the body against infections, aberrations of the immune response that enhance its aggressiveness can also be life-threatening. Autoimmune diseases and allergies occur due to imbalances in immune responses. The prevalence of these conditions makes them a global health concern. In the United States alone, up to eight percent of the population suffers autoimmune disease. It is the third major cause of death in the country, following heart disease and cancer (National Institutes of Health, 2005). Moreover, an estimate of thirty-to-forty percent of the population worldwide is affected by at least one allergic condition (Pawankar et al., 2012). The incidence of both autoimmune conditions and allergies is expected to increase. It is essential to understand what induces the development of these ailments in order to better treat and/or prevent them.

T CELL TOLERANCE

T cells develop in the thymus and then migrate into peripheral lymphoid compartments. In the periphery, a T cell's chief task is to respond to foreign pathogenic antigens while maintaining unresponsiveness to self and harmless environmental and microbial antigens. By definition, T cells attacking self-antigens can cause autoimmune disease while those attacking environmental antigens can induce allergies. The mechanisms that prevent T cells from attacking these harmless antigens are termed T cell tolerance. In some cases, a

break in T cell tolerance can lead to minor pathological consequences. However, in some circumstances, uncontrolled T cell responses can induce severe disease and, even, death (Sakaguchi et al., 1995). T cell tolerance controls the pathophysiology of autoimmune diseases and prevents allergic diseases through various mechanisms detailed below.

Central T cell Tolerance

The thymus, the site of T cell development, provides the first barrier against unwanted T cell responses. In the thymus, T cell-precursors, or thymocytes, develop T cell receptors (TCRs) with mostly random specificity (Davis, 1990). Thus, a screening process is needed to discriminate against (reword and include a statement for what happens with nTregs) and eliminate thymocytes with TCR specificity against self-antigens. In this process, antigen-presenting cells (APCs), such as thymic epithelial cells (TECs), present self-antigens to developing thymocytes (Derbinski et al., 2001). Thymocytes that interact strongly with these self-antigens do not join the mature T cell repertoire (Kappler et al., 1987). Instead, self-reactive thymocytes are deleted, anergized or undergo receptor editing, (Kappler et al., 1987; McGargill et al., 2000; Ramsdell et al., 1989). The importance of these processes is demonstrated by the fact that the inability to present self-antigens in the thymus, and the subsequent presence of self-reactive T cells in the periphery, results in autoimmune disease (Anderson et al., 2002; Liston et al., 2004). As the elimination of self-reactive TCRs occurs in this

central site of T cell development, this mechanism is termed central T cell tolerance.

Peripheral T cell Tolerance

Central T cell tolerance is not sufficient to eliminate all T cell responses to self-antigens. Some self-reactive T cells escape tolerance mechanisms in the thymus (Liu et al., 1995; Zehn and Bevan, 2006). In part, this is because developing T cells are not exposed to full repertoire of self-peptides present in the periphery (Bouneaud et al., 2000; Kyewski and Klein, 2006). Thus, additional means of immune regulation of T cells are needed in the periphery to prevent responses against self. The control of T cell responses outside of the thymus is also required for other reasons: to control the immune-mediated damage to healthy tissue after infection (Powrie et al., 2003) and to prevent responses against harmless extraneous antigens (Ling et al., 2004; Tiemessen et al., 2004; Verhagen et al., 2006). All together, these mechanisms are referred to as peripheral T cell tolerance.

Regulatory T cells

Regulatory T cells are crucial mediators of peripheral T cell tolerance. Contrary to conventional T cells (Tconv), which promote pathogen clearance and inflammation, regulatory T cells suppress immune activity. Thus, they maintain

immune homeostasis and prevent unwanted responses. Different types of regulatory T cells have been characterized. The best-defined ones are the type 1 regulatory T cells (Tr1) and those that express the forkhead box P3 transcription factor, or Foxp3$^+$ Tregs.

Foxp3$^+$ Tregs are required to prevent the development of fatal multi-organ inflammation in both mice and humans (Godfrey et al., 1994; Lyon et al., 1990; Wildin et al., 2001). These Tregs can control the function of immune cells by different means: suppressor cytokine production (TGF-β, IL-10), expression of CTLA-4, sequestration of IL2 and blocking antigen presentation to Tconv, among others (Asseman et al., 1999; de la Rosa et al., 2004; Huang et al., 2004; Paust et al., 2004; Powrie et al., 1996). The list of mechanisms by which Foxp3$^+$ Tregs suppress T cell responses keeps expanding.

Foxp3$^+$ Tregs can develop in the thymus or in the periphery. Foxp3$^+$ Tregs that develop in the thymus are referred to as natural Tregs, or nTregs, and those generated outside of the thymus are termed inducible Tregs, or iTregs (Apostolou et al., 2002; Apostolou and von Boehmer, 2004; Chen et al., 2003; Thorstenson and Khoruts, 2001). Evidence suggests that while the naïve T cell pool in the periphery is mostly depleted of self-reactive TCRs, nTregs possess TCR specificity to self-antigens (Itoh et al., 1999; Jordan et al., 2001). iTregs, on the other hand, having come from naïve T cells, have TCR specificity against

non-self antigens or self-antigens that are not effective at deleting T cell precursors. Thus, the combination of nTregs and iTregs helps assure a more effective means of preventing autoimmune responses and allergies, respectively.

The dissimilar conditions for the development of nTregs and iTregs are likely a clever way the immune system has developed to respond against what is endogenous and what is foreign. The suppression of immune responses against self-antigens is mandatory to health. Thus, nTregs, which suppress immune activity against self, are functionally competent as they exit the thymus. Yet, our bodies do not know beforehand whether to respond or not to the extensive amounts of foreign antigens they are exposed to. Thus, particular cytokines determine whether naïve T cells become iTregs or not. This is likely dependent on the pathogenicity of extraneous antigens. The minimal requirements for iTreg differentiation *in vitro* are IL-2 and TGF-β (Chen et al., 2003; Cobbold et al., 2004; Mucida et al., 2005; Zheng et al., 2007). In addition to these, retinoic acid (RA) helps promote iTreg differentiation *in vivo (Benson et al., 2007)*. Conversely, inflammatory signals, such as those from IL-6 and IL-4, can prevent the generation of inducible Tregs (Dardalhon et al., 2008; Dominitzki et al., 2007). Therefore, signals that indicate an antigen's pathogenicity, or lack thereof, determine the differentiation program of the responding naïve T cells.

Inducible Tregs are not the only type of regulatory T cells that arise in the periphery. Just like $Foxp3^+$ iTregs, Tr1 cells are regulatory T cells that develop

from mature naïve T cells (Groux et al., 1997). They also use IL-10 to suppress immune responses (Vieira et al., 2004). However, the master transcription factor that modulates Tr1 differentiation and activity remains unknown (Groux et al., 1997; Pot et al., 2011). Tr1 cells are induced from naïve T cells in an IL-27 dependent manner, both *in vitro* and *in vivo (Wang et al., 2011; Wu et al., 2011)*. They counteract the development of autoimmune diseases such as colitis and experimental autoimmune encephalomyelitis (EAE) in mice (Barrat et al., 2002; Groux et al., 1997) and have been suggested to counteract the development of multiple sclerosis (MS) in humans (Sweeney et al., 2011; Zhang et al., 2009). In addition to playing a protective role against some autoimmune diseases, Tr1 cells have been linked to protection against allergies (Akdis et al., 2004; Cottrez et al., 2000). Thus, Tr1 cells are also important to achieve tolerance.

Activation-Induced Cell Death

T cell activity can be regulated through the induction of apoptosis. T cells can be instructed to apoptose through cell extrinsic signals, such as ligation of a death surface receptor, or cell intrinsic signals (Kischkel et al., 2000; Suda et al., 1993; Tartaglia et al., 1993). T cell deletion by apoptosis allows the contraction of activated T cells upon clearance of pathogen to maintain homeostasis (Strasser and Pellegrini, 2004). In central tolerance, it is the mechanism by which self-reactive thymocytes are eliminated (Bouillet et al., 2002; Lamhamedi-Cherradi et al., 2003). As these mechanisms of apoptosis are induced upon antigenic

stimulation, they are collectively referred to activation-induced cell death (AICD). AICD also holds the responsibility of helping conserve peripheral T cell tolerance.

AICD in immune tolerance is particularly relevant in preventing immune activity at sites of immune privilege. Sites of immune privilege are body tissues that are not routinely surveilled by immune cells as they are in tissues in which inflammation would cause severe detrimental consequences. These include the testes, the eyes and the brain (Green et al., 2003). To prevent an inflammatory scenario, which the body cannot afford, sites of immune privilege are equipped with ligands for death surface receptors (Griffith et al., 1995). This helps avoid autoreactivity of T cells with specificity at these sites. The need for AICD as a mechanism of tolerance is evidenced in patients with autoimmune lymphoproliferative syndrome (ALPS) (Siegel et al., 2000). ALPS patients develop severe autoimmunity due to defective lymphocyte apoptosis.

Two-signal model of T cell activation

Another approach to peripheral T cell tolerance is the intrinsic regulation of T cell activation. Antigen stimulation through the TCR induces T cell activation only when additional surface signals, or co-stimulatory signals, are received. These co-stimulatory signals usually come in the form of CD28 (Boise et al., 1995). Antigen presenting cells (APCs) cells provide co-stimulatory ligands to CD28 in the form of CD80 or CD86 (Linsley et al., 1991). These surface molecules are

upregulated in the presence of pathogen-associated molecular patterns (PAMPs) or inflammatory cytokines (Santini et al., 2000; Takeuchi and Akira, 2007), indicating the presence of pathogens. Instead, an antigen being presented in the absence of these signals is likely to belong to self or to be a harmless foreign antigen. Therefore, both *in vitro* and *in vivo*, TCR signaling in the absence of co-stimulatory signals renders T cells unresponsive (Jenkins and Schwartz, 1987; Quill and Schwartz, 1987). This co-stimulatory requirement for T cell activation is termed the two signal model of T cell activation (Lafferty and Woolnough, 1977). T cells that receive TCR stimulation (signal one) in the presence of CD28 co-stimulatory signals (signal two) produce IL2, elevate IL2R-α surface levels (part of the IL-2-receptor complex) and proliferate (Boise et al., 1995; Jenkins et al., 1991; Martin et al., 1986; Thompson et al., 1989). On the other hand, *in vitro* and *in vivo* systems of T cell activation have corroborated that T cells stimulated in the absence of CD28 co-stimulation are poor IL-2 producers, do not maintain elevated IL2R-α surface levels, proliferate only modestly and undergo apoptosis (Groux et al., 1992; Jenkins and Schwartz, 1987; Quill and Schwartz, 1987). Supporting an active regulatory mechanism, upon *in vitro* challenge with CD28 co-stimulation, these cells remain unable to make IL2. This inability to make IL-2 upon challenge, both in naïve or effector differentiated cells, is termed T cell anergy (Harding et al., 1992; Jenkins et al., 1991; Tan et al., 1993). The physiological relevance of T cell anergy remains controversial. However, it suggests that T cell unresponsiveness due to CD28-deficient TCR stimulation is

an active process that suppresses future T cell encounters with potentially harmless antigens.

While the factors that induce T cell activation in the presence of CD28 co-stimulatory signals are fairly well understood, those that prevent T cell activation when CD28 co-stimulation is absent are only now starting to be revealed. CD28 co-stimulation provides a quantitative enhancement of the signaling pathways that allow for factors such as IL2 and IL2R-α to be expressed. Conversely, these pathways are weakly recruited in the absence of CD28 co-stimulation (Smith-Garvin et al., 2009). Evidence suggests that many E3 ubiquitin ligases, responsible for protein ubiquitylation, actively suppress T cell activation in the absence of CD-28 co-stimulation. E3 ubiquitin ligases are also involved in many other processes that regulate peripheral T cell tolerance. In order to discuss these processes in detail it is first necessary to understand the protein ubiquitylation cascade.

PROTEIN UBIQUITYLATION

Ubiquitylation is a post-translational modification that occurs when a substrate is covalently conjugated with ubiquitin. Ubiquitin is conjugated into a substrate via an iso-peptide bond between a glycine at the C-terminus of ubiquitin and a lysine on the substrate (Hershko and Ciechanover, 1982).

Originally, protein ubiquitylation was identified based on its role in the degradation of substrates by the proteasome (Finley et al., 1994; Sadowski and Sarcevic, 2010). It is now known that ubiquitylation can produce many different outcomes depending on the nature and quantity of ubiquitin attachment. Substrates can be mono-, multi- or poly-ubiquitylated. Mono-ubiquitylation specifies the addition of a single ubiquitin moiety at a lysine residue in the substrate, while multi-ubiquitylation refers to the addition of a single ubiquitin at different lysine residues of the substrate. Furthermore, the addition of ubiquitin chains is termed poly-ubiquitylation. As with the bond formed between the substrate and ubiquitin, ubiquitin moieties are linked to each other via lysine residues. Ubiquitin contains seven lysine residues: K6, K11, K27, K29 and K33, K48 and K63. Thus, different types of ubiquitin chains can be formed. K48 chains initiate the degradation by the proteasome. The outcome of other types of chains is less clearly defined. In some cases, they can lead to changes as diverse as DNA damage repair, signal transduction and apoptosis (Deng et al., 2000; Galan and Haguenauer-Tsapis, 1997; Hofmann and Pickart, 1999; Jesenberger and Jentsch, 2002; Petroski and Deshaies, 2003; Schnell and Hicke, 2003; Spence et al., 2000). The formation of one type of chain over another can determine the fate of the substrate.

Protein ubiquitylation cascade

Ubiquitylation requires three main types of proteins: E1 activating enzymes, E2 conjugating enzymes and E3 ubiquitin ligases (Passmore and Barford, 2004). The ubiquitylation cascade is illustrated in Figure 1. E1 activating enzymes initiate the ubiquitylation cascade by activating ubiquitin and transferring it to the E2. The E2 binds to an E3 and, either directly or through the transient movement to the E3, ubiquitin is passed onto the substrate. The E3 ubiquitin ligases confer specificity to the reaction by selecting the target to be ubiquitylated.

Two types of E3 ubiquitin ligases exist, RING (Really Interesting New Gene) type and HECT (homologous to E6-AP carboxyl terminus) type ligases (Joazeiro and Weissman, 2000). RING type E3 ligases contain varying domains for substrate binding and possess a RING domain through which they bind to the E2 conjugating enzyme. RING-type E3 ligases work as scaffold proteins that facilitate substrate ubiquitylation. E3 ubiquitin ligases from the HECT-type family possess intrinsic catalytic activity. Thus, in addition to binding to both the E2 and the substrate, HECT-type E3 ligases directly pass ubiquitin to the substrate.

Ubiquitylation by E3 ubiquitin ligases is a specific and highly regulated method of post-translational protein modification. Members of both the RING and HECT type E3 ligases orchestrate some of the processes that make up peripheral T cell tolerance. They are active participants in the induction of regulatory T cells and the control of T cell activation.

E3 UBIQUITIN LIGASES IN PERIPHERAL T CELL TOLERANCE

Substrate ubiquitylation by several E3 ligases provides a means of inhibiting unwanted T cell responses towards self and harmless foreign antigens. As an example, the E3 ligases Cbl-b, Itch and GRAIL are involved in both the control of T cell activation and iTreg function. Corroborating this, T cells lacking any of these three E3 ligases are hyperresponsive to antigen stimulation. Additionally, mice lacking Cbl-b, Itch or GRAIL develop varying degrees of spontaneous inflammatory diseases (Bachmaier et al., 2000; Nurieva et al., 2010; Venuprasad et al., 2008). While the type and extent of disease varies among knockout mice, it is clear that proper control of E3 ligase function helps ensure peripheral tolerance.

GRAIL

GRAIL (Gene related to anergy in lymphocytes) is a RING type E3 ligase involved in T cell activation. GRAIL downregulates TCR surface expression and, thus, limits signaling upon antigen stimulation. T cells lacking GRAIL are hyperresponsive to TCR stimulation (Nurieva et al., 2010). In addition to this, GRAIL is also necessary for effective iTreg function. Regulatory T cells that lack GRAIL have reduced suppressive function. This is also due to poor TCR-downregulation upon signaling in the absence of GRAIL. Mice lacking GRAIL do not develop spontaneous autoimmunity. They do, however, possess

compromised tolerance to antigens presented through the oral route (Nurieva et al., 2010)

Cbl-b

Just like GRAIL, Cbl-b is a RING type E3 ligase. Cbl-b is associated to many processes that limit T cell activation. It ubiquitylates many pro-activation signals in both naïve and effector-differentiated T cells upon antigen stimulation. Some of its targets include PLC-γ, PKC-θ, p85 and Vav-1. Ubiquitylation by Cbl-b induces their degradation or uncouples their interaction with TCR and CD28 signaling pathways (Bachmaier et al., 2000; Fang and Liu, 2001; Heissmeyer et al., 2004; Jeon et al., 2004; Krawczyk et al., 2000). Unlike mice lacking GRAIL, Cbl-b-deficient mice develop spontaneous autoimmunity (Bachmaier et al., 2000). Cbl-b acts in many of its substrates in collaboration with the E3 ligase Itch.

Itch

The HECT type E3 ligase Itch, along with Cbl-b, induces the proteolysis of PLC-γ and PKC-θ in anergized Th1 T cells (Heissmeyer et al., 2004). In the absence of Itch, these cells are hyperactive. Itch is also required for the differentiation of inducible regulatory T cells (iTregs). This E3 ligase is dispensable for the generation of nTregs. Nevertheless, T cells lacking Itch convert poorly into iTregs

(Venuprasad et al., 2008). Interestingly, mice lacking Itch develop spontaneous atopic disease and not autoimmunity (Fang et al., 2002; Perry et al., 1998).

The three E3 ligases described, GRAIL, Cbl-b and Itch, regulate similar processes: iTreg induction and T cell activation. Additionally, Itch and Cbl-b share some functions in T cell activation (Heissmeyer et al., 2004). However, the very dissimilar phenotypes between mice lacking these E3s indicate that these enzymes do not compensate for each other. This exemplifies the need to better understand the complexity of programs that control peripheral T cell tolerance.

THE REGULATION OF ITCH ACTIVITY IN PERIPHERAL T CELL TOLERANCE BY NDFIP1

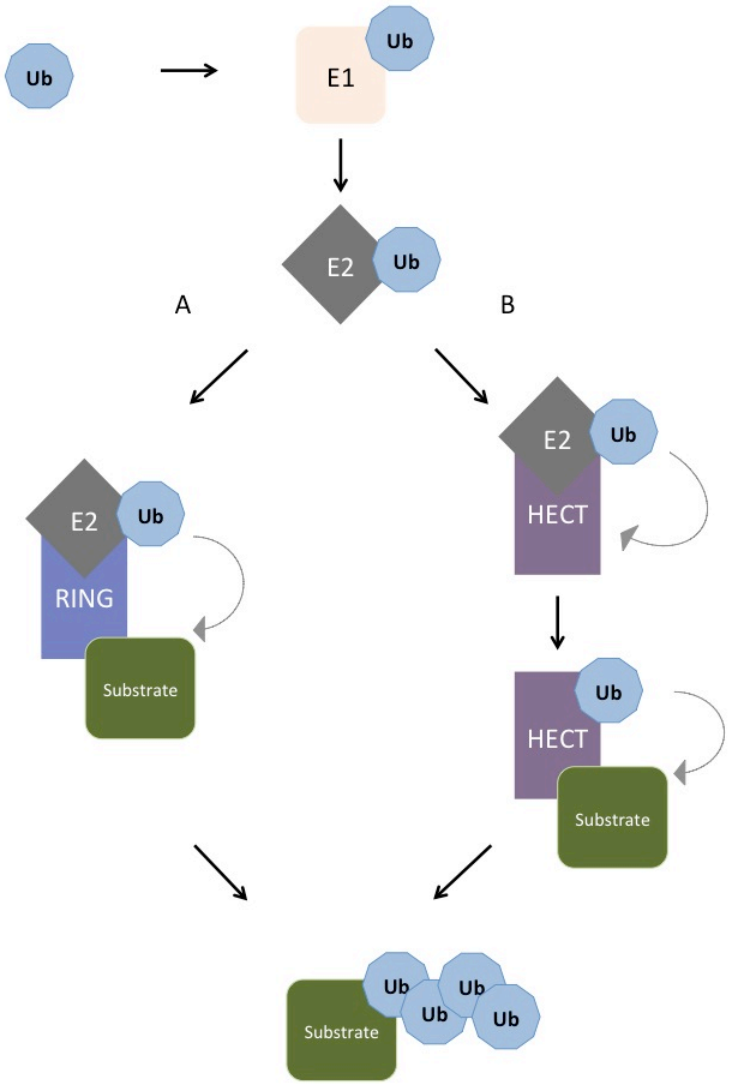
Understanding how the activity of E3 ligases is regulated could provide insight into their role in peripheral T cell tolerance. Studies suggest that the adaptor protein Ndfip1 (Nedd4 family-interacting protein 1) can control the function of the E3 ligase Itch in T cells. Ndfip1 is a 26kDa protein with three C-terminal transmembrane motifs as well as three N-terminal PY motifs; two PPXY- motifs and one LPXY- motif (Harvey et al., 2002). *In vitro*, Ndfip1 binds to the WW domains Itch and enhances its enzymatic activity (Harvey et al., 2002; Jolliffe, 2000; Mund and Pelham, 2009). The interaction between Ndfip1 and Itch *in vivo* is still under investigation. In effector T cells, Ndfip1 aids Itch in the ubiquitylation and degradation of Jun-B. Ndfip1-deficient T cells accumulate Jun-B and,

consequently, are more Th2-prone than their Ndfip1-sufficient counterparts (Oliver et al., 2006). These studies suggest that Ndfip1 works as an adaptor for Itch *in vivo*. It remains unknown whether Ndfip1 regulates other functions of Itch.

Mice that lack the adaptor protein Ndfip1 develop spontaneous inflammation of the skin, lungs and GI tract (Oliver et al., 2006; Ramon et al., 2011). The inflammation can be detected as early as 6 weeks of age. Ndfip1$^{-/-}$ mice die as early as 12 weeks of age. The disease is characterized by eosinophilic and lymphocytic infiltrates at sites of inflammation and goblet cell hyperplasia in the lungs (Oliver et al., 2006). At 4 weeks of age, and prior to the onset of inflammation, Ndfip1$^{-/-}$ mice present increased frequencies of antigen-experienced T cells in peripheral lymphoid organs. Moreover, Ndfip1$^{-/-}$ Rag1$^{-/-}$ mice, which lack a lymphoid compartment, do not present overt signs of inflammation at that same age (Ramon et al., 2011). The contribution of B cells to the phenotype remains to be assessed. However, this suggests the presence of a T cell intrinsic defect in Ndfip1$^{-/-}$ mice. Interestingly, Itchy-mutant mice develop a strikingly similar phenotype (Fang et al., 2002; Perry et al., 1998). This raises the question of whether Ndfip1, like Itch, functions in the maintenance of peripheral T cell tolerance.

This thesis explores the roles of Ndfip1 in peripheral T cell tolerance by supporting iTreg differentiation (Chapter 2) and limiting T cell activation (Chapter 3).

Figure 1



The ubiquitylation cascade Three proteins participate in the substrate ubiquitylation pathway: E1 activating enzymes, E3 conjugating enzymes and E3 ubiquitin ligases. Two types of E3 ubiquitin ligases exist. A. RING-type E3 ligases act as scaffold proteins that facilitate the movement of ubiquitin to the substrate by an E2 conjugating enzyme. B. HECT-type E3 ligases directly transfer ubiquitin to the substrate.

CHAPTER 2[1]

Ndfip1 controls IL-4 expression in inducible Tregs to allow Foxp3 expression

[1]Figures of this chapter are a modified version of the manuscript entitled "TGF-β induces the expression of the adaptor Ndfip1 to silence IL-4 production during iTreg cell differentiation", which was published in *Nature immunology* (2012) 13:77-85, with the following authors, Allison M Beal, Natalia Ramos-Hernández, Chris R Riling, Erin A Nowelsky and Paula M Oliver

Introduction

Regulatory T cells provide immune tolerance by suppressing the activity of other immune cells. They dampen immunopathology and prevent immune activity against self-antigens and harmless foreign antigens (Josefowicz et al., 2012a). Two main types of regulatory T cells expressing the forkhead box P3 (Foxp3) transcription factor have been characterized. Natural regulatory T cells (nTregs) are $Foxp3^+$ $CD4^+$ Tregs that develop in the thymus (Bonomo et al., 1995; Hori et al., 2003; Ohki et al., 1987; Saoudi et al., 1996). In addition, naïve $CD4^+$ T cells can differentiate into inducible Tregs (or iTregs) in peripheral lymphoid compartments under various circumstances (Chen et al., 2003; Curotto de Lafaille et al., 2004; Mucida et al., 2007; Sun et al., 2007). The need for Foxp3 expression in healthy immune function is evidenced in subjects with Foxp3 deficiency.

Both humans and mice with Foxp3 mutations develop a lethal multi-organ autoimmune disorder. Humans with Foxp3 mutations develop an Immunodysregulation Polyendocrinopathy Enteropathy X-Linked (IPEX) syndrome, whereas mice with Foxp3 mutations are termed scurfy mice (Godfrey et al., 1991; Wildin et al., 2001). Moreover, transfer of Tregs into Foxp3-deficient mice rescues the disease (Fontenot et al., 2003; Haribhai et al., 2011). These data stress the major task that $Foxp3^+$ regulatory T cells carry out to maintain immune tolerance.

nTregs and iTregs develop at different sites and through different mechanisms. TCR signaling strength towards self-antigens in the thymus determines Foxp3 expression in nTregs. Therefore, nTregs exit the thymus aware of their identity and function. This is not the case for iTregs. Naïve T cells in the periphery rely on environmental cues to become iTregs. Just as with effector T cell differentiation, the cytokine milieu determines whether a naïve T cell becomes an iTreg. Whereas certain cytokine signals promote iTreg differentiation, others prevent it and, instead, promote effector differentiation. This system helps channel naïve T cell resources towards the T cell responses as required.

The presence of both nTregs and iTregs in the immune system supports a division of labor to efficiently control immune responses. nTregs are "educated" to respond against self-antigens and suppress autoimmune reactions once outside the thymus. In contrast, iTregs develop from naïve T cells in the periphery. They can, potentially, suppress immune responses against self and non-self antigens. *Foxp3$^{\Delta CNS1}$ mice* have an iTreg deficiency and, yet, unaltered nTreg population. *Foxp3$^{\Delta CNS1}$ mice* do not show signs of autoimmunity. Instead, *Foxp3$^{\Delta CNS1}$* mice develop spontaneous Th2-mediated inflammation in the GI and respiratory tracts, sites of constant exposure to non-self antigens (Josefowicz et al., 2012b). This suggests that nTregs and iTregs possess specialized suppressor functions.

Mice that lack the Nedd4 family-interacting protein 1 (Ndfip1) develop a systemic inflammatory disease similar to the one that *Foxp3*$^{\Delta CNS1}$ mice develop. Inflammation in Ndfip1$^{-/-}$ mice is characterized by lymphadenopathy and splenomegaly as well as immune cell infiltrates in the skin, lungs and gastrointestinal tract (Oliver et al., 2006; Ramon et al., 2011). Ndfip1$^{-/-}$ mice die prematurely by 12 weeks of age. Ndfip1 interacts with and modulates the activity of the E3 ligase Itch (Harvey et al., 2002; Oliver et al., 2006). It is necessary for the degradation of the transcription factor JunB in Th2 cells by Itch. Consequently, Ndfip1-deficient and Itchy mutant Th2 cells accumulate JunB and overproduce IL-4 and IL-5 (Fang et al., 2002). It has been shown more recently that, additionally, Itchy mutant naïve T cells are resistant to iTreg differentiation (Venuprasad et al., 2008). Thus, it is possible that Ndfip1 also assists Itch in in this effort. Here we test the hypothesis that Ndfip1 plays a role in inducible Treg differentiation.

Results

Ndfip1 is dispensable for the development of natural regulatory T cells

As an initial approach to assess a possible defect in regulatory T cells, we sought to determine whether Ndfip1$^{-/-}$ mice have normal frequencies of Tregs in the thymus, the site of nTreg development, and whether Tregs were found in peripheral lymphoid compartments. We found that, compared to WT mice, 4-to-6 week old Ndfip1-deficient mice contained comparable percentages of CD4^{+} CD25^{+} Foxp3^{+} T cells in their spleen, lymph nodes and Peyer's patches (data not shown). However, in the thymus, Ndfip1$^{-/-}$ mice had increased percentages of CD25^{+} Foxp3^{+} Tregs (Figure 2a and 2c). The increased frequency of Tregs in the thymi of Ndfip1$^{-/-}$ mice could be due to an intrinsic defect in the development of nTregs or, alternatively, it could be an indirect result of inflammation (Liu et al., 2008). Analysis of nTregs in 9 day old-mice, prior to the onset of inflammation, showed similar frequencies of Foxp3^{+} T cells in the thymi of WT and Ndfip1$^{-/-}$ mice (data not shown). Additionally, the thymi of mixed bone marrow chimeras contained similar frequencies of nTregs from WT and Ndfip1$^{-/-}$ sources (data not shown). Together, these data suggest that the elevated frequencies of nTregs in the thymi of Ndfip1$^{-/-}$ mice are due to inflammation and that Ndfip1 is not required for nTreg development. We also assessed the frequencies of CD25^{+}

Foxp3^{+} Tregs in the small bowel of Ndfip1$^{-/-}$ mice. The small bowel is a site of preferential accumulation of iTregs (Sun et al., 2007). Moreover, the small bowel of Ndfip1$^{-/-}$ mice is a site of severe inflammation (Ramon et al., 2011). This hinted towards a possible defect in iTreg differentiation. Contrary to what was found in the thymus, the small bowel of Ndfip1$^{-/-}$ mice contained lower frequencies and absolute numbers of CD4^{+} Tregs (Figure 2b and 2d and data not shown). This suggests that Ndfip1 has a role in inducible Treg differentiation.

Ndfip1 is necessary for the generation of iTregs *in vitro*

In vitro, Naïve CD4^{+} T cells differentiate into iTregs when stimulated in the presence of TGF-β (Chen et al. 2003). In order to investigate if Ndfip1 regulates iTreg differentiation, we tested whether Ndfip1$^{-/-}$ CD4^{+} T cells could differentiate into iTregs *in vitro*. Naïve (CD4^{+} CD25^{-} CD62L ^{hi} CD44^{lo}) T cells from Ndfip1$^{-/-}$ mice were stimulated in the presence of TGF-β for five days and percentages of iTregs were determined. Ndfip1-deficient cultures contained significantly lower percentages of iTregs when compared to WT cultures at all concentrations of TGF-β assessed (Figure 3a and 3b). These data suggest that Ndfip1$^{-/-}$ T cells are less responsive to iTreg differentiation conditions *in vitro*. Moreover, it supports a key role for Ndfip1 in iTreg differentiation.

Ndfip1-deficient T cells differentiate poorly into iTregs *in vivo*

Having shown that Ndfip1 regulates iTreg differentiation *in vitro*, we next sought to test *in vivo* iTreg differentiation of naïve Ndfip1$^{-/-}$ T cells. For this, we adopted a previously published model of *in vivo* iTreg differentiation in the GI tract. iTreg differentiation can be analyzed by transferring Ovalbumin (Ova)-specific (OTII^{+}) T cells into WT (congenic) mice and then feeding the recipient mice low concentrations of Ova (Sun et al. 2007). Thus, to test iTreg conversion, we transferred Ndfip1$^{-/-}$ Rag1$^{-/-}$ OTII^{+} CD45.2^{+} or Ndfip1$^{+/+}$ Rag1$^{-/-}$ OTII^{+} CD45.2^{+} T cells into CD45.1^{+} recipients. Prior to transfer, T cells from Ndfip1$^{+/+}$ as well as Ndfip1$^{-/-}$ Rag1$^{-/-}$ OTII^{+} mice were Foxp3^{-} and naïve (data not shown). Recipient mice were 'fed' Ova for five consecutive days, and then transferred T cells were analyzed for iTreg differentiation. Significantly fewer of the Ndfip1$^{-/-}$ cells became Foxp3^{+} T cells in the mesenteric lymph nodes (Figure 3c) and Peyer's patches (Figure 3d). From these data we conclude that Ndfip1 is necessary for iTreg differentiation *in vitro* and *in vivo*.

Ndfip1 and Itch are needed for iTreg differentiation

Knowing that both Ndfip1 and Itch are needed for iTreg differentiation and that Ndfip1 regulates the activity of Itch in Th2 cells, it seemed possible that they also

cooperate to allow iTreg differentiation (Oliver et al., 2006; Venuprasad et al., 2008). To test whether Ndfip1 and Itch are similarly involved in iTreg differentiation, Ndfip1$^{-/-}$ and Itchy-mutant T cells were stimulated under iTreg conditions side by side. Consistent with figure 3 as well as published results, Ndfip1$^{-/-}$ T cells and Itchy mutant T cells were impaired in iTreg differentiation (Figure 4). Thus supporting that Ndfip1 cooperates with Itch in iTreg differentiation. However, Ndfip1$^{-/-}$ T cells were less able to differentiate into iTregs than Itch-deficient T cells. Thus, while both Itch and Ndfip1 are necessary for iTreg differentiation, these results suggest that Ndfip1 can also promote iTreg differentiation via mechanisms independent of Itch.

Ndfip1 limits IL-4 production to promote iTreg differentiation

Inflammatory cytokines can interfere with iTreg differentiation (Dardalhon et al., 2008; Dominitzki et al., 2007). Such is the case for IL-4. Under Th2 conditions, T cells lacking Ndfip1 are more prone to make IL-4 (Oliver et al., 2006). Thus, we assessed whether Ndfip1$^{-/-}$ T cells were also making IL-4 under conditions that promote iTreg differentiation and whether this was affecting Foxp3 expression. Our first finding was that Ndfip1$^{-/-}$ T cells made IL-4 under conditions of iTreg differentiation (data not shown). Additionally, IL-4 blocking antibody rescued iTreg differentiation in Ndfip1$^{-/-}$ T cells (Figure 5a). Thus, even in the presence of

TGF-β, naïve Ndfip1$^{-/-}$ T cells were making IL-4 and, consequently, IL-4 receptor signaling was inhibiting iTreg differentiation. To confirm these findings, we assessed the ability of naïve T cells from Ndfip1$^{-/-}$IL-4$^{-/-}$ mice to differentiate into iTregs *in vitro*. As expected, naïve Ndfip1$^{-/-}$IL-4$^{-/-}$ T cells had normal Foxp3 expression compared to Ndfip1$^{+/+}$IL-4$^{-/-}$ cells (Figure 5b and 5c). Hence, IL-4 production and autocrine signaling prevent iTreg differentiation in Ndfip1$^{-/-}$ mice.

Additional studies (Beal et al., 2012) characterized the mechanism by which Ndfip1 controls IL-4 production and, thus, promotes iTreg differentiation. Under conditions that support Th2 cell differentiation, JunB protein levels accumulate in Ndfip1$^{-/-}$ T cells, driving IL-4 expression (Oliver et al., 2006). Similarly, JunB protein was more abundant in Ndfip1$^{-/-}$ T cells under iTreg conditions. Confirming a direct connection between JunB and IL-4 production, JunB protein was binding to the IL-4 promoter in Ndfip1$^{-/-}$ T cells. However, what was responsible for defective iTreg differentiation were not the levels of JunB per se, but the consequent expression of IL-4. Even with more JunB protein, Ndfip1$^{-/-}$ T cells differentiated into iTregs when IL-4 blocking antibody was present. Further studies also assessed whether Ndfip1$^{-/-}$ T cells were able to suppress once they have acquired the regulatory program. Ndfip1$^{-/-}$ T cells differentiated into iTregs in the presence of IL-4 blocking antibody were able to suppress the proliferation

of WT cells just as well as Ndfip1$^{+/+}$ cells. This suggests that Ndfip1 plays a role in iTreg differentiation but not in iTreg function. Finally, at 4 to 6 weeks of age, Ndfip1$^{-/-}$ IL-4$^{-/-}$ mice had less GI pathology and longer lifespans than Ndfip1$^{-/-}$ mice (Beal et al., 2012). Together, these data show that in the absence of IL-4, Ndfip1$^{-/-}$ cells can become iTregs and, consequently, significantly decrease immunopathology.

Discussion

Ndfip1 limits the production of IL-4 under conditions that promote Th2 cell differentiation and iTreg differentiation. T cells lacking Ndfip1 accumulate more JunB and secrete more IL-4 than their WT counterparts and are more biased to differentiate into Th2 cells. Ndfip1 promotes the degradation of JunB under Th2 conditions and, thus, controls IL-4 expression (Oliver et al., 2006). Our results here show that Ndfip1 uses a similar mechanism to promote iTreg differentiation. Supporting this, Ndfip1-deficient T cells produce IL-4 under conditions that are meant to support iTreg differentiation. Autocrine IL-4 signaling caused by increased JunB protein levels blocked Ndfip1$^{-/-}$ iTreg differentiation. Since Tregs are indispensable for proper immune function, poor iTreg development in Ndfip1$^{-/-}$ mice is likely contributing to the tissue inflammation that develops at mucosal barrier sites.

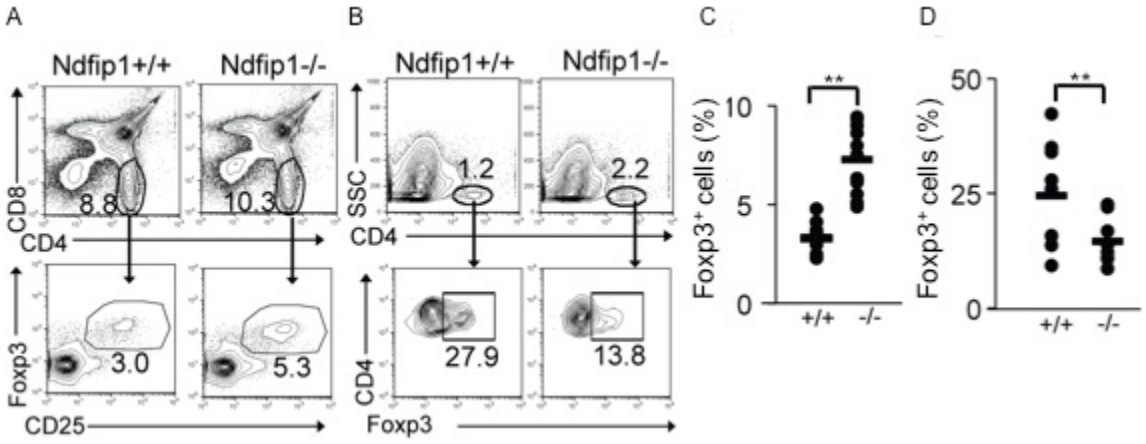
Both Ndfip1 and Itch control iTreg differentiation. Ndfip1$^{-/-}$ T cells and Itchy mutant T cells do not differentiate into iTregs as well as WT T cells. Also, IL-4 blocking antibody restores iTreg differentiation in both Ndfip1$^{-/-}$ and Itchy mutant T cells (Beal et al., 2012). Thus, both Ndfip1 and Itch are involved in the control of IL-4 expression under conditions that promote iTreg differentiation. In Th2 cells, Ndfip1 aids Itch in the control of IL-4 expression. Ndfip1 and Itch are also collaborating to limit the expression of IL-4 in iTregs. Surprisingly, Ndfip1$^{-/-}$ cells

are more impaired in iTreg differentiation than Itchy mutant T cells. Thus, Ndfip1 must promote iTreg differentiation through mechanisms that do not involve Itch. The only known role of Ndfip1 is regulating Nedd4 family E3 ubiquitin ligase activity. This suggests that Ndfip1 might collaborate with another Nedd4 family member to suppress IL-4 expression and promote iTreg differentiation.

By controlling the expression of IL-4, Ndfip1 prevents the development of a fatal inflammatory disease. Ndfip1$^{-/-}$ mice develop a Th2-type pathology that leads to death by 12 weeks of age (Oliver et al., 2006). It is difficult to measure how much the iTreg deficiency contributes to the pathology. However, mice with impaired iTreg differentiation develop a similar allergic inflammation (Josefowicz et al., 2012b). Thus, impaired iTreg differentiation, in addition to a Th2-bias, is likely contributing to the inflammation in Ndfip1$^{-/-}$ mice. Nonetheless, there is mounting evidence that Ndfip1 has functions beyond IL-4 production. When Ndfip1$^{-/-}$ mice cannot make IL-4, they outlive IL-4-sufficient Ndfip1$^{-/-}$ mice. Yet, Ndfip1$^{-/-}$ IL-4$^{-/-}$ mice still have infiltrating T cells in the small bowel and lungs (Beal et al., 2012). One of these functions is in the regulation of T cell activation, detailed in chapter 3.

Figures and Legends

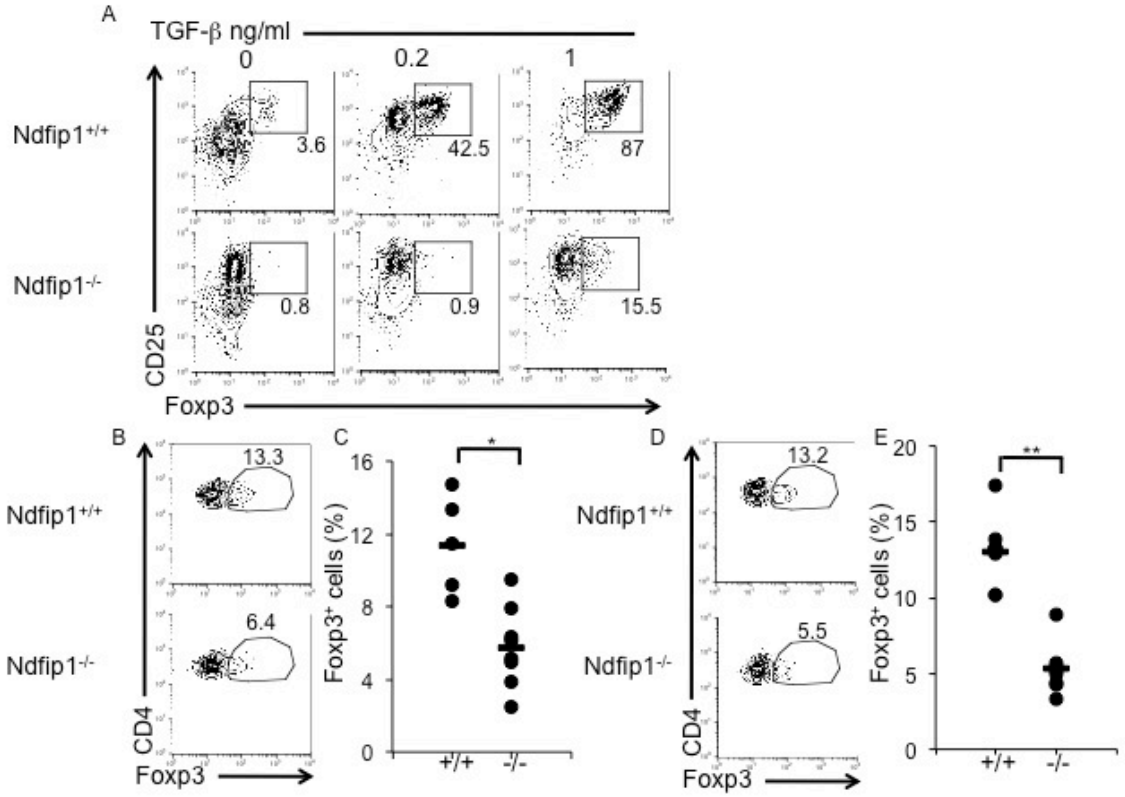
Figure 2



Decreased frequency of regulatory T cells in the small bowel of Ndfip1$^{-/-}$ mice

The thymi (A, C) and small bowel (B, D) from Ndfip1$^{+/+}$ and Ndfip1$^{-/-}$ mice (4 to 8 weeks of age) were analyzed for CD4^{+} percentages and CD25^{+} Foxp3^{+} T cell expression by flow cytometry. Percentages of CD25^{+} Foxp3^{+} Tregs are shown for the (C) thymus and (D) small bowel. Each dot represents a single mouse. Bars represent the mean. Two-tailed student t-test was performed. **P<0.01. Data representative of 8 experiments.

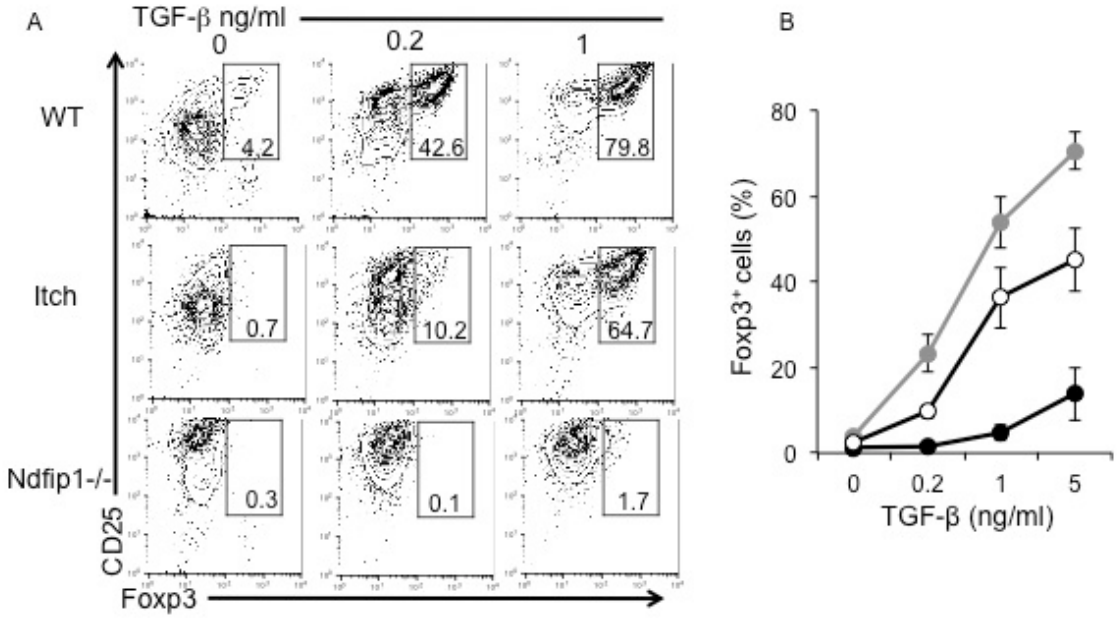
Figure 3



Ndfip1-deficient T cells convert poorly into iTregs *in vitro* and *in vivo*

(A) Sorted naïve $CD4^+$ T cells were cultured for 5 days with IL-2, anti-CD3, anti-CD28 and varying concentrations of TGF-β. After incubation, the expression of CD25 and Foxp3 was analyzed by flow cytometry. (B-E) Flow cytometry of $CD45.2^+$ iTregs in the spleen (B, C) and mLNs (D, E) of $CD45.1^+$ recipient mice after 5 days of Ova feeding. Each dot represents a single mouse. Bars represent the mean (C, E). Two-tailed student t-test was performed. *P<0.05 **P<0.01. Data representative of 3 experiments (A) or six experiments (B-E).

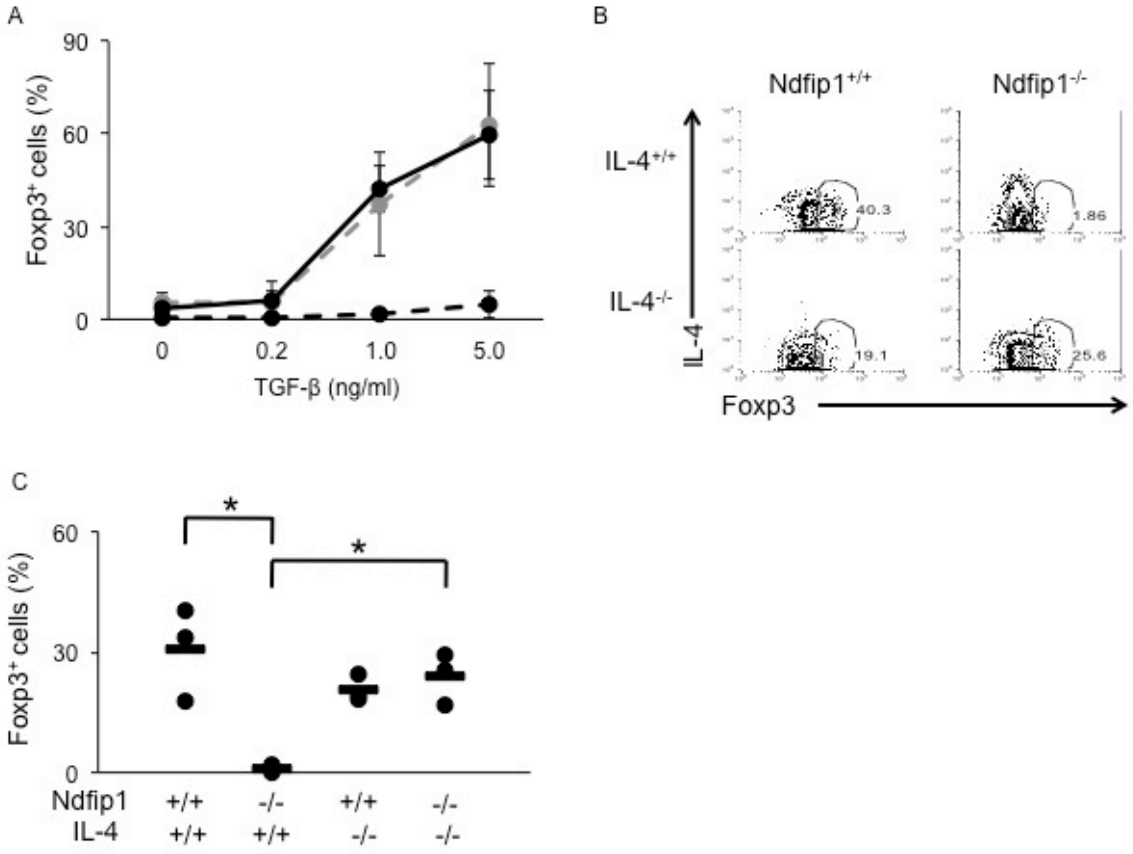
Figure 4



Ndfip1-deficient and Itch-deficient CD4$^+$ T cells convert poorly into iTregs *in vitro*

(A) Sorted naïve CD4$^+$ T cells were cultured for 5 days with IL-2, anti-CD3, anti-CD28 and varying concentrations of TGF-β. After incubation, the expression of CD25 and Foxp3 was analyzed by flow cytometry. (B) Mean percentage of CD25$^+$ Foxp3$^+$ iTregs upon *in vitro* conversion at increasing concentrations of TGF-β (mean ± s.e.m.). Grey dots represent Ndfip1$^{+/+}$ mice, black dots represent Ndfip1$^{-/-}$ mice and white dots are Itchy-mutant mice. Data representative of 8-12 mice.

Figure 5



Ndfip1$^{-/-}$IL-4$^{-/-}$ T cells have restored iTreg conversion

(A) Sorted naïve CD4^{+} T cells were cultured for 5 days with IL-2, anti-CD3, anti-CD28, varying concentrations of TGF-β with or without anti-IL-4 blocking antibody. After incubation, the expression of CD25 and Foxp3 was analyzed by flow cytometry and mean percentages of iTregs were plotted. Black line represents Ndfip1$^{+/+}$ T cells, black dashed line represents Ndfip1$^{-/-}$ T cells and the grey dotted line represents Ndfip1$^{-/-}$ T cells with anti-IL-4 blocking antibody. (B, C) Sorted naïve T cells from Nfip1$^{+/+}$, Ndfip1$^{-/-}$, IL-4$^{-/-}$ and Ndfip1$^{-/-}$IL-4$^{-/-}$ mice were cultured under iTreg conditions (as in (A)). (C) Each dot represents a

single mouse. Bars represent the mean. Two-tailed student t-test was performed. *P<0.05. Data representative of two-to-four experiments with three or more mice (A) or three experiments with 3 mice (B, C).

Materials and Methods

Mice

Ndfip1$^{-/-}$ and Itchy mutant mice have been previously described (Fang et al., 2002; Oliver et al., 2006). CD45.1 (C57BL6.SJL-Ptprc^{a} Pepc^{b}/BoyJ mice, #002014), IL-4$^{-/-}$ (B6.129P2-IL-4^{tm1Cgn}/J, #002253), OT-II^{+} (B6.Cg-Tg (TcraTcrb) 425Cbn/J, #004194) and Rag1$^{-/-}$ (B6.129S7-Rag1^{tm1Mom}/J, #002216) mice were purchased from the Jackson Laboratory. All mice were housed in a barrier facility at the Children's Hospital of Philadelphia in accordance with the Institutional Animal Care and Use Committee protocol.

Flow cytometry, cell sorting and antibodies

Flow cytometry was performed using a FACSCalibur (BD Biosciences) and results were analyzed using FlowJo (Tree Star). Naïve CD4^{+} T cells (CD4^{+} CD25^{-} CD44^{lo} CD62L^{hi}) were sorted using a FACSAria (BD Biosciences) or MoFlo (Beckman Coulter, Inc.).

The following antibodies and conjugates were used for flow cytometry and/or cell sorting: anti-CD4 (GK1.5, BioLegend), anti-CD44 (IM7, BioLegend), anti CD62L (MEL-14, eBioscience), anti-CD25 (PC61.5, eBioscience), anti-Foxp3 (FJK-16s, eBioscience), Streptavidin (Invitrogen), anti-CD8 (53-6.7, BioLegend), anti-

CD45.2 (104, BioLegend) and anti-Thy1.2 (53-2.1, BD Biosciences). Foxp3 staining was done using a Fixation/Permeabilization Kit (00-5521-00, eBioscience).

Tissue processing

Spleens, thymi and lymph nodes were harvested and mashed through 70mm filters in cold Hank's Balanced Salt Solution (HBSS). Cell suspensions from spleens were treated with ACK lysis buffer to lyse red blood cells.

A 3-4-inch section of small bowel was flushed with cold PBS. Peyer's patches were removed. Tissue was minced with scissors and treated with DNAse (20ug/ml, Sigma D5025), collagenase type I (.8mg/ml, C0130, Sigma) and collagenase type Ia (.9mg/mL, C2674, Sigma) in DMEM for 1 hour in end-over-end rotation at room temperature. Cell suspensions were filtered through a 100mm filter, then a 40mm filter and 10% FCS was added.

***In vivo* iTreg differentiation**

$2x10^6$ $Thy1.2^+$ sorted cells from either $Ndfip1^{-/-}$ $Rag1^{-/-}$ $OTII^+$ or $Ndfip1^{+/+}$ $OTII^+$ mice were transferred intravenously into $Ndfip1^{+/+}$ $CD45.1^+$ mice. Recipient mice were fed 1.5% Ova (grade III) in water for 5 consecutive days after which cells from the mesenteric lymph node and Peyer's Patches were stained for flow cytometry analysis.

In vitro iTreg conversion

0.5 x10^6 sorted naïve T cells were stimulated with 5μg/mL of plate-bound anti-CD3e (145-2C11, BD Biosciences) and anti-CD28 (37.51, BD Biosciences) antibodies in the presence of 50 U/mL of IL-2 (R&D Systems), varying concentrations of TGF-β (Peprotech) and with or without anti-IL-4 (11B11, BioLengend). Cells were grown in complete Dulbecco's Modified Eagle Medium (DMEM) (11960, Invitrogen).

Statistics

All statistical analyses were performed using Student's *t*-tests. A *P*-value of equal or less than 0.05 was used to determine statistical significance.

CHAPTER 3

Ndfip1 negatively regulates $CD4^+$ T cell activation by limiting IL-2 production

Introduction

Upon T cell receptor (TCR) stimulation, various signaling cascades are initiated that instruct T cells towards the appropriate response. For example, when $CD4^+$ T cells see their cognate antigen in the presence of co-stimulation they produce and secrete IL-2 (Linsley et al., 1991; Thompson et al., 1989). Autocrine IL-2 receptor signaling initiates a positive feedback loop that further increases IL-2 and IL-2Rα expression, and triggers proliferation (Kim et al., 2001). Co-stimulatory signals are key to this process by complementing the signals received from the T cell receptor, thus, boosting IL-2 production.

In contrast, $CD4^+$ T cells that receive signals exclusively from the TCR produce poor amounts of IL-2 (Jenkins et al., 1990; Quill and Schwartz, 1987). This is partly due to a lack of co-receptor signals that supplement the production of IL-2 and, thus, allow proper $CD4^+$ T cell activation. But, in addition, $CD4^+$ T cells also recruit mechanisms that actively suppress IL-2 expression in the absence of co-stimulatory signals (Jeon et al., 2004; Macian et al., 2002; Soto-Nieves et al., 2009). While the pathways regulating $CD4^+$ T cell activation and promoting IL-2 production have been characterized, less is known about the pathways actively repressing IL-2 production.

One way to repress IL-2 production and secretion is by reducing the levels or

functions of signaling proteins by E3 ubiquitin ligases. E3 ubiquitin ligases that restrain $CD4^+$ T cell activation include Casitas B cell lymphoma-b (Cbl-b), gene regulating anergy in lymphocytes (Grail) and Itch (Heissmeyer et al., 2004; Macian et al., 2004). These factors can dampen signaling downstream of the T cell receptor by blocking protein-protein interactions or by ubiquitylating and degrading signaling proteins (Chiang et al., 2000; Heissmeyer et al., 2004; Huang et al., 2010; Krawczyk et al., 2005). For example, Itch and Cbl-b have been shown to increase the rate of degradation of PKCθ and PLCγ1 in effector $CD4^+$ T cells stimulated in the absence of co-stimulation (Heissmeyer et al., 2004).

Itch is a homologous to the E6-AP carboxyl terminus (HECT)-type E3 ubiquitin ligase of the Neural-precursor cell expressed and developmentally downregulated 4 (Nedd4)-family. While Itch, WWP2 and Nedd4 have known functions in $CD4^+$ T cells (Chen et al., 2009; Fang et al., 2002; Heissmeyer et al., 2004; Yang et al., 2008), a role for the other 6 Nedd4 family members in $CD4^+$ T cells has yet to be defined. Most members of this family have been shown to associate with the membrane-tethered adaptor Nedd4 family-interacting protein 1 (Ndfip1) and its only relative Ndfip2 (Harvey et al., 2002; Mund and Pelham, 2009; Oliver et al., 2006) *in vitro*.

While Ndfip1 has been shown to bind most Nedd4 family members *in vitro*

(Harvey et al., 2002), to date it has only been shown to interact with Itch in primary $CD4^+$ T cells (Oliver et al., 2006). Both Itchy-mutant (mice lacking Itch) and $Ndfip1^{-/-}$ mice develop Th2-mediated inflammation at barrier surfaces, including the skin, gastrointestinal (GI) tract and lung (Fang et al., 2002; Oliver et al., 2006). This is in part because, in antigen experienced $CD4^+$ T cells, both Ndfip1 and Itch are required for ubiquitylation and degradation of JunB, a transcription factor that promotes IL-4 and IL-5 production (Fang et al., 2002; Oliver et al., 2006). Accumulation of JunB in these cells leads to excessive IL-4 production and promotes the differentiation of $CD4^+$ T cells into Th2 cells (Oliver et al., 2006). Moreover, iTreg differentiation is also blocked in Itch or Ndfip1-deficient T cells due to IL-4 production (Beal et al., 2012). These findings may help explain why both $Ndfip1^{-/-}$ and Itchy- mutant mice develop Th2-mediated inflammation.

In contrast to Itchy-mutant mice, which exhibit inflammation at 5 months of age, $Ndfip1^{-/-}$ mice develop inflammation by 6 weeks of age and do not survive beyond 14 weeks of age. Furthermore, *in vivo* analysis of $CD4^+$ T cells from mice lacking Ndfip1 at 4-6 weeks of age shows that these cells display markers characteristic of activation (Ramon et al., 2011), while $CD4^+$ T cells from Itchy-mutant mice do not (unpublished observation). This suggests that Ndfip1 might regulate other Nedd4 family members in T cells.

Due to the increased frequency of $CD4^+$ T cells with an activated phenotype in

Ndfip1$^{-/-}$ mice we hypothesized that Ndfip1-deficient CD4^{+} T cells lack a negative regulatory circuit that limits T cell activation. Here we show that naïve Ndfip1$^{-/-}$ CD4^{+} T cells are hyperactive in response to TCR stimulation due to a T cell intrinsic defect. Loss of Ndfip1 leads to increased IL-2 production, elevated levels of CD25 expression, and proliferation in the absence of CD28 co-stimulation. Our data provide evidence that NFAT and Erk, which are essential for the expression of IL-2, also drive the expression of Ndfip1. Once expressed, Ndfip1 regulates the duration of IL-2 production and, thus, prevents CD4^{+} T cells from becoming fully activated in the absence of co-stimulation.

Results

CD4^{+} T cells lacking Ndfip1 require antigen exposure to become CD44^{hi} *in vivo*

Mice lacking Ndfip1 develop a fatal atopic inflammatory disease. Ndfip1 controls JunB levels and, subsequently, IL-4 expression. Thus, Ndfip1$^{-/-}$ CD4^{+} T cells are Th2 biased (Oliver et al., 2006). This, in part, explains the phenotype of these mice. However, this does not explain the increased proportion of CD44^{hi} CD4^{+} T cells present at secondary lymphoid organs of Ndfip1$^{-/-}$mice (Oliver et al., 2006; Ramon et al., 2011). In mature CD4^{+} T cells, CD44 is upregulated in response to antigen stimulation (Lesley et al., 1993). This suggests that these Ndfip1$^{-/-}$

$CD44^{hi}$ $CD4^{+}$ T cells have seen antigen. However, certain other conditions, such as a lymphopenic environment or defective means of T cell quiescence, can induce the upregulation of CD44 and, thus, lead to the spontaneous activation of T cells in the absence of antigen presentation (Buckley et al., 2001; Goldrath et al., 2000; Murali-Krishna and Ahmed, 2000). To evaluate whether $Ndfip1^{-/-}$ $CD4^{+}$ T cells were spontaneously activated, we generated mice in which T cells were not exposed to antigen, as they were specific to the Ova antigen, which is not expressed in mice. Thus, we generated $Ndfip1^{-/-}$ $Rag1^{-/-}$ $OTII^{+}$ mice. We then analyzed the levels of CD44 in the spleens of Ndfip1-suficient and -deficient $Rag1^{-/-}$ $OTII^{+}$ mice. We compared the levels of CD44 to the levels on polyclonal $CD4^{+}$ T cells from WT and $Ndfip1^{-/-}$mice. As shown previously, polyclonal $CD4^{+}$ T cells from the spleens of $Ndfip1^{-/-}$mice had increased frequencies of $CD44^{hi}$ T cells (Oliver et al., 2006; Ramon et al., 2011). However, T cells from both $Ndfip1^{+/+}$and $Ndfip1^{-/-}Rag1^{-/-}$ $OTII^{+}$ mice remained $CD44^{lo}$ (Figure 6). Additionally, we did not detect any overt signs of inflammation in Ndfip1-/- Rag1-/- $OTII^{+}$ mice (data not shown). Thus, this suggests that polyclonal Ndfip1-/- $CD4^{+}$ T cells are more easily activated than WT T cells, and $Ndfip1^{-/-}$ $CD4^{+}$ T cells that are Ova-specific are not activated, as they are not exposed to antigen. From this, we infer that $CD4^{+}$ T cells from Ndfip1-/- are not spontaneously

becoming $CD44^{hi}$; instead, $Ndfip1^{-/-}$ $CD4^{+}$ T cells need to see antigen to become activated.

T cell specific deletion of Ndfip1 leads to increased percentages of activated $CD4^{+}$ T cells and eosinophilic inflammation

Ndfip1 could be regulating $CD4^{+}$ T cell activation through T cell intrinsic or T cell extrinsic mechanisms. To determine whether Ndfip1-/- mice had more activated $CD4^{+}$ T cells as a result of the absence of Ndfip1 in T cells, we generated mice lacking Ndfip1 only in cells of the T cell lineage ($Ndfip1^{CD4\text{-}CKO}$). $Ndfip1^{CD4\text{-}CKO}$ mice were generated through the crossing of CD4-Cre mice with Ndfip1-floxed mice (Figure 7A). Mice were analyzed for the presence of both Cre and the floxed Ndfip1 locus (Figure 7B). Additionally, we analyzed Ndfip1 mRNA expression in T cells (Figure 7C). T cells from $Ndfip1^{CD4\text{-}CKO}$ mice did not express Ndfip1 mRNA. Therefore, $Ndfip1^{CD4\text{-}CKO}$ mice lack Ndfip1 in T cells.

We then analyzed the $CD4^{+}$ T cells in the spleens of $Ndfip1^{CD4\text{-}CKO}$ mice for CD44 levels. We found that a T cell specific deletion of Ndifp1 was sufficient to cause an elevation in the percentages of $CD44^{hi}$ $CD4^{+}$ T cells in spleens (Figure 7D and 7E). Interestingly, $Ndfip1^{CD4\text{-}CKO}$ mice showed comparable percentages of $CD44^{hi}$ $CD4^{+}$ T cells as age-matched $Ndfip1^{-/-}$ mice (Figure 7D). Thus, we can

conclude that the increased $CD4^+$ T cell activation in $Ndfip1^{-/-}$ mice is due to a T cell intrinsic defect in the absence of Ndfip1.

Inflammation in $Ndfip1^{-/-}$ mice is characterized in the lungs, GI tract and skin by infiltrating T cells and eosinophils in these organs (Oliver et al., 2006; Ramon et al., 2011). Thus, we next sought to determine whether $Ndfip1^{-/-}$ T cells were contributing to this disease. Histological analysis of the esophagus showed inflammatory cell infiltrates and thickening of the epithelial layer of $Ndfip1^{CD4\text{-}CKO}$ mice (Figure 7F). Additionally, flow cytometry of the esophagus showed increased eosinophilia and $CD4^+$ T cells (Figure 7G). Analysis of the small bowel, skin and the lung of $Ndfip1^{CD4\text{-}CKO}$ mice found increased inflammation in these organs as well (Figure 7H-K and data not shown). Thus, $Ndfip1^{-/-}$ T cells are sufficient to induce disease. It is important to note that the pathology in $Ndfip1^{CD4\text{-}CKO}$ mice was less severe than that observed in $Ndfip1^{-/-}$ mice (data not shown). This suggests that cells outside the T cell compartment contribute to the pathology of Ndfip1-/- mice. Nonetheless, we conclude that a T cell specific loss of Ndfip1 is responsible for the increased T cell activation and this defect contributes to the pathology in $Ndfip1^{-/-}$ mice.

$Ndfip1^{-/-}$ $CD4^+$ T cells are less dependent on CD28 co-stimulation than $Ndfip1^{+/+}$ counterparts

The data presented thus far suggest that Ndfip1-/- $CD4^+$ T cells have an activation defect. In order to test this, we stimulated T cells using an *in vitro* approach and assessed markers of activation. Naïve (CD4+, $CD44^{lo}$, $CD62L^{hi}$ CD25-) Ndfip1-/- T cells were stimulated through the TCR in the presence of CD28 co-stimulatory signals for different time points, and surface levels of CD44 (data not shown) and IL2ra, a subunit of the IL2 receptor and a transient marker for T cell activation, were analyzed. Both WT and $Ndfip1^{-/-}$ $CD4^+$ T cells stimulated through their TCR and CD28 presented similar levels of IL2ra and CD44 at all the time points evaluated (Figure 8A and data not shown). However, when stimulated through their TCR in the absence of CD28 costimulation, WT and $Ndfip1^{-/-}$ T cells responded differently (Figure 8B). After 1 day of stimulation, WT T cells upregulated their levels of IL2ra. However, after 3 days of stimulation, these cells downregulated IL2ra levels. After 5 days of stimulation, few live cells remained in the cultures (Figure 8B and data not shown). Although $Ndfip1^{-/-}$ T cells appeared similar to WT T cells after 1 day of stimulation, after 3 days of TCR signaling they maintained elevated IL2ra and CD44 levels. After 5 days of stimulation these cells persisted in culture and looked very much like cells that had received co-stimulation (Figure 8B and data not shown). These results suggest that $Ndfip1^{-/-}$ $CD4^+$ T cells are hyperresponsive to TCR stimulation in the absence of co-stimulatory signals.

In addition to elevating the surface expression of CD44 and IL-2Rα, $CD4^+$ T cells receiving signals through the TCR and CD28 secrete IL-2 and proliferate. Thus, we wanted to assess whether $Ndfip1^{-/-}$ T cells were also more prone to secrete IL-2 and proliferate upon TCR stimulation in the absence of co-stimulatory signals. Consistent with published data, WT $CD4^+$ T cells stimulated through the TCR alone made minimal amounts of IL-2 and proliferate poorly. However, $Ndfip1^{-/-}$ T cells made significant amounts of IL-2 and proliferated after 3 days of TCR stimulation despite the lack of co-stimulation (Figure 8C and 8D). Thus, upon receipt of TCR stimulation, $Ndfip1^{-/-}$ $CD4^+$ T cells upregulated surface expression of CD44 and IL-2Rα, made more IL-2 and proliferated.

Increased production of IL-2 by $Ndfip1^{-/-}$ T cells could be due to increased IL-2 expression or due to increased survival of these cells. We analyzed the percentage of live cells after 3 days of culture with TCR signals alone. No significant differences in live cell frequencies were observed between WT and $Ndfip1^{-/-}$ cells (data not shown). However, after 5 days of culture, $Ndfip1^{-/-}$ T cells survived in culture, whereas most WT T cells had died. Thus, a significant difference in survival was observed between WT and $Ndfip1^{-/-}$ T cells by 5 days of TCR only stimulation. Since an increase in IL-2 is seen at a time when there is no change is survival, this suggests that increased IL-2 production by $Ndfip1^{-/-}$ $CD4^+$ T cells is not due to their increased survival. Instead, increased IL-2

production by Ndfip1$^{-/-}$ T cells is likely supporting their survival after TCR only stimulation.

Ndfip1$^{-/-}$ CD4^{+} T cells might be hyperactive only after reaching a certain threshold of TCR stimulation. Alternatively, these cells could be hyperactive regardless of the strength of TCR stimulation received. To assess this, we stimulated cells with varying concentrations of anti-CD3 stimulating antibody and measured IL-2 production and IL-2Rα levels after 3 days of stimulation. CD28 co-stimulatory signals can, in some cases, come from ligands on T cells (Sabzevari et al., 2001). To ensure that T cells were not receiving CD28-co-stimulatory signals, we used naïve CD4^{+} T cells from Ndfip1$^{-/-}$ CD28$^{-/-}$ mice and compared them to CD28$^{-/-}$ controls. At all concentrations of anti-CD3 tested, Ndfip1$^{-/-}$ CD28$^{-/-}$ CD4^{+} T cells made more IL-2 than CD28$^{-/-}$ T cells (Figure 8E). Likewise, increased frequencies of Ndfip1$^{-/-}$ CD28$^{-/-}$ T cells had high levels of IL-2Rα when compared to CD28$^{-/-}$ T cells (data not shown). Interestingly, Ndfip1$^{-/-}$ CD28$^{-/-}$ and CD28$^{-/-}$ T cells showed similar patterns of IL-2 production, with peak expression at 5ug/mL of anti-CD3. Thus, Ndfip1$^{-/-}$ T cells are hyperactive regardless of the strength of signal given.

Ndfip1$^{-/-}$ CD4^{+} T cells become activated and differentiate into IL-4 producing cells in the absence of CD28 co-stimulation *in vivo*

We next wanted to assess whether T cells in Ndfip1$^{-/-}$ CD28$^{-/-}$ mice become activated *in vivo* and induce disease. We first analyzed the activation status of CD4^{+} T cells in the spleen of 5-10 week old Ndfip1$^{-/-}$ CD28$^{-/-}$ mice. Like Ndfip1$^{-/-}$mice, Ndfip1$^{-/-}$ CD28$^{-/-}$ mice contained significantly increased frequencies of CD44^{hi} CD4^{+} T cells in their spleens (Figure 9A). This supports that CD4^{+} T cells lacking Ndfip1 do not require CD28 co-stimulation to become activated. Furthermore, IL-4 was detected from the splenocyte cultures of Ndfip1$^{-/-}$ CD28$^{-/-}$ mice. Anti-CD3 stimulated splenocytes from Ndfip1$^{-/-}$ CD28$^{-/-}$ mice made significant amounts of IL-4 ex vivo. Conversely, CD28$^{-/-}$ splenocytes made little to no IL-4 (Figure 9B). This suggested that, even in the absence of CD28 co-stimulatory signals, Ndfip1-/- CD4^{+} T cells could differentiate into cytokine-producing effector cells.

Having detected IL-4 from Ndfip1$^{-/-}$ CD28$^{-/-}$ cells, we next sought to determine if in the absence of CD28 signaling, Ndfip1$^{-/-}$ T cells could also induce pathology. Tissue analysis showed mucosal inflammation in Ndfip1$^{-/-}$ CD28$^{-/-}$ mice. Histological analysis and flow cytometry of the lungs and esophagi of Ndfip1$^{-/-}$ CD28$^{-/-}$ mice revealed epithelial hypertrophy and increased CD4^{+} T cell infiltrates in both organs (Figure 9C and 9F-9H and data not shown). Eosinophilia

was not significantly increased in the esophagi of Ndfip1$^{-/-}$ CD28$^{-/-}$ mice (Figure 9C). Eosinophilia was, however, significantly increased in the small bowel of these mice by both histological analysis and flow cytometry (Figure 9D, 9J and 9L). Therefore, Ndfip1$^{-/-}$ CD28$^{-/-}$ mice develop inflammation at mucosal sites. The pathology that Ndfip1$^{-/-}$ CD28$^{-/-}$ mice develop is, however, delayed compared to Ndfip1$^{-/-}$mice. While Ndfip1$^{-/-}$ mice presented signs of inflammation at 6 weeks of age, overt signs of inflammation in the lungs, GI tract or skin of Ndfip1$^{-/-}$ CD28$^{-/-}$ mice were not detected until about 9 weeks of age (Figure 9 and data not shown). Based on these data we conclude that, even in the absence of CD28 signaling, Ndfip1-/- CD4^{+} T cells are able to become activated, differentiate into effectors, make IL-4, and induce disease.

Ndfip1 restricts CD4^{+} T cell activation by limiting the expression of IL-2

Different primary defects could be causing the increased IL-2 production, elevated levels of surface IL-2Rα and proliferation of Ndfip1$^{-/-}$ CD4^{+} T cells. Upon TCR and CD28 stimulation, CD4^{+} T cells increase the surface expression of IL-2Rα (Figure 8A and 8B). Upregulation of IL-2Rα increases the affinity of the IL-2 receptor. IL-2 receptor signaling leads to an increase in the expression of both IL-2 and IL-2Rα and also promotes proliferation. TCR and CD28 stimulation also directly induce the expression of IL-2. IL-2, then, acts in an autocrine

manner signaling preferentially through the high affinity IL-2 receptor. This feedback loop leads to the subsequent increase in IL-2 levels, IL-2Rα levels and proliferation. Since Ndfip1$^{-/-}$ T cells had increased levels of both IL-2 and IL-2Rα (Figure 8), we wanted to investigate whether Ndfip1 was causing CD4^{+} T cell hyperactivity by directly regulating the levels of one or both of these proteins. To test this we co-cultured naïve Ndfip1$^{+/+}$ or Ndfip1$^{-/-}$ CD4^{+} T cells (CD45.2) with equal numbers of naïve WT (CD45.1) CD4^{+} T cells and assessed the proliferation and the levels of IL-2Rα of WT T cells after anti-CD3 stimulation. As shown previously (Figure 8B and 8D), Ndfip1$^{-/-}$ T cells proliferated more (Figure 10A) and had higher levels of IL-2Rα (Figure 10C) than Ndfip1$^{+/+}$ T cells under the same conditions. Interestingly, WT T cells stimulated in the presence of Ndfip1$^{-/-}$ T cells proliferated more (Figure 10A) and had higher levels of IL-2Rα (Figure 10C) than WT T cells stimulated in the presence of Ndfip1$^{+/+}$ T cells. This suggested that a soluble factor produced by Ndfip1$^{-/-}$ T cells was acting in trans on the WT T cells. Therefore, we repeated the experiment stimulating the cells with anti-CD3 and in the presence of IL-2 blocking antibody. Under such conditions, the proliferation of WT T cells was abrogated irrespective of whether in the presence of Ndfip1$^{+/+}$ or Ndfip1$^{-/-}$ T cells (Figure 10B). The levels of IL-2Rα of WT T cells were also similarly decreased under both conditions (Figure 10D). This suggested that IL-2 production by Ndfip1$^{-/-}$ T cells was causing the

proliferation and the increase in IL-2Rα levels on the WT T cells. However, in the presence of IL-2 blocking antibody, Ndfip1$^{-/-}$ T cells still proliferated more and had higher levels of IL-2Rα than the WT T cells with which they were co-cultured. This could indicate that IL-2 expression is not fully responsible for the increased levels of IL-2Rα and the proliferation of Ndfip1$^{-/-}$ T cells. Alternatively, it is possible that the IL-2 blocking antibody was not able to fully block autocrine IL-2 signaling. Supporting this, the IL-2 receptor has a much higher affinity for IL-2 than the IL-2 blocking antibody does. However, our results suggest that IL-2 production by Ndfip1$^{-/-}$ CD4^{+} T cells can induce the proliferation and the increase in IL-2Rα levels of WT T cells in trans.

In an effort to determine whether Ndfip1 directly controls IL-2 production and/or IL-2Rα surface levels, we assessed the mRNA expression of IL-2 and IL-2Rα in Ndfip1$^{+/+}$ and Ndfip1$^{-/-}$ naïve CD4^{+} T cells at early time points following TCR-stimulation. As shown in Figure 11A, by 12 hours after TCR stimulation, IL-2 transcription was evident in Ndfip1$^{+/+}$ T cells. However, levels of IL-2 transcripts were significantly decreased by 24 hours. This suggested that transcription of IL-2 is transient and quickly terminated. While IL-2 levels declined by 24 hours of stimulation in Ndfip1$^{+/+}$ T cells, under the same conditions IL-2 was still highly expressed in Ndfip1$^{-/-}$ cells. Hence, Ndfip1 is not regulating initial IL-2 production in T cells but rather is limiting its duration. Conversely, the levels of IL-2Rα

expression in WT and Ndfip1$^{-/-}$ T cells were similar at all measured time points (Figure 11B). Thus, Ndfip1 is not directly affecting the expression of IL-2Rα. This would appear to contradict our data in Figure 8B, in which we showed that on day 3 of stimulation the levels of IL-2Rα were increased. However, the RNA expression data showed that this likely due to the downstream consequences of increased IL-2 production. Thus, these data support the argument that Ndfip1 is preventing the full activation of T cells by limiting IL-2 production at the transcriptional level.

NFAT and Erk induce the expression of Ndfip1 to limit IL-2 production in the absence of co-stimulation

Having shown that Ndfip1 limited the duration of IL-2 production (Figure 11A), we next wanted to determine how Ndfip1 expression is regulated in CD4^{+} T cells and how this might impact IL-2 expression. Thus, we stimulated Ndfip1$^{+/+}$ CD4^{+} T cells through the TCR and analyzed expression of Ndfip1 at different time points. Prior to stimulation of naïve T cells little, if any, Ndfip1 was expressed. However, expression of Ndfip1 was upregulated after 12 hours of TCR-stimulation (Figure 12A), dropped after 24 hours of TCR signaling, and continued declining by 36 hours. Interestingly, the expression pattern of Ndfip1 was strikingly similar to that of IL-2 in TCR-stimulated T cells (Figure 11A). The similarity between the transcriptional patterns of Ndfip1 and IL-2 suggested that

factors that induce IL-2 expression upon TCR-stimulation might also play a role in regulating the expression of Ndfip1, which will then limit IL-2 transcription.

TCR signaling promotes IL-2 expression through the cooperation of various proteins including Jnk, NFAT, Erk and PI3K (reviewed in Smith-Garvin et al. 2009). While co-stimulatory signals, such as those delivered from CD28, can significantly enhance signaling, TCR-stimulation alone can support IL-2 expression to some extent (Figure 11A and Diehn et al. 2002). It is not known, however, how Ndfip1 expression is affected by TCR signaling and whether the factors that promote IL-2 expression also play a role in its expression.

To determine whether Jnk, NFAT, Erk or PI3K also regulate Ndfip1 expression, we stimulated naïve Ndfip1$^{+/+}$ CD4^{+} T cells through the TCR in the presence of inhibitors for these different factors. We then analyzed Ndfip1 mRNA levels after overnight stimulation. Ndfip1 expression increased following TCR stimulation (Figure 12B) but this was modestly reduced when either Jnk or PI3K were inhibited. In contrast, the expression of Ndfip1 was almost completely abrogated in the presence of inhibitors for either NFAT or Erk. Thus, NFAT and Erk are required for Ndfip1 expression. Taken together, these data suggest that two key factors that induce IL-2 production, NFAT and Erk, are also major inducers of Ndfip1, a protein that will later attenuate IL-2 expression. This suggests that NFAT and Erk induce Ndfip1 upon T cell stimulation to create a negative feedback loop that restricts IL-2 transcription.

Negative regulation of IL-2 production by Ndfip1 is not dependent on the E3 ubiquitin ligase Itch

We have shown previously that Ndfip1 interacts with the HECT-type E3 ubiquitin ligase Itch to promote the ubiquitylation and degradation of JunB and limit IL-4 production (Oliver et al., 2006; Beal et al., 2011). Thus, we postulated that Ndfip1 might cooperate with Itch to limit IL-2 production. This seemed particularly likely since Itchy-mutant T cells have been shown to produce more IL-2 under certain conditions (Fang et al., 2002; Venuprassad et al., 2006). To test this, we analyzed IL-2 production by naïve $CD4^+$ T cells lacking either Ndfip1 or Itch following anti-CD3 stimulation. As in previous experiments, T cells lacking Ndfip1 produced more IL-2 at day 3 and day 5 following stimulation through the TCR alone (Figure 13A). However, under the same conditions, T cells lacking Itch produced no more IL-2 than their WT counterparts. These data were surprising considering previously published data showing that upon TCR-only stimulation, Itch-deficient T cells become activated (Venuprassad et al., 2006). However, these prior experiments were performed using differentiated effector cells. Thus we performed experiments as described by Venuprassad and colleagues using $Ndfip1^{-/-}$ or Itchy-mutant $CD4^+$ T cells differentiated *in vitro*. As shown in Figure 13B, WT T cells differentiated *in vitro* produced less IL-2 upon TCR-only stimulation with ionomycin. In contrast, effector cells lacking either Ndfip1 or Itch were still able to make IL-2 after ionomycin treatment (Figure 13B). Taken

together, these data suggest that there are different mechanisms that negatively regulate T cell activation in naïve and effector $CD4^+$ T cells. Our data support a role for Itch and Ndfip1 in controlling IL-2 production in effector T cells, whereas Ndfip1 negatively regulates activation and IL-2 production in naïve T cells via an Itch independent mechanism.

Increased IL-2 production by Ndfip1$^{-/-}$ CD4$^+$ T cells is independent of IL-4

The inability to control IL-4 expression and, thus, the propensity that Ndfip1$^{-/-}$ T cells have to become Th2 cells is likely contributing to the pathology of the mice. Moreover, either directly or indirectly, IL-4 could be altering the unresponsiveness of T cells. As an example, while IL-4 has not been shown to directly alter IL-2 expression, it can enhance T cell survival (Vella et al., 1998). In order to determine if IL-4 is responsible for the increased expression of IL-2 by Ndfip1$^{-/-}$ T cells we examined IL-2 production by $CD4^+$ T cells from Ndfip1$^{-/-}$ IL-4$^{-/-}$ mice. Naïve T cells from Ndfip1$^{-/-}$ IL-4$^{-/-}$ mice made more IL-2 than IL-4$^{-/-}$ T cells upon stimulation through the TCR alone (Figure 14A). Thus, IL-4 signaling is not responsible for the increased production of IL-2 by Ndfip1$^{-/-}$T cells. Instead, this supports a model in which Ndfip1$^{-/-}$ T cells are hyperactive due to intrinsic defects in their regulation of IL-2 expression.

We also investigated the frequencies of activated T cells in the spleens of Ndfip1$^{-/-}$ IL-4$^{-/-}$ mice. Interestingly, while there was a trend towards increased

frequencies of CD44^{hi} T cells in the spleens of Ndfip1$^{-/-}$ IL-4$^{-/-}$ mice at 12 weeks of age, it did not reach significance (data not shown). Thus, IL-4 production and, consequently, defective iTreg differentiation are partly responsible for the significant increase in CD44^{hi} T cells found in Ndfip1$^{-/-}$ mice (discussed in chapter 2). This may be direct due to regulatory T cells suppressing T cell activation, or indirect due to barrier damage and increased exposure of T cells to antigens.

We next sought to investigate Ndfip1$^{-/-}$ IL-4$^{-/-}$ mice for signs of mucosal inflammation. At 6 weeks of age, Ndfip1$^{-/-}$mice are beginning to show signs of inflammation in the skin, lungs and GI tract (Oliver et al., 2006; Ramon et al., 2011). At that same age, Ndfip1$^{-/-}$ IL-4$^{-/-}$ mice have more infiltrating cells in the lungs and GI tract than their WT counterparts; however, they appear to be generally healthy (Beal et al., 2012). Yet, at 12 weeks of age, there was evidence of developing inflammation in the lungs and the esophagi of Ndfip1$^{-/-}$ IL-4$^{-/-}$ mice (Figure 14B and 14C). The esophagi of Ndfip1$^{-/-}$ IL-4$^{-/-}$ mice showed signs of epithelial hyperplasia (Figure 14B). Additionally, both eosinophilia and neutrophilia was detected in the lungs and esophagi of Ndfip1$^{-/-}$ IL-4$^{-/-}$ mice (Figure 14B and C and data not shown). Infiltrating T cells were present in both tissues as well (Figure 14D and 14E). Thus, while CD44^{hi} cells were not significantly increased in the spleens of Ndfip1$^{-/-}$ IL-4$^{-/-}$ mice, we observed

increased frequencies of T cells in tissues. Additionally, while delayed in comparison to Ndfip1$^{-/-}$ mice, the inflammatory disease that Ndfip1$^{-/-}$ IL-4$^{-/-}$ mice developed, ultimately, lead them to premature death (data not shown). These results show that, while IL-4 strongly contributes to pathology of Ndfip1$^{-/-}$ mice, even in its absence, T cells can become activated, move to tissues and induce pathology. This is likely due to the uncontrolled expression of IL-2 by T cells that lack Ndfip1.

Discussion

The data presented in this chapter support a role for the adaptor protein Ndfip1 in the negative regulation of naïve CD4^{+} T cell activation. After stimulation through the T cell receptor but in the absence of CD28 signaling, Ndfip1 prevents the expression of IL-2 and, thus, naïve T cell activation. Ndfip1$^{-/-}$ CD4^{+} T cells upregulate IL-2Rα surface levels, make IL-2 and proliferate much more than Ndfip1-suficient T cells upon TCR signaling alone. By controlling T cell activation in the absence of CD28 co-stimulation, Ndfip1 also prevents inflammation in mice. T cells from Ndfip1$^{-/-}$ CD28$^{-/-}$ mice become effectors and induce pathology much like T cells from Ndfip1$^{-/-}$ mice. These pathologic consequences are due to intrinsic defects in T cells lacking Ndfip1 since a comparable inflammation results when mice lack Ndfip1 only in cells of the T cell lineage. Thus, through the

regulation of IL-2 expression, Ndfip1 prevents T cell hyperactivity and promotes immune tolerance.

We have shown previously that Ndfip1 promotes Itch mediated ubiquitylation and degradation of JunB, thus dampening IL-4 production (Oliver et al., 2006). JunB accumulation and, hence, overproduction of IL-4 explain the Th2 bias of T cells lacking Ndfip1. However, this mechanism does not account for the increased IL-2 production. Supporting this, Ndfip1$^{-/-}$ T cells lacking IL-4 produce IL-2 when stimulated via their TCR in the absence of CD28 co-stimulation (Figure 14). Also, we do not observe increased levels of JunB in Ndfip1$^{-/-}$ T cells stimulated under these conditions (data not shown). Furthermore, Itch does not regulate the expression of IL-2 in naïve T cells after TCR stimulation (Figure 13A). Thus, Ndfip1 regulates IL-2 production in naïve T cells independently of JunB, IL-4 and Itch. Conversely, when T cells are differentiated into effectors *in vitro*, both Ndfip1$^{-/-}$ and Itch-deficient cells show more IL-2 production than their WT counterparts. Thus, Itch and Ndfip1 both regulate IL-2 production in effector differentiated CD4^{+} T cells. Based on our other studies, we propose that they collaborate in this task. It is interesting that Ndfip1 plays a role in dampening IL-2 production in both naive and antigen experienced cells, while Itch only impacts the latter. This suggests that Ndfip1 might be working through different mechanisms to control IL-2 expression in naïve and antigen-experienced T cells. As Ndfip1 can modulate the activity of many E3 ligases of the Nedd4 family,

future studies will be needed to identify whether Ndfip1 is regulating the activity of other Nedd4 family members in naïve T cells after TCR stimulation.

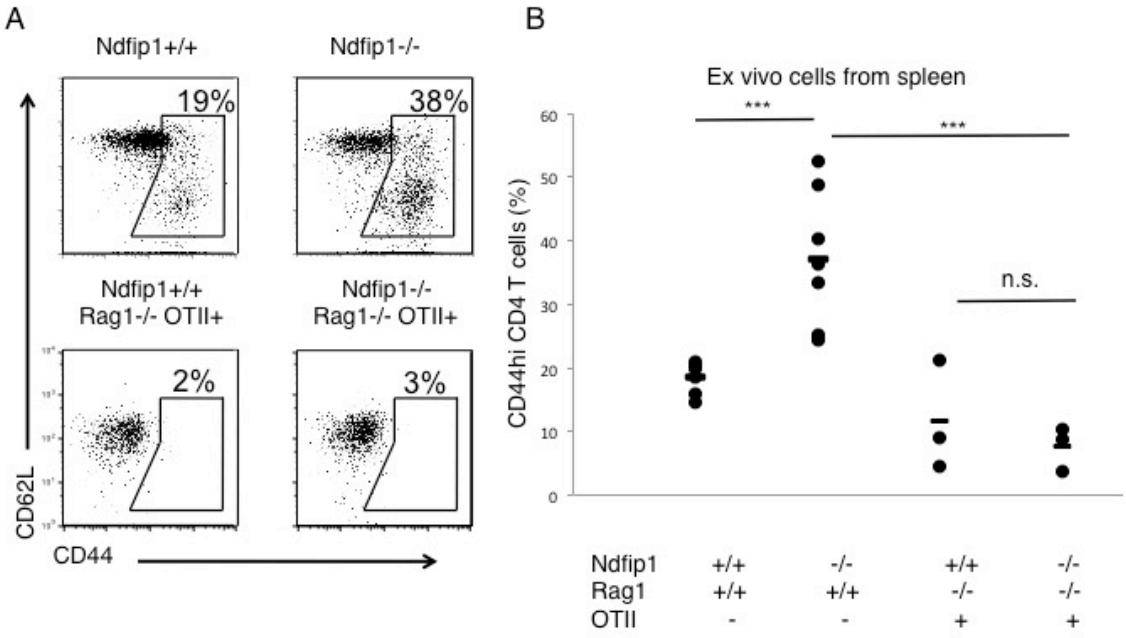
In naïve T cells, Ndfip1 limits IL-2 at the transcriptional level. Interestingly, Ndfip1 does not regulate initial transcription of IL-2. Instead, Ndfip1 restricts the duration of IL-2 transcription (Figure 11A). Both calcium-induced NFAT and signaling via Erk induce Ndfip1 expression upon TCR stimulation (Figure 12B). Given that IL-2 and Ndfip1 have similar expression patterns after TCR signaling, and that they rely on common transcription factors, suggests a negative feedback loop through which TCR signaling activates IL-2 production but also limits IL-2 expression via Ndfip1.

However, not all of the signaling proteins that induce IL-2 expression are evenly contributing to the expression of Ndfip1. Of the factors assessed, NFAT and Erk were more strongly involved in the expression of Ndfip1 compared to JNK and Pi3K (Figure 12B). It is possible that NFAT and Erk were more involved in the expression of Ndfip1 because the activation of these two proteins, and their respective signaling pathways, is less dependent on co-stimulatory signals than the other factors (Adachi and Davis, 2010, Macián et al., 2002). When T cells are stimulated in the absence of co-receptor signaling, NFAT opposes T cell activation. It does so by inducing the expression of genes that promote unresponsiveness, such as Grail (Soto-Nieves et al. 2009) and Ikaros (Macián et al., 2002), among others. Our results suggest that in addition to these factors,

NFAT interferes with T cell activation by increasing expression of Ndfip1. Interestingly, the Ndfip1 promoter contains multiple putative sites for NFAT binding (data not shown), which suggests that NFAT may directly regulate Ndfip1 expression. Based on these data, we propose that the negative regulatory factor Ndfip1 limits IL-2 production in the absence of co-stimulation, thereby preventing T cells from responding to low affinity signals such as those coming from self-peptides or environmental antigens.

Figures and Legends

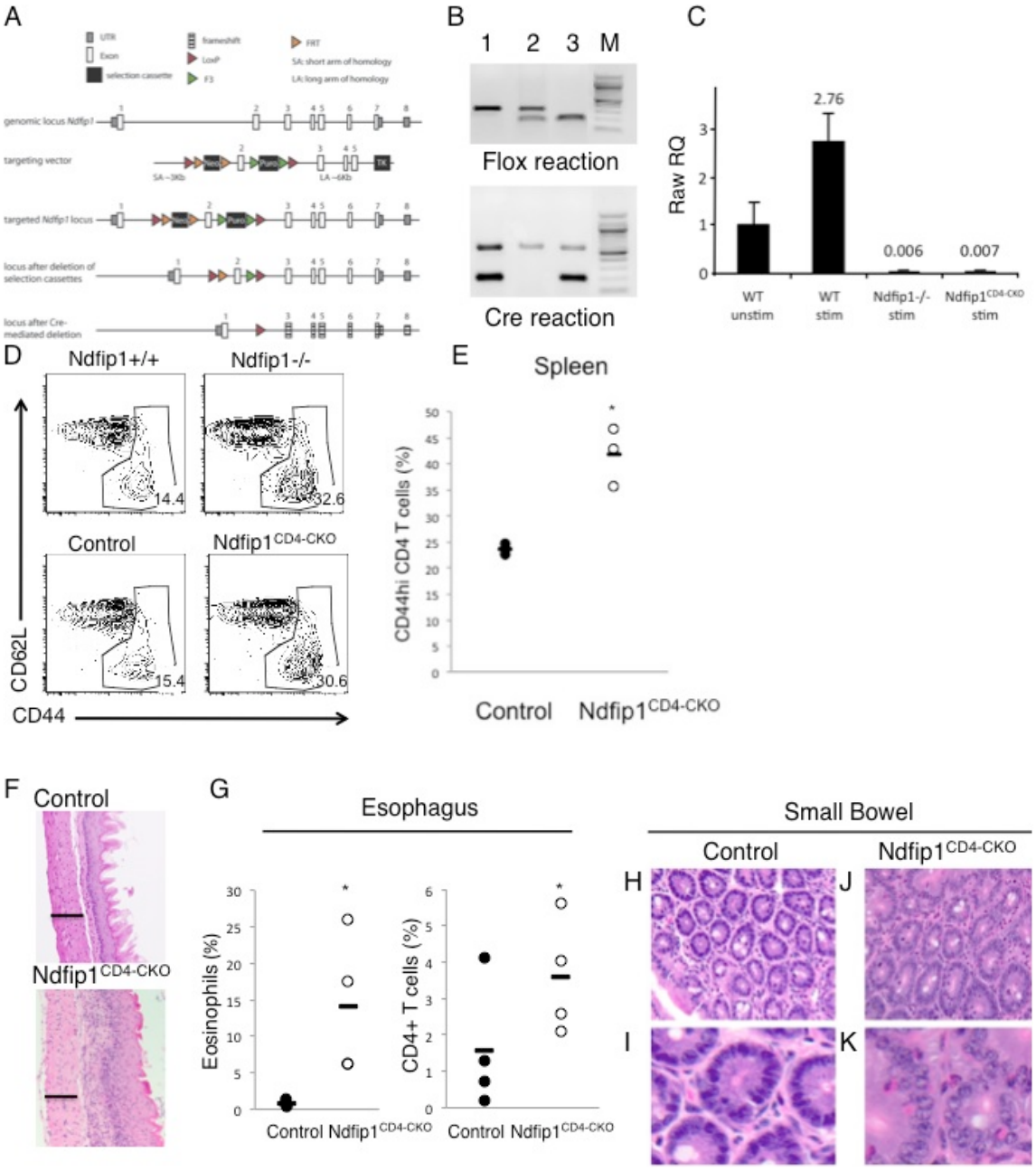
Figure 6



Activated phenotype of T cells in mice lacking Ndfip1 is antigen-dependent

(A, B) Spleen cells were isolated from mice and analyzed by flow cytometry. Representative plots (A) or combined data (B) of $CD4^+$ T cells from 3-7 mice per genotype. (A) Gate shown in plot illustrates how we define the percent of "activated" T cells. Numbers represent percentage of cells in gate. (B) Graph representing 'activated' phenotype cells illustrated in A. Each dot represents the percentage of cells in the spleen of a single mouse. Lines represent mean for each sample population and P values are for samples connected by the line. *** represents a P value of <0.001. n.s. stand for non-significant, with a P value greater than 0.05 based on a paired T test. This analysis was performed in at least three independent experiments.

Figure 7



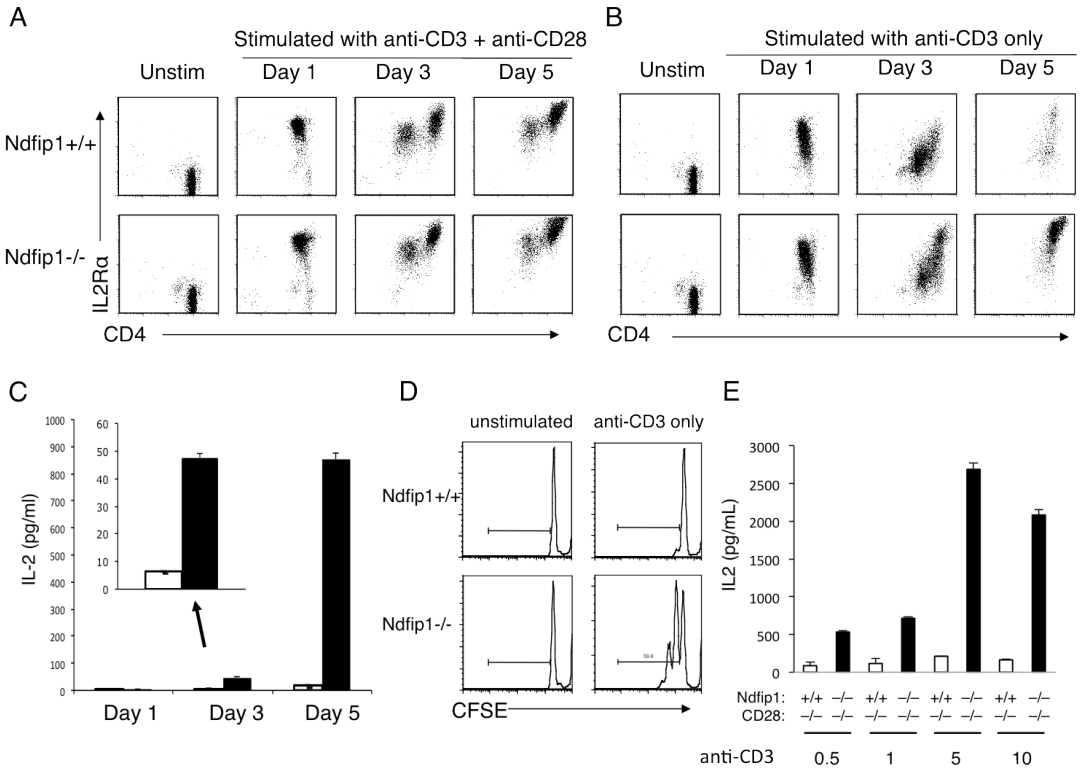
Activation of T cells in the absence of Ndfip1 is T cell intrinsic

(A) Model illustrating T cell specific deletion of Ndfip1. The targeting vector was designed to introduce loxP sites on either side of exon 2 of the gene encoding Ndfip1. Correct insertion of the vector into the Ndfip1 locus is shown as ‘targeted Ndfip1 locus’. Selection cassettes were removed using flp recombinase. Mice

harboring the floxed exon 2 were crossed to CD4-cre mice to generate mice with T cells lacking Ndfip1 as illustrated at bottom of panel. In this Ndfip1 locus, Cre-mediated deletion results in loss of Exon 2 and the remaining exons are out of frame (illustrated by frameshift). (B) Mice harboring the floxed allele were analyzed by PCR and representative results are shown in upper panel. The upper (slower migrating) band shows the WT locus and the faster migrating band shows the floxed locus. Presence of Cre was also determined by PCR and is shown in the lower panel. The slower migrating band is an internal positive control while the faster migrating band reflects the presence of Cre. Lane 3 shows the results indicating a mouse with a T cell specific deletion of Ndfip1 (designated by $Ndfip1^{CD4\text{-}CKO}$). (C) Loss of Ndfip1 expression in $Ndfip1^{CD4\text{-}CKO}$ mice was analyzed using qPCR. WT and $Ndfip1^{CD4\text{-}\ CKO}$ $CD4^{+}$ T cells were stimulated with anti-CD3 and anti-CD28 for 24 hours and analyzed using primers for Ndfip1. Ndfip1 mRNA levels were normalized to an internal control and data shown are relative to WT unstimulated levels. Bars represent the mean and lines show standard deviation of triplicate samples. Data are representative of 2 different experiments using a total of 4 mice per genotype. (D) Activation profiles of $CD4^{+}$ gated T cells from spleens of mice of the indicated genotypes are shown. Gates indicate “activated” phenotype cells and numbers indicate the percentage of cells falling within that gate. (E) Percentages of activated phenotype T cells in spleens of mice as determined by flow cytometry in (D). Data are representative of mice from at least three independent experiments. At least 5 mice of each genotype were analyzed. Each dot represents percentages

of cells from a single mouse, bars represent the mean (F) Sections of the esophagus of 6-week old mice were analyzed by histology using H and E stains. Bar illustrates 100 microns. (G) Cells were isolated from the esophagus and analyzed by flow cytometry for eosinophils ($SiglecF^{+}$) or T cells ($CD4^{+}$). Each dot represents percentages of cells from a single mouse, bars represent the mean. At least four mice were analyzed from each genotype. (H-K) Small bowel sections from control mice (H and I) or $Ndfip1^{CD4\text{-}CKO}$ mice (J and J). Line represents 100 microns. I and K show inset from panels H and J, respectively. Eosinophils can be seen in the inset from $Ndfip1^{CD4\text{-}CKO}$ mouse. (F, H-K) Images are representative of at least three mice from each genotype from two independent experiments. * represents a P value of <0.05.

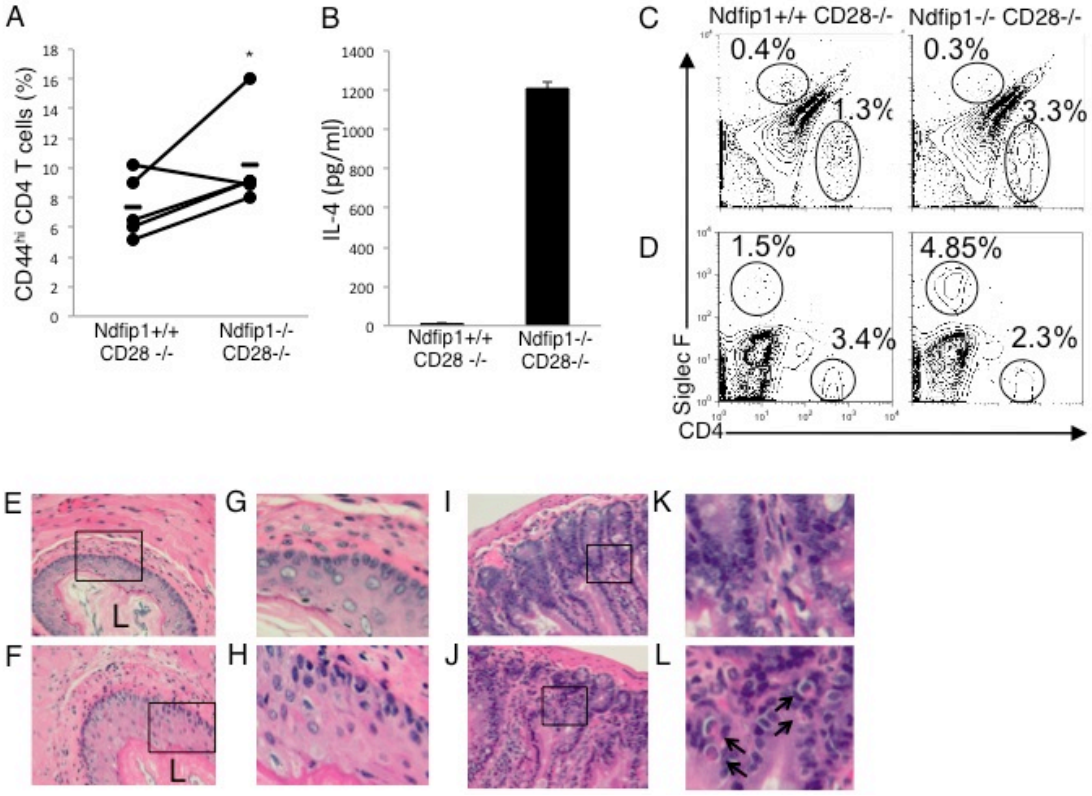
Figure 8



T cells lacking Ndfip1 can produce IL-2 and proliferate in the absence of CD28 co-stimulation (A-F) Naïve ($CD44^{lo}$ $CD62L^{hi}$ $CD25^{-}$) CD4+ T cells were isolated from $Ndfip1^{-/-}$ mice or $Ndfip1^{+/+}$ littermate controls and stimulated *in vitro* with anti-CD3 and anti-CD28 (A, E) or anti-CD3 only (B-E). (A and B) Cells were analyzed for levels of IL-2Rα by flow cytometry at the indicated time points. Plots are representative of three independent experiments from 3 mice of each genotype. (C) Supernatants were analyzed for IL-2 by ELISA at time points indicated. Graphs are of representative data from three independent experiments, showing a single comparison of genotypes performed in triplicate. White bars represent cultures of $Ndfip1^{+/+}$ T cells and black bars show $Ndfip1^{-/-}$

T cells. Bars represent the mean and lines show the standard deviation of the triplicate samples. (D) Cells described in panel B were labeled with CFSE immediately prior to stimulation and analyzed at day three for proliferation. Histograms showing CFSE dilution are representative of at least three independent experiments. (E) IL-2 levels in cultures of Ndfip1$^{+/+}$CD28$^{-/-}$ and Ndfip1$^{-/-}$CD28$^{-/-}$ T cells stimulated with various concentrations of anti-CD3 as noted and analyzed at day three. White bars are Ndfip1$^{+/+}$CD28$^{-/-}$ T cells and black bars are Ndfip1$^{-/-}$CD28$^{-/-}$ T cells. Bars represent the mean and lines show the standard deviation of the triplicate samples.

Figure 9

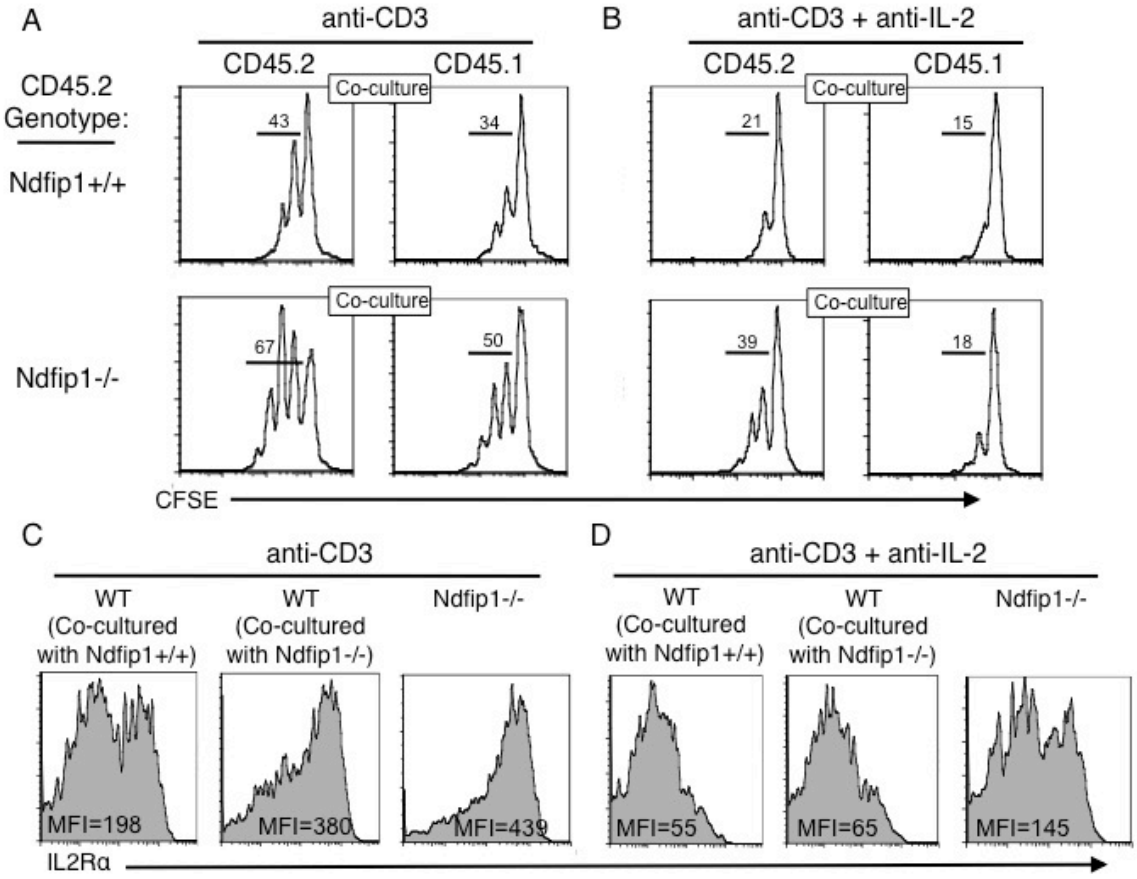


T cells in mice lacking both Ndfip1 and CD28 become activated, make IL-4 and migrate into the gastrointestinal tract

(A) Percentages of activated phenotype cells among the $CD4^{+}$ cells in the spleens of 5-10 wk old $Ndfip1^{-/-}$ $CD28^{-/-}$ mice or $Ndfip1^{+/+}$ $CD28^{-/-}$ littermate controls. Each dot represents a single mouse and data are representative of 4 independent experiments. Dots connected by lines show age matched littermate pairs in each experiment. * represents a P value of <0.05 based on a paired T test. (B) Cells were isolated from spleens of mice and stimulated overnight with anti-CD3. IL-4 in the supernatants was determined using ELISA. Data are representative of three independent experiments from three mice of each genotype. Bars represent the mean and error bars indicate standard deviation of

three triplicate samples. (C and D) Cells isolated from esophagus (C) and small bowel (D) following collagenase treatment were analyzed by flow cytometry for eosinophils (SiglecF^{+}) or T cells (CD4^{+}). Representative plots from at least three independent experiments are shown. (E-H) Sections of esophagus from Ndfip1$^{+/+}$CD28$^{-/-}$ mice (E and G) or Ndfip1$^{-/-}$CD28$^{-/-}$ mice (F and H). E and F were taken at 20X magnification, while G and H are insets depicted by boxed regions in E and F. Epithelial thickening can be seen in G and H. L indicates the lumen for visual reference. Representative of three mice of each genotype from three independent experiments. (I-L) Sections of small bowel from Ndfip1$^{+/+}$CD28$^{-/-}$ mice (I and K) or Ndfip1$^{-/-}$CD28$^{-/-}$ mice (J and L). I and J were taken at 20X magnification, while K and L are insets depicted by boxed regions in I and J. Eosinophils can be seen in insets and are indicated by arrows. Representative of three mice of each genotype from three independent experiments.

Figure 10

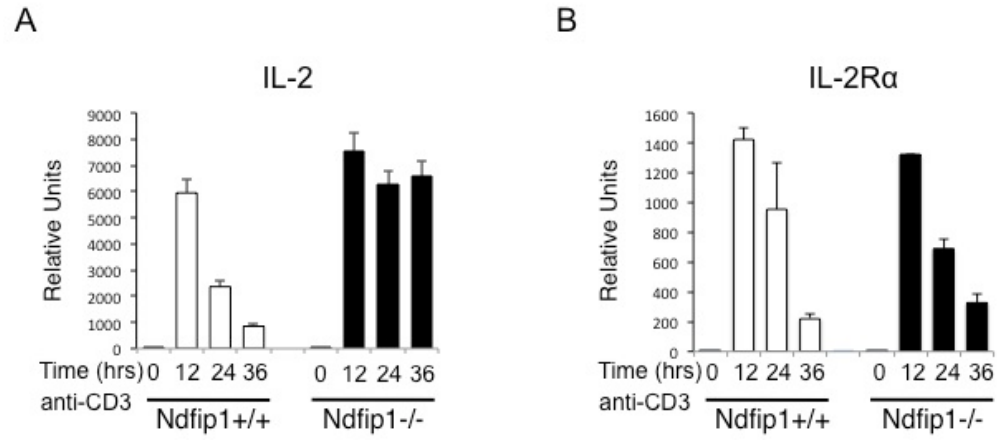


IL-2 production by Ndfip1$^{-/-}$ T cells can increase IL-2Rα levels and allow proliferation of WT T cells

(A-D) Naïve Ndfip1$^{+/+}$ or Ndfip1$^{-/-}$ T cells (both are CD45.2) were mixed with equal numbers of WT (CD45.1) T cells, labeled with CFSE, and cultured in the presence of anti-CD3. Data are representative of two independent experiments, which together represent 4 mice from each genotype. (A) After 3 days, cells were analyzed for proliferation as determined by CFSE dilution and results are shown in histograms. (B) Anti-IL-2 was added to the cultures at day 0 and cells were again analyzed at day three for proliferation as described for panel A. (C) IL-2Rα levels were analyzed on the cells in panel A. Mean fluorescence intensities

(MFI) for the population are shown within each histogram. (D) IL-2Rα levels were analyzed on the cells in panel B. The MFI for the population are shown within each histogram.

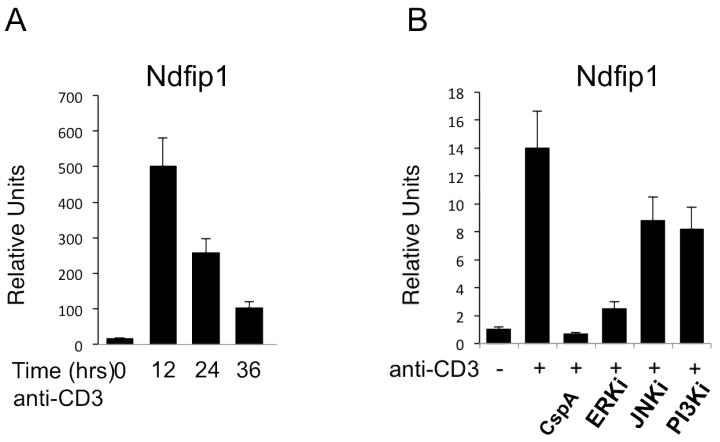
Figure 11



Ndfip1$^{-/-}$ T cells express more IL-2 mRNA upon TCR stimulation

(A-B) Naïve CD4^{+} T cells were stimulated with anti-CD3 and mRNA expression for IL-2 (A) and IL-2Rα (B) was analyzed at the indicated time points. mRNA levels were normalized to an internal control and data shown are relative to WT unstimulated levels. Bars represent the mean and lines show standard deviation of triplicate samples. Data is representative of 2 different experiments using at least total of 4 mice per genotype.

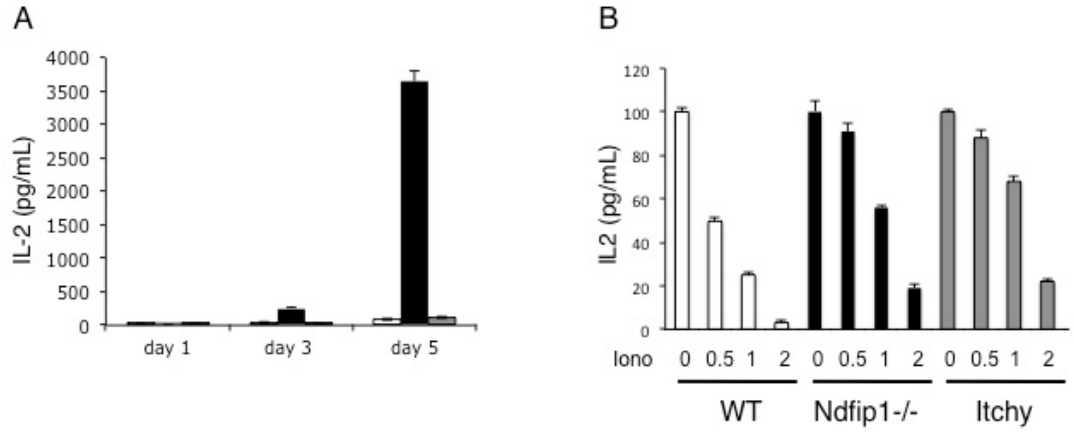
Figure 12



Inhibitors for NFAT and Erk prevent the expression of Ndfip1 in TCR-stimulated cells

(A-B) Naïve $CD4^+$ T cells were stimulated with anti-CD3 and Ndfip1 mRNA levels were measured at the indicated time points (A) or after overnight stimulation (B). (B) Cells were cultured in the presence of the indicated inhibitors. Cyclosporine A (cspA) inhibits NFAT. (A-B) mRNA levels were normalized to an internal control and data shown are relative to unstimulated levels. Bars represent the mean and lines show standard deviation of triplicate samples. Data is representative of 3 different experiments using three or more mice per genotype.

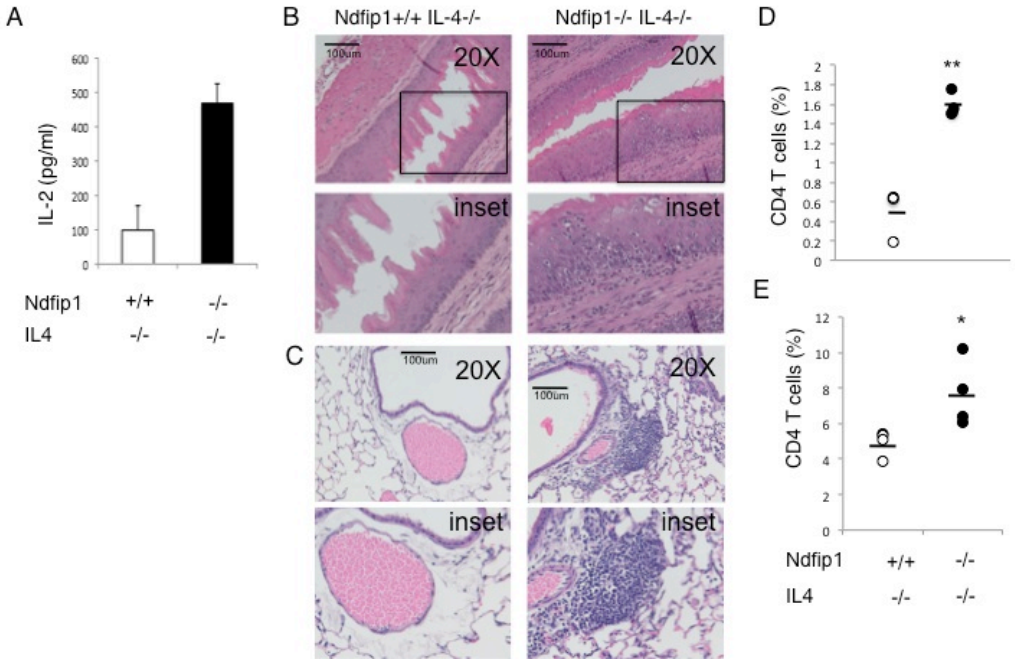
Figure 13



Ndfip1 limits IL-2 production after activation of naïve T cells via an Itch independent mechanism

(A) Naïve T cells from WT, Ndfip1$^{-/-}$ and Itch-deficient mice (Itchy) were sorted and stimulated with anti-CD3. Supernatants were analyzed for IL-2 at the various time points by ELISA. (A-B) Data are representative of at least three independent experiments. White bars represent cultures of Ndfip1$^{+/+}$ T cells, black bars show Ndfip1$^{-/-}$ T cells and gray bars represent Itch-deficient T cells. Bars represent the mean and error bars indicate standard deviation of three triplicate samples. (B) CD4^{+} sorted as described in methods as described in methods were stimulated with the various concentrations of ionomycin. Cells were rested for 24 hours and then restimulated with anti-CD3 and anti-CD28. The amount of IL-2 in the supernatants was analyzed by ELISA.

Figure 14



Ndfip1$^{-/-}$ IL-4$^{-/-}$ T cells produce high levels of IL-2 and promote inflammation

(A) IL-2 production from Ndfip1$^{+/+}$ IL-4$^{-/-}$ or Ndfip1$^{-/-}$ IL-4$^{-/-}$ T cells stimulated with anti-CD3 as measured by ELISA. Data are representative of three independent experiments from at least three mice of each genotype. (B-D) Cells isolated from the esophagus of Ndfip1$^{+/+}$ IL-4$^{-/-}$ and Ndfip1$^{-/-}$ IL-4$^{-/-}$ mice analyzed by flow cytometry. (B and C) Representative sections of esophagus (B) and lung (C) from Ndfip1$^{+/+}$ IL-4$^{-/-}$ and Ndfip1$^{-/-}$ IL-4$^{-/-}$ mice. Bars illustrate 100 microns and lower panels represent inset shown in upper panels. Similar results were observed in the sections from three mice analyzed from each genotype. (D) Cells were isolated from esophagi from Ndfip1$^{+/+}$ IL-4$^{-/-}$ and Ndfip1$^{-/-}$ IL-4$^{-/-}$ mice. Cells were analyzed using flow cytometry for percentages of T cells among live-gated

cells. Data were combined from three independent experiments using a total of three mice of each genotype. Bars are mean values of T cells ($CD4^+$) (E). Cells isolated from lungs from $Ndfip1^{+/+}$ $IL\text{-}4^{-/-}$ and $Ndfip1^{-/-}$ $IL\text{-}4^{-/-}$ mice were analyzed by flow cytometry for percentages of T cells among live-gated cells. Data were combined from three independent experiments using a total of four mice of each genotype. Bars are mean values of T cells ($CD4^+$) Each dot represents a single mouse. * represents a P value of <0.05 based on a paired T test while ** represents P<0.01.

Materials and Methods

Mice

Ndfip1$^{-/-}$ and Itchy mutant mice have been described previously (Oliver et al., 2006; Fang et al., 2002). CD45.1 (C57BL6.SJL-Ptprc^{a} Pepc^{b}/BoyJ mice, #002014), IL-4$^{-/-}$ (B6.129P2-Il4^{tm1Cgn}/J, #002253), CD28$^{-/-}$ (B6.129S2-*Cd28*^{tm1Mak}/J, #002666) OT-II^{+} (B6.Cg-Tg (TcraTcrb) 425Cbn/J, #004194) and Rag1$^{-/-}$ (B6.129S7-Rag1^{tm1Mom}/J, #002216) mice were purchased from the Jackson Laboratory. CD4-cre transgenic mice (B6.Cg-Tg (CD4-cre) 1Cwi N9, 4196) were purchased from Taconic. Ndfip1$^{CD4\text{-}CKO}$ mice were generated as described in Figure 7. All mice were housed in a barrier facility at the Children's Hospital of Philadelphia in accordance with the Institutional Animal Care and Use Committee protocol. For genotyping, DNA from tail biopsies was amplified by PCR using the following primers: Ndfip1 WT Forward: 5'TAGGCCAAGGTGAAAACTGG3'; Ndfip1 WT Reverse: 5'AGAGGTGGGTTCAACAGTGG3'. Ndfip1 knockout Forward: 5'CGACTTCCAGTTCAACATCAGC3'; Ndfip1 knockout Reverse: 5'GTCTGTTGTGCCCAGTCATAGC3'. Primers for IL-4$^{-/-}$, CD28$^{-/-}$, Rag1$^{-/-}$ and CD4-cre Tg mice are available on the Jackson Laboratories website (www.jaxmice.jax.org)

Tissue processing and cell isolation.

Spleen and lymph nodes were harvested and mashed through 70mm filters in cold Hank's Balanced Salt Solution (HBSS). Cell suspensions from spleens were treated with ACK Lysis Buffer to lyse red blood cells.
Esophagus and a 3-4" section of small bowel were flushed with cold PBS. Peyer's patches were removed from small bowel. Organs were minced with scissors and treated with DNAse (20ug/ml, Sigma D5025), collagenase type I (.8mg/ml, Sigma C0130) and collagenase type Ia (.9mg/mL, Sigma C2674) in DMEM for 1 hour in end-over-end rotation at room temperature. Cell suspensions were filtered through a 100mm filter, then a 40mm filter and 10% FCS was added. Cells were incubated for 10 minutes at 4°C with Fc Block (2.4G2, BD Biosciences) prior to antibody staining.

Flow Cytometry, Cell Sorting and antibodies

Flow cytometry was performed using a FACSCalibur or a BD LSR II(BD Biosciences, San Diego, CA). For flow cytometry, cells were stained with fluorescently labeled antibodies in 3% fetal bovine serum in PBS for 30 minutes at 4°C and washed. Naïve $CD4^{+}$T cells were isolated by sorting spleen and lymph node cells for $CD4^{+}CD25^{-}$ $CD44^{lo}$ and $CD62L^{hi}$ cells on the FACS Aria (BD Biosciences). The frequency of live cells was generated by gating live cells

on flow cytometric forward scatter (FSC)-side scatter (SSC) plots. CD25 (PC61.5) and CD62L (MEL-14) antibodies were obtained from eBiosciences (San Diego, CA). CD4 (GK1.5) and CD44 (IM7) antibodies were obtained from BioLegend (San Diego, CA). Siglec F (E502440) antibody was obtained from BD Biosciences (San Diego, CA).

Histology

Esophagus and sections of small bowel were dissected and fixed in 10% formalin for at least 24 hours. All organs were then embedded in paraffin, sectioned and stained with hematoxylin and eosin.

Enzyme-Linked Immunosorbent Assay (ELISA)

IL-2 and IL-4 ELISAs were performed on supernatants harvested at the indicated times from *in vitro* cell cultures. Assays were performed using Ready-Set-Go ELISA kits (eBiosciences) in Nunc-Immuno MicroWell 96 well solid plates (Thermo Scientific). Results were analyzed using a Synergy HT Microplate Reader (Bio Tek).

Co-Cultures, stimulation and CFSE

Naïve sorted $CD4^+$ T cells were stimulated with plate-bound anti-CD3 (145-

2C11, BD Biosciences) with or without anti-CD28 (37.51, BD Biosciences) antibodies (as indicated) (5µg/mL) for time points as indicated. Percentage of live cells was determined using flow cytometry by live-cell gating of events on *forward scatter* by *side scatter*. For figure 13B, $CD4^+$ T cells (not sorted for naïve) were stimulated with plate-bound anti-CD3 and anti-CD28 (5µg/mL) for 3 days and left resting for 2 days in IL-2 (50 u/mL) (ro 23- 6019, Hoffman-LaRoche). Cells were then stimulated with ionomycin (0-3 uM) for 16 hours, rested for 4 hours and re-stimulated with anti-CD3 and CD28 antibodies (5µg/mL). Culture supernatants were collected 24 hours after re-stimulation. For figure 12, the following inhibitors were used: Cyclosporine A (NFAT inhibitor) (239835, EMD Millipore), PI3K inhibitor (LY294002) (PHZ1144, Invitrogen), JNK inhibitor (S5567, Sigma) and Erk inhibitor (#513001, Calbiochem). Inhibitors were added to cultures after the first 24 hours of stimulation. Carboxyfluorescein succinimidyl ester (CFSE) labeling: Cells were re-suspended at a 1x10^7/mL concentration in PBS at room temperature and mixed at a 1:1 ratio with CFSE (C-1157, Invitrogen) in PBS for 4 minutes with constant agitation. Labeling process was quenched with FCS. Co-culture assays: CD45.1 and CD45.2 cells were mixed in a 1:1 ratio and CFSE-labeled as described above. Cells were cultured in the presence of anti-IL-2 (S4B6, BD Biosciences) and IL4 (11B11, Biolegend) antibodies where specified.

qPCR

RNA from harvested cells was isolated with the RNeasy Mini Kit (Qiagen). RNA-to-cDNA reactions were done using the High Capacity RNA-to-cDNA Kit (Applied Biosystems). For qPCR reactions, TaqMan Gene Expression Master Mix was used (4370048, Applied Biosystems). The Ndfip1 primer/probe set has been previously described (Beal et al., 2011). FAM dye, MGB primer/probes sets for IL-2 (Mm00434256_m1), IL-2Rα (Mm01340213_m1) and ACTB (4352933E) were obtained from Applied Biosystems. Samples were amplified in triplicate using the 7500 Real-Time PCR system (Applied Biosytems). Data were analyzed using the 7500 software v2.0 (Applied Biosystems).

Statistical Analysis

All statistical analyses were performed using Student's *t*-tests unless stated otherwise. A *P*-value of equal or less than 0.05 was used to determine statistical significance.

CHAPTER 4

DISCUSSION

Ndfip1 restrains the levels of IL-4 in differentiated T cells (Figure 15) and limits IL-2 expression in naïve T cells (Figure 16). By limiting the expression of these two pro-inflammatory cytokines, both directly and indirectly, Ndfip1 supports T cell tolerance and ensures the balance between responses that promote pathogen clearance and those that control immunopathology.

Ndfip1 controls IL-4 expression in effector CD4$^+$ T cells

Previously, our lab showed that the adaptor protein Ndfip1 limits IL-4 expression under Th2 stimulating conditions and, consequently, Th2 cell differentiation. (Oliver et al., 2006). The E3 ubiquitin ligase Itch ubiquitylates and degrades the transcription factor JunB in activated CD4$^+$ T cells (Fang et al., 2002). Ndfip1 binds to Itch and facilitates this process. As JunB promotes the transcription of IL-4, activated CD4$^+$ T cells lacking Ndfip1 secrete IL-4 and are more prone to adopt a Th2 phenotype.

Here we show that, by limiting IL-4 production, Ndfip1 regulates biological processes beyond Th2 differentiation. For example, in chapter 2 we show that Ndfip1 also limits the production of IL-4 under conditions that promote the generation of iTregs. Naïve T cells that lack Ndfip1 cannot become Foxp3$^+$ iTregs. Instead, these cells make IL-4 upon iTreg differentiation, preventing them from expressing Foxp3. Supporting this, T cells that lack Ndfip1 and IL-4 restore

iTreg differentiation. Additionally, Ndfip1$^{-/-}$ IL-4$^{-/-}$ mice are not as sick as Ndfip1$^{-/-}$ mice. This supports resumed iTreg function. These results are in agreement with previous findings showing that IL-4 hinders iTreg conversion of WT naïve T cells (Dardalhon et al., 2008) but provide new mechanistic details of how this is controlled. Additionally, these results show that Ndfip1 restrains the levels of IL-4 in circumstances beyond Th2 cell differentiation. Ndfip1 provides an intrinsic method to limit IL-4 production and guarantee Foxp3 expression under iTreg conditions.

The inability of these naïve Ndfip1$^{-/-}$ T cells to differentiate into iTregs is not the only threat to peripheral T cell tolerance imposed by IL-4 overproduction. Naïve T cells from WT mice stimulated in the presence of TGF-β and IL-4 can become pathogenic Th9 cells (Dardalhon et al., 2008). Mice that lack Ndfip1 have increased frequencies of Th9 cells, whereas, Ndfip1$^{-/-}$ IL-4$^{-/-}$ mice do not (Ramon et al., 2012). Thus, naïve Ndfip1$^{-/-}$ T cells stimulated under iTreg conditions are likely becoming Th9 cells as well. If so, by controlling IL-4 expression in the presence of TGF-β, Ndfip1 is promoting tolerance in at least to ways: it is supporting suppression by iTregs and, also, preventing the generation of pathogenic Th9 cells.

It is reasonable to hypothesize that Ndfip1 limits IL-4 production upon differentiation into all CD4^{+} T cell subtypes. The cytokine environment

determines T cell differentiation into particular subtypes. Cytokine signaling, thus, directs T cells towards the responses appropriate for pathogen clearance while minimizing self-damage. By limiting IL-4 expression, Ndfip1 promotes iTreg differentiation and controls differentiation into Th2 cells and, possibly, into Th9 cells. Also, data from our lab shows that Ndfip1 controls IL-4 production to promote Th17 differentiation (Ramon et al., 2012). Naïve Ndfip1$^{-/-}$ T cells stimulated under Th17 conditions make little IL-17 in comparison to WT cells. However, Th17 conversion is restored in Ndfip1$^{-/-}$ T cells differentiated in the presence of IL-4 blocking antibody. Nonetheless, together, these data suggest that, by limiting IL-4 production, Ndfip1 controls the differentiation of CD4^{+} T cell subtypes and, consequently, promotes a balance between responses against pathogens and tolerance.

T cells are not the only producers of IL-4 among immune cells. Cells from the myeloid compartment, such as basophils and eosinophils, can also secrete IL-4 (Kasaian et al., 1996; La Flamme et al., 2012; Sabin et al., 1996). Thus, it is possible that Ndfip1 is controlling IL-4 expression in the myeloid cell compartment as well as in T cells. This possibility has not been assessed. However, as detailed in chapter 3, mice with a systemic deletion of Ndfip1 and mice with a T cell specific deletion of Ndfip1 possess very similar phenotypes. This suggests that Ndfip1 is preventing pathogenicity primarily by controlling functions in the T cell compartment.

By limiting the expression of IL-4, Ndfip1 prevents the development of pathology. Studies shown in chapter 3 confirm that there is more to Ndfip1 than the control of IL-4 expression. However, disease development at mucosal surfaces is greatly prevented by this role. This is evidenced when comparing mice that lack Ndfip1 and mice that lack both Ndfip1 and IL-4 (detailed in chapter 3). We hypothesize that the protection of barrier surfaces by Ndfip1-suficient iTregs strongly contributes to this. Thus, by limiting the expression of IL-4, Ndfip1 does beyond controlling cell differentiation. Ndfip1 promotes immune tolerance and supports healthy mucosal surfaces.

Ndfip1 limits IL-2 expression in naive CD4$^+$ T cells

Co-stimulatory signals, such as those from CD28, supplement the signals provided by the T cell receptor to enhance the activation of naive CD4$^+$ T cells. APCs provide co-stimulation to naïve CD4$^+$ T cells in the form of CD80 and CD86, to which the CD28 molecule on the surface of the T cell can bind (Linsley et al., 1991). APCs become activated and upregulate CD80 and CD86 in response to microbial or viral products. Hence, APCs, via CD80 and CD86, activate T cells. Co-stimulation allows activated T cells to make significant amounts of IL-2. APCs can also present antigen to naïve T cells in the absence of pathogen invasion. In this scenario, antigen presentation results in tolerance rather than immunity (Jenkins and Schwartz, 1987). The lack of co-stimulatory signals leads to little production of IL-2. Under these circumstances, the antigen

belongs either self or is a harmless environmental antigen. This allows T cells to remain tolerant to such antigens, and prevents harmful T cell responses (Josefowicz et al., 2012a).

T cells have intrinsic mechanisms of tolerance that limit the production of IL-2 after antigen presentation in the absence of co-stimulation. Signaling proteins that instruct IL-2 expression are weakly recruited to TCR signal transduction pathways in the absence of co-stimulatory signals (Smith-Garvin et al., 2009). Additionally, TCR signaling in the absence of co-stimulation can, instead, actively interfere with further IL-2 expression. In the absence of co-stimulation, some signaling proteins activated upon TCR signaling instruct the expression of proteins that, through diverse methods, prevent IL-2 from being transcribed (Macian et al., 2002; Soto-Nieves et al., 2009). Consequently, the expression of IL-2 under these conditions is decreased.

Ndfip1 controls the activation of naive $CD4^{+}$ T cells by limiting the production of IL-2 upon TCR signaling in the absence of CD28 co-stimulation. While Ndfip1-suficient naïve T cells require CD28 co-stimulation to express IL-2, $Ndfip1^{-/-}$ T cells do not. Additionally, T cells that lack Ndfip1 and CD28 become activated, secrete IL-4 and move into tissues, where they can induce inflammation. Finally, our data show that Ndfip1 is required to limit IL-2 expression upon TCR stimulation. Ndfip1 does not restrain the initial amount of IL-2 expressed but

limits the duration of its expression. By limiting IL-2 expression in this manner, Ndfip1 promotes T cell tolerance.

Together these data support a model in which TCR signaling drives a negative feedback mechanism to control the duration of IL-2 expression via Ndfip1. Like IL-2, the expression of Ndfip1 is induced upon TCR stimulation and then declines after 24 and 36 hours of stimulation (Figure 12A). That Ndfip1 is needed to limit the duration of IL-2 expression and that Ndfip1 and IL-2 present similar patterns of decreased expression upon TCR stimulation suggest that Ndfip1, in addition to regulating the expression of IL-2 could also be regulating its own expression. However, whether through self-regulation or alternate processes, it remains unknown why the levels of Ndfip1 decline following the initial increase (Figure 12A). One possibility is that, by limiting the expression of Ndfip1, IL-2 expression could occur if TCR stimulation takes place together with CD28 co-stimulation. This scenario assumes that Ndfip1 is not needed when T cells are stimulated in the presence of CD28 co-stimulation. In this case, by controlling the expression of Ndfip1, a T cell would be establishing a balance between tolerance and activation, subject to whether TCR stimulation occurs by itself or in the presence of CD28 co-stimulation, respectively.

While our data investigates the role of Ndfip1 in IL-2 expression of naïve T cells stimulated through the TCR alone, results from chapter 3 do not assess whether Ndfip1 also controls the levels of IL-2 in naïve T cells stimulated through the TCR

in the presence of CD28 co-stimulatory signals. As explained above, IL-2 expression occurs at its fullest in the presence of co-stimulation. Thus, if the factors that induce the expression of IL-2 are also inducing the expression of Ndfip1, it is possible that Ndfip1 is also expressed and controlling IL-2 expression in cells that receive co-stimulation. Further supporting this hypothesis, the Ndfip1 promoter contains binding sites for both NFAT and the AP-1 complex (data not shown). These transcription factors work together and induce the expression of IL-2 (Smith-Garvin et al., 2009). While NFAT can be activated in the absence of co-stimulation, this is not the case for AP-1. Co-stimulatory signals supplement TCR signals for the effective formation of the AP-1 complex (Lenschow et al., 1996). Thus, through the formation of the AP-1 complex, co-stimulation would enhance the expression of both IL-2 and Ndfip1. Moreover, Ndfip1 would limit the expression of IL-2 whether CD28 co-stimulation is given or not.

An alternate possibility is that Ndfip1 limits IL-2 production only when co-stimulatory signals are absent. There is precedence for this. For example, the DNA-binding protein Ikaros is expressed to hinder IL-2 expression only when co-stimulation is not received (Macian et al., 2002). Interestingly, NFAT is required for both the expression of Ndfip1 and Ikaros. Thus, upon TCR signaling alone, NFAT could be inducing the expression of Ndfip1 to control IL-2 transcription.

Ndfip1 limits the expression of IL-2 independent of its ability to restrain JunB levels. In Th2 cells and iTregs, Ndfip1 aids in the degradation of JunB.

Consequently, Ndfip1 controls the expression of IL-4, a target of JunB, in these cells (Beal et al., 2012; Oliver et al., 2006). However, Ndfip1 limits IL-2 through a mechanism other than the regulation of JunB levels. Supporting this, JunB protein does not accumulate in naïve Ndfip1$^{-/-}$ T cells more than in WT cells following TCR stimulation alone (data not shown). Therefore, we hypothesize that Ndfip1 is regulating IL-2 expression via other mechanisms. The only known binding partners of Ndfip1 are Nedd4-family E3 ubiquitin ligases, which can induce protein degradation. Thus, we speculate that, in the absence of CD28 signals, Ndfip1 is promoting the degradation of proteins that induce IL-2 expression via interaction with a Nedd4-family E3 ligase. Alternatively, Ndfip1 could be causing a decrease in the mRNA levels of IL-2 upon TCR engagement by controlling IL-2 mRNA stability or IL-2 locus accessibility. Further studies will be needed to determine this.

Ndfip1 regulates T cell function by promoting the activity of Itch and related E3 ligases

Ndfip1 regulates the activity of Itch under circumstances besides Th2 cell differentiation. *In vitro,* Ndfip1 binds to and regulates the activity of many members of the Nedd4 family of E3 ligases. In T cells, specifically in Th2 cells, it has only been shown that Ndfip1 regulates the activity of the Nedd4 family member Itch (Oliver et al., 2006). In the presence of Ndfip1, Itch binds to JunB, ubiquitylates it and, thus, instructs its degradation. By degrading JunB, Itch

controls the expression of IL-4. Here, I present data showing that Ndfip1 and Itch also work together to restrict IL-4 production in iTregs. T cells that lack Itch and T cells that lack Ndfip1 differentiate poorly into iTregs. However, defective iTreg differentiation in Ndfip1$^{-/-}$ T cells is much more profound that in Itchy-mutant T cells (Beal et al., 2012). Thus, Ndfip1 may be regulating iTreg differentiation through collaboration with Itch as well as with other Nedd4 family members.

Naïve T cells do not require Itch to control the expression of IL-2, either. Naïve Ndfip1$^{-/-}$ CD4^{+} T cells make abundant IL-2 upon TCR stimulation in the absence of CD28 signals. Under the same conditions, Itchy mutant T cells control the expression of IL-2 and behave as WT T cells. Thus, while its ability to control IL4 expression is, at least, partially dependent on its regulation of Itch, Ndfip1 controls the expression of IL-2 in naïve T cells fully independent of Itch. These results suggest another scenario where Ndfip1 regulates cytokine expression through its collaboration with another E3 ligase of the Nedd4 family.

While Itch does not limit the expression of IL-2 in naïve CD4^{+} T cells, both Ndfip1 and Itch prevent the hyperactivity of antigen-experienced T cells following stimulation. In the absence of either Itch or Ndfip1, differentiated T cells make more IL-2 than WT T cells. It is possible that Ndfip1 and Itch collaborate to control the activation of antigen-experienced T cells. JunB overexpression can induce T cell hyperactivity (Li et al., 1999). Thus, it is possible that, by controlling the levels of JunB in differentiated cells, Ndfip1 and Itch collaborate to limit T cell

activation. Another possibility is that Ndfip1 is limiting effector T cell activation by promoting the ubiquitylation of additional targets of Itch that support T cell activation, such as TCR-zeta, PLC-γ and PKC-θ (Heissmeyer et al., 2004; Huang et al., 2010). Whether through the regulation of any of these factors or not, the results presented here support the idea that Ndfip1 controls the activation of naïve T cells and differentiated T cells through different mechanisms. This idea is not entirely new. For example, it has been suggested that the E3 ubiquitin ligase Cbl-b regulates T cell activation by different mechanisms in naïve T cells and differentiated T cells (Bachmaier et al., 2000; Chiang et al., 2000; Jeon et al., 2004).

In naïve T cells, the most likely scenario is that Ndfip1 is limiting IL-2 production by interacting with another E3 ligase of the Nedd4 family. Nedd4 family E3 ligases are the only known interacting partners of Ndfip1 (Mund and Pelham, 2009). Yet, prior to the studies detailed in chapter 3, Itch was the only known interacting partner of Ndfip1 in $CD4^+$ T cells. In addition to Itch, two E3 ligases of the Nedd4 family have been linked to T cell activation, WWP2 and Nedd4 (Chen et al., 2009; Yang et al., 2008). However, these two E3 ligases are thought to promote T cell activation. $CD4^+$ T cells that lack either WWP2 or Nedd4 are, thus, hyporesponsive to stimulation. It is possible that, by interacting with either one of these two ligases, Ndfip1 is preventing their function. Nedd4 promotes T cell activation by inducing the degradation of Cbl-b. Thus, by interacting with Nedd4, Ndfip1 might be preventing the degradation of Cbl-b and, hence,

preventing T cell activation. While a rather complex scenario, this might explain why Cbl-b deficient T cells and Ndfip1 deficient T cells have similar hyperactive phenotypes (Jeon et al., 2004). Another possibility is that Ndfip1 is preventing IL-2 expression in naïve T cells by interacting with a protein not previously considered, such as another Nedd4 member with unknown roles in T cell activation or a protein that does not belong to this family of E3 ligases. Future studies are needed to investigate the cooperation of Ndfip1 with other proteins to limit IL-2 expression in naïve $CD4^+$ T cells.

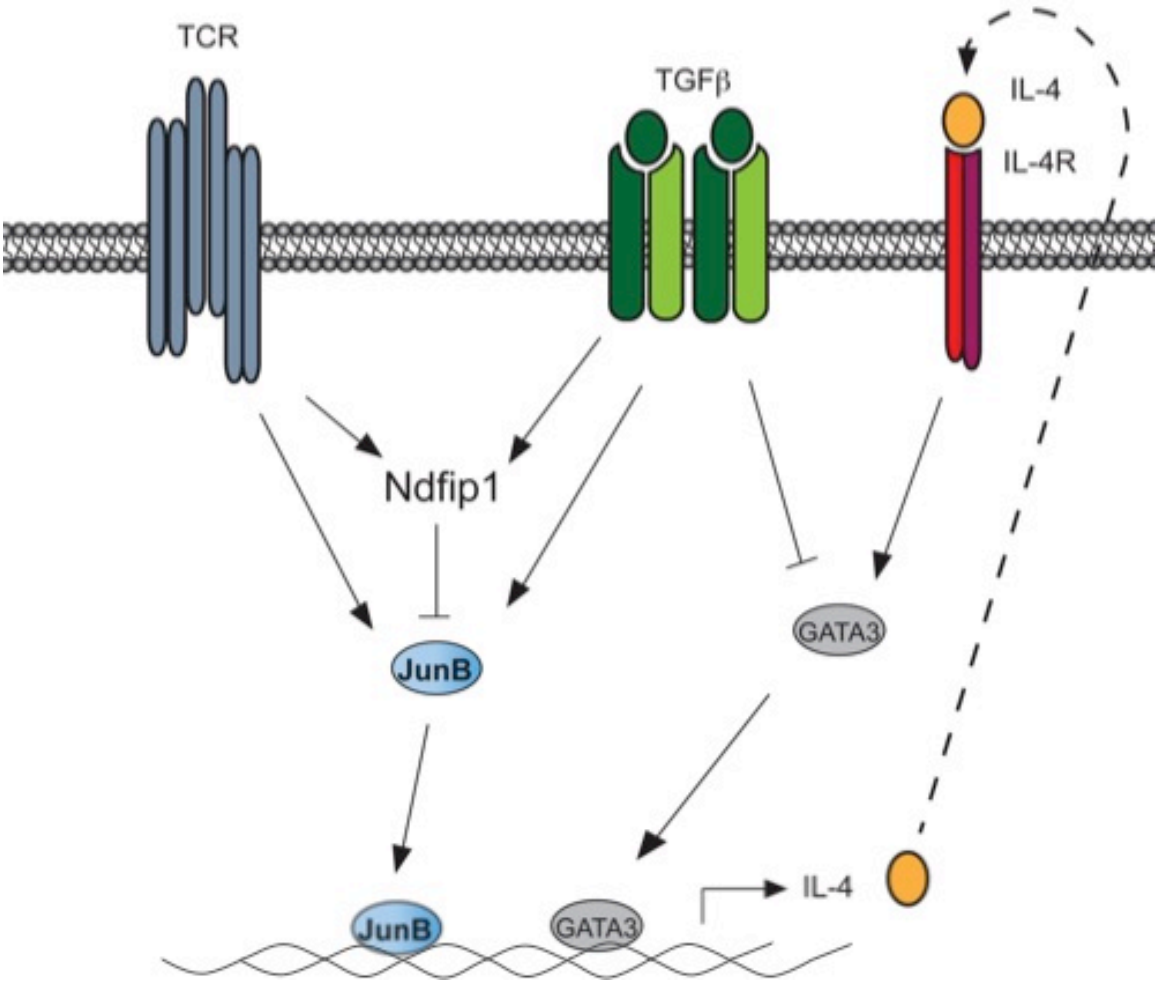
Ndfip1, T cell tolerance and disease

The adaptor protein Ndfip1 protects against atopic disease in mice and, most likely, in humans. Mice that lack Ndfip1 develop inflammation in the skin, gastrointestinal tract and lungs. Also, single nucleotide polymorphisms (SNPs) in the human Ndfip1 locus associate with inflammatory bowel disease, asthma and atopic dermatitis. However, SNPs in the Ndfip1 locus do not associate with the autoimmune diseases rheumatoid arthritis, type-1 diabetes or celiac disease (data not shown). This raises the question of whether Ndfip1 supports T cell tolerance by protecting against the development of autoimmune diseases as well. While not studied in detail yet, we have no reason to believe that Ndfip1 affects central T cell tolerance. Central T cell tolerance is largely responsible for the elimination of self-reactive T cells (Anderson et al., 2002). Thus, the normal function of mechanisms of central tolerance might explain the absence of

obvious signs of autoimmune disease in both Ndfip1-/- mice and individuals with SNPs in the Ndfip1 locus. However, functional central tolerance does not guarantee that self-reactive T cells do not exist in the periphery (Xing and Hogquist, 2012). Peripheral T cell tolerance mechanisms protect against such situations. Thus, both in mice and in humans, further study will be needed to evaluate whether Ndfip1 also promotes tolerance to self-antigens.

Ndfip1 is regulating the regulators of T cell tolerance. Here we propose that one or more members of the Nedd4 family of HECT-type E3 ligases, in addition to Itch, facilitate T cell tolerance. Several E3 ligases, including both RING- and HECT-type, have been linked to T cell tolerance (Paolino and Penninger, 2009). It is notable, however, that Ndfip1, a 221 amino acid long trans-membrane protein with no known catalytic activity, is responsible for so many tasks in T cells. Its ability to activate multiple members of the same family of enzymes puts it in a unique position. Its relevance is evidenced by the young age at which Ndfip1 mice develop disease and in its fatal course. Ndfip1 and its multiple roles in T cells provide an example of the complexity that characterizes and restrains the proper function of the immune system.

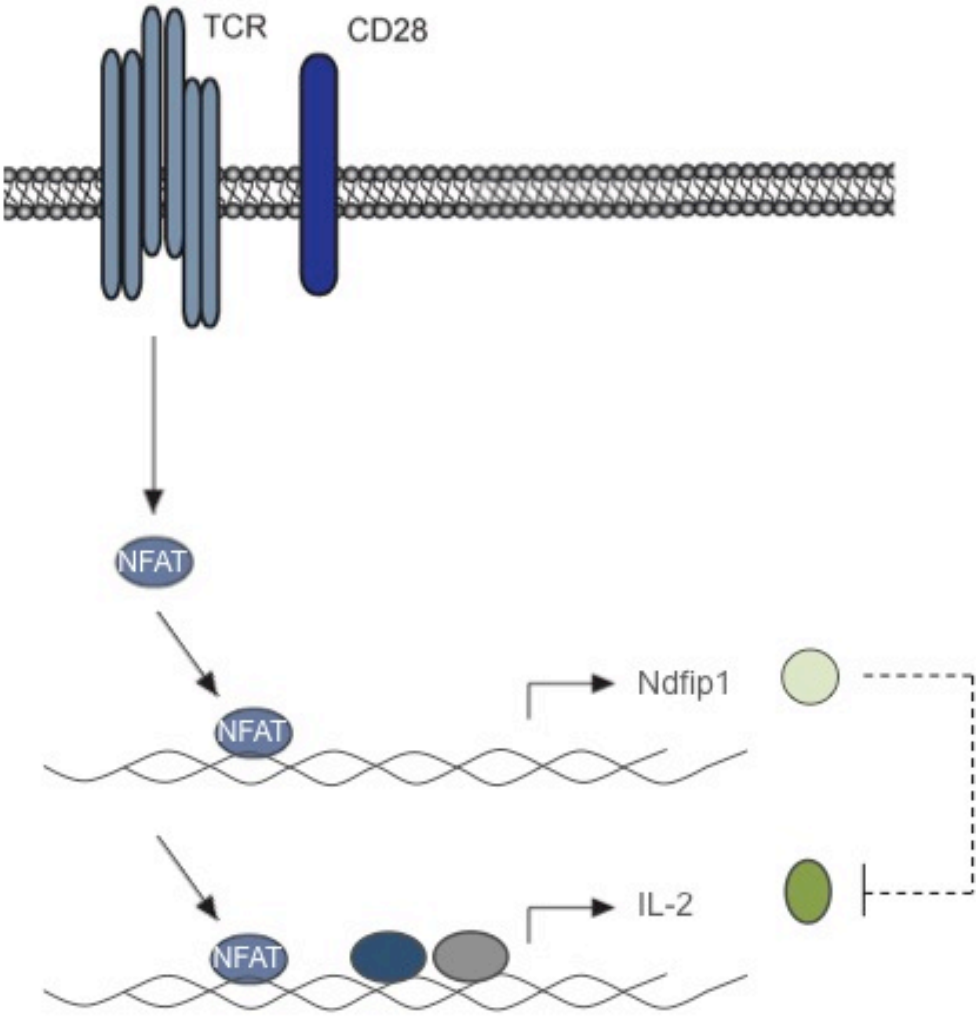
Figure 15



Ndfip1 limits the expression of IL-4 in differentiating iTregs

Ndfip1 is expressed under conditions that support iTreg differentiation. Ndfip1 assists Itch in the degradation of JunB. As JunB induces the expression of IL-4, by promoting its degradation, Ndfip1 prevents the expression of IL-4. This allows iTreg development.

Figure 16



Ndfip1 limits IL-2 expression in naïve T cells

Upon TCR signaling alone, NFAT induces the expression of IL-2 and Ndfip1. By promoting the expression of Ndfip1, TCR signaling creates a negative regulatory system to limit the extent of IL-2 expression and, thus, control T cell activation.

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