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Roles for the E3 Ligase Itch in Immune Response Modulation

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Roles for the E3 ligase Itch in Immune Response Modulation

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by

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Dedicated to

Hayley Elizabeth Thomson,

for reminding me why I started this journey in the first place.

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Table of Contents

List of Figures:

List of Tables:

Abstract

Cloning of the inversion breakpoints in the agouti mutant strain a^{18H} has implicated the E3 ligase Itch in the spontaneous inflammation that occurs in these mice. Anaylsis of a^{18H} mice suggests that the inflammatory disease is due to a dysregulated T helper type-2 immune response. In order to verify the importance of Itch inactivation in the spontaneous inflammation of a^{18H} mice and to determine which cell type is responsible for the pathology, the Itch gene has been targeted for conditional deletion in mice using the Cre-loxP system.

The results presented in this thesis provide the first direct evidence that Itch deficient CD4+ T cells are sufficient to cause spontaneous inflammation. The function of CD4+CD25+ regulatory T cells was found to be intact *in vitro*. Instead, sensitivity to the immunomodulatory cytokine TGF-β was reduced in Itch deficient CD4+ T cells. Inadequacies in peripheral tolerance mechanisms were observed based on oral tolerance studies.

Itch deficiency was found to directly contribute to the CD4+ bias towards T helper type-2 (Th2) differentiation observed in a^{18H} mice. Itch modulation of Th2 differentiation occurs in a Stat6 dependent pathway. Itch fl/fl CD4 Cre Stat 6 -/- CD4+ T cells were found to be incapable of differentiating into Th2 cells *in vitro*. Itch fl/fl CD4 Cre Stat 6 -/- did not develop spontaneous inflammation disease, underscoring the importance of Th2 differentiation in the pathology of a^{18H} mice.

Itch fl/fl CD4 Cre mice were found to accumulate memory phenotype CD8+ cells in their secondary lymphoid organs. The inflammatory pathology of Itch fl/fl CD4 Cre

mice was not required for accumulation of Itch deficient memory-like CD8+ cells. The cause for this accumulation remains unclear, although an increase in homeostatic proliferation of the memory-like cells is considered unlikely.

The findings of this thesis provide definitive support for several speculations regarding the function of Itch. In addition, they provide novel venues of study for the role of Itch in the immune system. Collectively, they argue that further study of this E3 ligase will be a fruitful endeavor for immunologists.

Chapter 1: Introduction

1.A Tolerance in the immune system:

In a well-regulated immune system, immune reactions occur in response to pathogenic antigens but not in response to self or harmless foreign antigens. This acceptance of both self-antigens and antigens to which the immune system is constantly exposed (through inhalation or ingestion) is defined as tolerance. The study of tolerance mechanisms is an integral field of immunology, and the manipulation of tolerance has extensive clinical applications. Problems with tolerance are linked to a wide spectrum of clinical pathologies, including allergies and autoimmune diseases. Furthermore, the ability to tolerize or break tolerance to specific antigens has ramifications in both tumor immunology and transplantation.

1.A.1 Deletion:

One of the key mechanisms for maintaining tolerance against self-antigens is clonal deletion of self-reactive T cells, either in the thymus or in the periphery (Miller and Basten 1996). The importance of thymic deletion has been underscored by the recent description of AIRE deficient mice (Anderson, Venanzi et al. 2002), which develop a multi-organ autoimmune disease due to an inability to delete self-reactive T cells in the thymus. The existence of autoreactive T cells in the periphery of healthy individuals, however, indicates that clonal deletion does not completely account for tolerance (Fowell and Mason 1993).

1.A.2 Ignorance:

Another straightforward mechanism for tolerance is immunological ignorance. In this situation, immune cells cannot localize to the environment in which their antigen is

found (Zinkernagel, Ehl et al. 1997). The blood-brain barrier is a classic example of this type of tolerance, as lymphocytes are not found in the central nervous system of otherwise healthy individuals. Signaling pathways involved in T cell mobility may play a key role in this mode of tolerance.

1.A.3 T cell anergy:

Sometimes T cells do encounter their cognate antigen but do not elicit an immune response through proliferation or cytokine release (Schwartz 1996). This cell-autonomous state of unresponsiveness is defined as anergy and can be induced when a T cell is stimulated by antigen in the absence of co-stimulation (Jenkins, Chen et al. 1990) or in a specific immunomodulatory cytokine milleu (Groux, Bigler et al. 1996). The molecular basis of anergy remains poorly defined, but heterkaryon studies involving fusion of anergic and nonanergic T cells have made it clear that the anergic state is maintained by the dominant action of one or more regulatory molecules, rather than the simple absence of signaling components (Telander, Malvey et al. 1999).

1.A.3.1 Clonal anergy:

Anergy can be further subdivided into two potentially distinct experimental groups: clonal anergy and *in vivo* anergy (Schwartz 2003). Clonal anergy arises in antigen-experienced cells and occurs in response to partial stimulation without appropriate co-stimulatory signals. It should be noted that clonal anergy can only be induced in previously stimulated cells, with most studies of clonal anergy involving T cell lines. It has not been successfully described for naïve T cells, making this form of anergy a purely *in vitro* model system for tolerance (Davis and Lipsky 1993; Schwartz 2003). Once cells become clonally anergic, they do not proliferate in response to

subsequent antigenic stimulation; however, their effector functions are not affected. This form of anergy can be reversed by the addition of exogenous interleukin 2 (IL-2) (Essery, Feldmann et al. 1988; Powell, Bruniquel et al. 2001).

The biochemical pathways involved in regulating clonal anergy have been worked out in some detail. Treatment of T helper type 1 (Th1) cell lines with cyclosporine A inhibits clonal anergy, implying that the calcium/calmodulin/calcineurin pathway is critical for anergy induction (Jenkins, Chen et al. 1990). Further proof that this pathway is important comes from studies in which treatment of T cells with the calcium ionophore ionomycin mimics clonal anergy (Jenkins, Pardoll et al. 1987). Critical differences between clonal anergy and ionomycin-induced anergy exist, however, as the latter takes a longer time to induce, abolishes all cytokine production, and is stable for only four days. Biochemically, the selective activation of NFAT without AP-1 is critical for anergy induction, as it leads to reduced IL-2 production as well as activation of a specific set of "anergy genes" (Macian, Garcia-Cozar et al. 2002). In addition, there is a loss of mitogen activated protein kinase (MAPK) signal transduction in anergized cells ((DeSilva, Feeser et al. 1996; Fields, Gajewski et al. 1996; Li, Whaley et al. 1996).

1.A.3.2 *In vivo* anergy:

In contrast to the induction of clonal anergy in antigen experienced cells, *in vivo* anergy is seen when naïve T cells are stimulated with antigen in the absence of co-stimulatory molecules or in the presence of high levels of co-inhibitory molecules. Cells undergoing *in vivo* anergy proliferate and differentiate to varying degrees but then fail to proliferate upon second exposure to the antigen. Historically, *in vivo* anergy was first noted in 1989 with the injection of Mls-1a splenic cells into Mls-1b mice (Rammensee,

Kroschewski et al. 1989). Most T cells proliferated and died, but a small proportion survived and did not proliferate in response to further stimulation with Msl-1b cells. The potential caveat in this model and subsequent superantigen model systems was that the surviving, non-proliferating cells may never have recognized the antigen. Jenkins and colleagues addressed this issue by devising a model of anergy involving adoptive transfer of transgenic T cells into nonirradiated syngenic mice followed by intraperitoneal injection of antigen (Pape, Merica et al. 1998). Cells that survived the initial proliferation and collapse were viable for several months and remained unresponsive to further antigen stimulation for several weeks. This tolerance was not due to bystander suppressor effects or a suppressor cytokine milleu, as mixed transgenic TCR transfers revealed that anergy was antigen specific (Pape, Merica et al. 1998).

Considering the different natures of clonal anergy and *in vivo* anergy, it is not surprising that the biochemical events regulating these two types of anergy seem to be very different. Whereas clonally anergic cells show a block at the level of MAPK signaling, changes in proximal tyrosine phosphorylation events and calcium mobilization upon antigen engagement have been described for *in vivo* anergy (Bhandoola, Cho et al. 1993; Migita, Eguchi et al. 1995; Tanchot, Guillaume et al. 1998; Utting, Teh et al. 2000).

1.A.4 Regulatory T cells:

Activation and proliferation of T cells can be actively suppressed by a dominantly acting subset of T cells known as regulatory T cells (Sakaguchi 2000; Shevach 2000; Shevach 2002). These cells have generated considerable excitement because of their dominant role in modulating immune responses, which has led to intriguing speculations

for their potential roles in autoimmune diseases, transplants, cancer, and infectious diseases. There are currently at least three classes of T cells for which regulatory or immunomodulatory functions have been described in detail, and the relationship between these three remains unclear **(Figure 1-1)**.

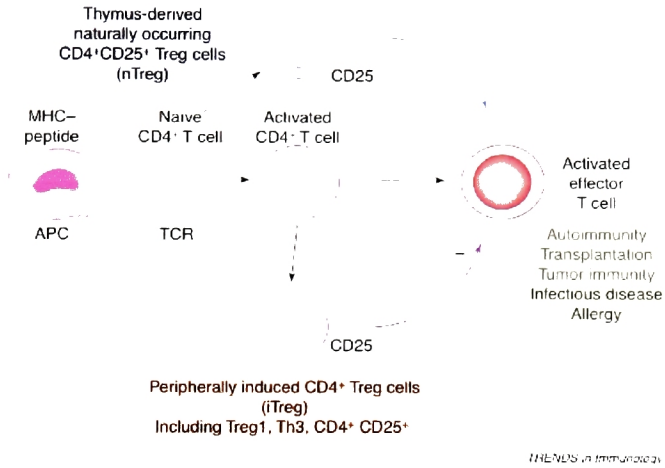


Figure 1-1. Regulatory T cell subtypes. Both naturally occurring (nTreg) and peripherally induced (pTreg) have been implicated in downregulating immune responses (yellow box). The lineage relationship between nTregs and pTregs is presently unclear. Figure taken from Piccirillo et al., Trends in Immunology, 2004.

A.4.1 CD4+CD25+ Regulatory T cells:

CD4+CD25+ regulatory T cells, which make up 5-10% of peripheral T cells, are a naturally occurring immunosuppressive T cell subpopulation (Sakaguchi, Sakaguchi et al. 1995). CD4+CD25+ cells behave like partially anergic cells *in vitro*, proliferating poorly in response to TCR stimulation unless provided with exogenous IL-2 (Papiernik, de Moraes et al. 1998; Takahashi, Kuniyasu et al. 1998). Depletion of these cells *in vivo* leads to multi-organ autoimmune disease characterized predominantly by gastritis, thyroiditis, and oopheritis (Takahashi, Kuniyasu et al. 1998). It is of note that though

more than one organ may be affected in a given mouse, this disease is always restricted to organs, with no evidence for the development of systemic autoimmune disease or anti-nuclear antibody formation (Takahashi, Kuniyasu et al. 1998; Shevach 2000). In addition to their role in maintaining immune homeostasis, CD4+CD25+ cells have also been implicated in acquired tolerance such as oral tolerance (Thorstenson and Khoruts 2001; Zhang, Izikson et al. 2001; Hauet-Broere, Unger et al. 2003). In addition CD4+CD25+ regulatory T cells can inhibit T helper type 2 (Th2) mediated airway eosinophilia in mice (Suto, Nakajima et al. 2001).

Characterization of CD4+CD25+ regulatory T cell is still in its infancy. Although markers other than IL-2 receptor alpha chain (CD25) have been identified for regulatory T cells (CD103, galectin 1, Ly6, OX-40, CTLA-4 (CD158), 4-1BB, glucocorticoid induced tumor necrosis factor receptor (GITR), TNFR2, TGF-β 1, programmed cell death 1 (PD1), and neutropilin-1) , they are also expressed on activated CD4+ cells (Gavin, Clarke et al. 2002; McHugh, Whitters et al. 2002; Bruder, Probst-Kepper et al. 2004). A functional role for CTLA-4 in regulatory T cell function has been described (Read, Malmstrom et al. 2000), but the role of CTLA-4 in suppressor function remains controversial. Classification of CD45RBlo cells as another, potentially overlapping, group of naturally occurring regulatory T cells causes further confusion in the literature, although it has recently been shown that only the CD4+CD25+ fraction of CD45RBlo have functional suppressive properties *in vivo* (Mottet, Uhlig et al. 2003). A critical discovery was the recent finding that regulatory T cell development is controlled by the transcription factor Foxp3. Foxp3 is selectively expressed in CD4+CD25+ cells (Fontenot, Gavin et al. 2003; Hori, Nomura et al. 2003) and over expression of Foxp3 in

non-suppressor cells leads to a suppressor phenotype both *in vitro* and *in vivo* (Hori, Nomura et al. 2003).

The mechanisms by which CD4+CD25+ cells suppress immune responses are still a subject of debate. Upon TCR mediated activation, CD4+CD25+ are capable of suppressing proliferation of other T cells by inhibiting *in vitro* IL-2 production in an antigen non-specific manner, but require direct cell-cell contact with T cells to do so (Thornton and Shevach 1998; Thornton and Shevach 2000). Interluekin 4 (IL-4), Interleukin 10 (IL-10), and Transforming Growth Factor β (TGF-β) are not required for this *in vitro* suppression (Takahashi, Kuniyasu et al. 1998; Thornton and Shevach 1998; Piccirillo, Letterio et al. 2002). *In vivo*, the mechanism by which CD4+CD25+ regulatory T cells exert their effect is less clear. It seems unlikely that the direct cell-contact mediated suppression seen *in vitro* is the sole mechanism by which such a relatively small percentage of CD4+CD25+ suppressor T cells can systemically regulate the effects of all T cells, but data supporting other mechanisms are still contradictory and unclear.

1.A.4.2 Tr1 Regulatory T cells:

A potentially distinct group of regulatory T cells,Tr1 cells, secrete IL-10 and TGF-β and dampen immune responses via these immunomodulatory cytokines (Roncarolo, Bacchetta et al. 2001). In contrast to CD4+CD25+ cells, which are naturally generated in the thymus, Tr1 cells are induced upon exposure to foreign or tissue specific antigen in the periphery (Bluestone and Abbas 2003). Similar to CD4+CD25+ suppressor cells, Tr1 cells are capable of suppressing T cell proliferation *in vitro* in an antigen non-specific manner; however, their effects are mediated primarily by IL-10 and

TGF-β. Both IL-10 and TGF-β are particularly important for regulating immune responses to mucosal antigens (Jutel, Akdis et al. 2003).

The role of IL-10 in modulating immune responses is well established. IL-10 deficient mice develop chronic cholitis due to dysregulation of Th1 mediated immune responses (Kuhn, Lohler et al. 1993). Conversely, IL-10 transgenic mice fail to mount proper Th1 and Th2 responses (Rouleau, Cottrez et al. 1999). IL-10 dampens immune responses via multiple mechanisms, including inhibition of antigen presentation by antigen presenting cells (APCs) (Enk, Angeloni et al. 1993; Steinbrink, Wolfl et al. 1997; Moore, de Waal Malefyt et al. 2001) and induction of T cell anergy (Groux, Bigler et al. 1996; Zeller, Panoskaltsis-Mortari et al. 1999).

A specific role for IL-10 in the function of regulatory T cells comes from several lines of experimental evidence. The existence of IL-10 secreting T cells has been associated with tolerance in SCID patients transplanted with HLA mis-matched hematopoetic stem cells (Roncarolo, Yssel et al. 1988; Bacchetta, Bigler et al. 1994). Induction of anergy to phopholipase A2, the major allergen of bee venom, in either patients undergoing immunotherapy or in naturally tolerant beekeepers, correlates with an increase in IL-10 producing T cells (Akdis, Blesken et al. 1998). In addition, antigen stimulation of T cells in the presence of IL-10 leads to the generation of poorly proliferating T cell clones that secrete high levels of IL-10, moderate levels of TGF-β, interferon gamma (IFN-γ) and Interleukin 5 (IL-5), and no IL-2 or IL-4 (Groux, O'Garra et al. 1997). These regulatory T cells, defined as Tr-1 cells, are capable of suppression *in vitro*. Suppression is predominantly mediated by IL-10 and TGF-β in an antigen non-specific manner.

In addition to their suppressive capabilities *in vitro*, Tr1 cells are capable of modulating immune responses *in vivo*. Tr1 cells can inhibit Inflammatory Bowel Disease (IBD) in severe combined immunodeficiency (SCID) mice (Groux, O'Garra et al. 1997). In contrast to the preventative role of CD25+ or CD45RBlo regulatory T cells, Tr1 cells are capable of suppressing IBD associated inflammation even after the inflammatory process has begun (Foussat, Cottrez et al. 2003). Tr1 cells have also been shown to inhibit airway hyperreactivity (Stock, Akbari et al. 2004) as well as Th2 mediated inflammation in an IL-10 dependent manner (Cottrez, Hurst et al. 2000). Taken together, these data suggest a role for IL-10 secreting Tr1 regulatory T cells in modulating immune responses.

The connection between suppression by CD4+CD25+ regulatory T cells and Tr1 T cells is still unclear. Since CD4+CD25+ regulatory T cells have been shown to lead to the differentiation of T cells into Tr1 cells *in vitro* (Dieckmann, Bruett et al. 2002), it is possible that the function of regulatory T cells in *vivo* is mediated by their ability to induce Tr1 cells **(Figure 1-2):**

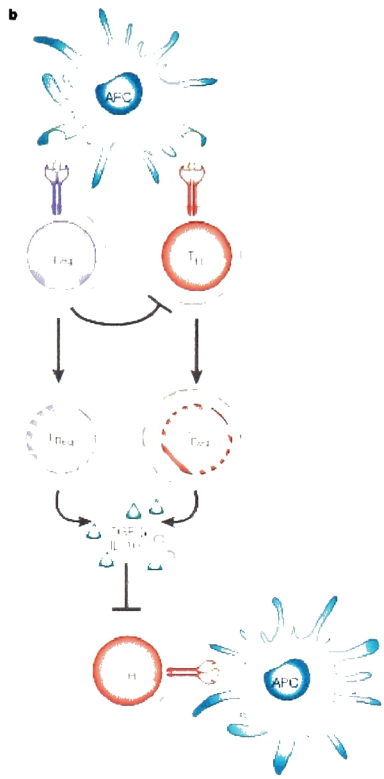


Figure 1-2. The lineage and functional relationships between CD4+CD25+ naturally occurring regulatory T cells and induced Tr-1 and Th3 regulatory T cells. Induced regulatory T cells (striped cells) can develop either from CD4+CD25+ cells (blue stripes) or CD4+CD25- cells (red stripes). CD4+CD25+ cells function to suppress either by direct cell contact mediated inhibition, or through the suppressive cytokines secreted by induced regulatory T cells. Figure taken from Bluestone et al., Nature Reviews Immunology, 2003.

1.A.4.3 Th3 regulatory T cells:

Another subset of induced suppressor cells is T helper type-3 (Th3) cells, which secrete TGF-β, IL-10, and IL-4. Th3 cells can be induced by stimulation of T cells in the presence of TGF-β *in vitro* (Zheng and McKeown-Longo 2002). Th3 cells are also induced in gut associated lymphoid tissue (GALT) in models of oral and mucosal tolerance (Chen, Kuchroo et al. 1994; Friedman and Weiner 1994; Neurath, Fuss et al. 1996; Weiner 1997; Weiner 2001). Th3 cells are capable of inhibiting Th2 immune responses (Garside, Steel et al. 1995; Russo, Jancar et al. 1998) and have also been shown to be important in models of colitis (Neurath, Fuss et al. 1996; Powrie, Carlino et al. 1996) and tracheal eosinophilia (Haneda, Sano et al. 1997; Russo, Jancar et al. 1998). TGF-β secretion was critical for the suppressive function of Th3 regulatory T cells in the

colitis model, as blocking TGF-βwith antibodies blocked the regulatory cells' suppressive capacity (Powrie, Carlino et al. 1996).

The TGF-β family is one of the most pleiotropic cytokine families known, with members playing a role in development, epithelial cell growth and differentiation, and carcinogenesis (Letterio and Roberts 1998). The three isoforms of TGF-β (TGF-β 1, TGF-β 2, TGF-β 3) are highly homologous and use the same serine-threonine kinase type I and type II receptors to signal (Massague 1990). TGF-β 1 is the predominant TGF-β isoform expressed in lymphoid organs, whereas TGF-β 2 and TGF-β 3 are highly expressed in mesyncymal tissues and bone (Millan, Denhez et al. 1991). Early embryonic lethality of TGF-β 2 and TGF-β 3 deficient mice has made studying their role in immune cell function difficult (Kaartinen, Voncken et al. 1995; Proetzel, Pawlowski et al. 1995). About 60% of TGF-β 1 deficient mice die *in utero*, but the remainder develop a severe autoimmune phenotype that leads to their death by 4 weeks of age due to cardiopulmonary infiltrations (Shull, Ormsby et al. 1992; Kulkarni, Ward et al. 1995; Yaswen, Kulkarni et al. 1996).

TGF-β plays a critical role in T cell function during an immune response. TGF-β deficient mice progressively develop a multi-organ autoimmune disease (Shull, Ormsby et al. 1992; Kulkarni, Huh et al. 1993), and a similar disease phenotype is seen in mice expressing a dominant negative TGF-β receptor II subunit in T cells (Gorelik and Flavell 2000). TGF-β inhibits proliferation, cytokine production, and cytotoxicity in T cells (Letterio and Roberts 1998). In addition, TGF-β secreting regulatory T cells are capable of regulating both Th1 and Th2 mediated immune responses (Bridoux, Badou et al. 1997). Retroviral transduction of T cells with TGF-β induces IL-10 production,

suggesting that part of TGF-β's role in modulating the immune response may be through IL-10 (Kitani, Fuss et al. 2003).

1.A.4.4 TGF-β, IL-10, and CD4+CD25+ regulatory T cells:

The role of IL-10 or TGF-β in the suppressor function of CD4+CD25+ regulatory T cells is still unclear and is one reason for the distinction between regulatory subtypes. Assays using cells deficient in TGF-β and IL-10 signaling have shown that TGF-β and IL-10 are not necessary for the suppressive effects of CD4+CD25+ regulatory T cells *in vitro* (Piccirillo, Letterio et al. 2002). These results have been used to suggest that CD4+CD25+ regulatory T cells do not exert their effect through either of these cytokines; however, it remains unclear if the mechanisms used by CD4+CD25+ cells to suppress T cell proliferation *in vitro* are the same as those used *in vivo*.

The role of IL-10 in mediating CD4+CD25+ suppression *in vivo* may depend on the context of the immune response. IL-10 deficient CD25+ cells are capable suppressing the autoimmune disease that occurs in post-natal day 3-thymectomized mice (Thornton and Shevach 1998). In contrast, CD25+ regulatory T cells prevent inflammatory bowel disease in the SCID colitis model in an IL-10 dependent manner (Asseman, Mauze et al. 1999). IL- 10-/- CD4+CD25+ T cells can suppress autoimmune gastritis but not bacterial driven inflammatory bowel disease, suggesting that the type of inflammatory response may also influence the role of IL-10 in the suppressive effects of CD4+CD25+ regulatory T cells (Suri-Payer and Cantor 2001).

The role of TGF-β in CD4+CD25+ regulatory T cell function *in vivo* is equally unclear. Studies using identical regiments of anti-TGF-β antibodies have provided contradictory results in different *in vivo* models of suppressor function (Powrie, Carlino

et al. 1996; Read, Malmstrom et al. 2000; Piccirillo, Letterio et al. 2002; Nakamura, Kitani et al. 2004). TGF-β is not required for suppression of autoimmune gastritis by CD4+CD25+ regulatory T cells (Piccirillo, Letterio et al. 2002). In contrast, TGF-β is required for CD4+CD45RBlo regulatory T cell suppression in the SCID model of colitis (Powrie, Carlino et al. 1996). CD4+CD45RBlo cells do not represent a different regulatory T cell subpopulation, as TGF-β is also required when CD4+CD45RBlo cells are further purified into CD4+CD25+CD45RBlo cells (Read, Malmstrom et al. 2000). CD4+ CD25+ regulatory T cell suppression of trinitrobenzene acid (TNBS) induced colitis in SJL/J mice (Fuss, Boirivant et al. 2002) is also TGF-β dependent. Finally, TGF-β has been implicated in CD4+CD25+ suppression of CD8+ cytotoxic T cells in a model of type-1 diabetes (Green, Gorelik et al. 2003). Thus, most existing data suggest that CD4+CD25+ suppress via a TGF-β dependent mechanism *in vivo*, but counterexamples exist which may imply a context dependent role for TGF-β as well.

The mechanism by which TGF-β exerts an effect on regulatory T cell functions is even less clear. Surface bound TGF-β is upregulated in CD4+CD25+ suppressor cells upon antigen stimulation (Nakamura, Kitani et al. 2001). The coordinate expression of TGF-β and TGF-βRII on suppressor cells may explain their anergic phenotype, as TGF-β negatively regulates IL-2 mediated proliferation (Kehrl, Wakefield et al. 1986). TGF-β has also been shown to play a role in the generation (Park, Paik et al. 2004) and expansion (Yamagiwa, Gray et al. 2001) of regulatory T cells. TGF-β may work in part by inducing Foxp3 expression and regulatory T cell function of naive T cells (Chen and Wahl 2003; Fantini, Becker et al. 2004) **(Figure 1-2)**. In addition, TGF-β plays a critical role in the suppressive capabilities of T helper cells that have been anergized and

converted to suppressor cells by CD25+ regulatory T cells (Jonuleit, Schmitt et al. 2002). This mechanism may provide a means by which local suppression can be spread to a more systemic level of tolerance. In summary, it is clear that the exact relationship between TGF-β and CD4+CD25+ suppressor cells requires further experiments.

1.B Atopic Diseases:

Mucosal immune responses are tightly regulated to allow activation of effector mechanisms against pathogens but prevent cell mediated inflammatory responses to non-pathogenic external antigens. Misregulation of these immune responses may lead to atopic diseases. Atopic diseases, including asthma and atopic dermatitis, are multifactorial and complex pathologies that effect approximately 20% of the world's population, according to the World Health Organization. These diseases share in common high IgE levels (referred to as atopy) as well as infiltration of effector cells at the site of the allergic response. The types of effector cells present can be diverse, ranging from T lymphocytes to eosinophils, monocytes/macrophages, and mast cells. Both genetic predisposition and environmental factors influence the type and extent of the pathology (Cookson 1999).

1.B.1 The role of Th2 immune responses in atopic diseases:

CD4+ helper T cells can differentiate into two well defined subsets called T helper type 1 (Th1) cells and T helper type 2 (Th2) cells. Th1 cells preferentially express the chemokine receptors CCR5, CXCR3, and CCR1 and produce inflammatory cytokines such as interferon gamma (IFNγ) and lymphotoxin alpha (LTα). Th1 cells generally mediate cellular immunity. Th2 cells express the chemokine receptors CCR3 and CCR4

and produce cytokines such as interleukins 4,5,9,10, and 13 (IL-4,IL-5,IL-9,IL-10,IL-13). These cytokines in turn regulate B cell proliferation and isotype switching (Rossi and Zlotnik 2000; Dong and Flavell 2001). Th2 immune responses are critical for humoral responses and for immune responses against extracellular parasites such as helminthes (Heinzel, Sadick et al. 1989; Finkelman, Pearce et al. 1991). The distinction between Th1 and Th2 immune responses is not only significant during an immune response against a pathogen, but is also important in immune system mediated pathologies. The dominance of either helper cell type shapes the outcome of immunologically mediated disease conditions, including autoimmune diseases and allergic responses (Abbas, Murphy et al. 1996).

There exists a strong correlation between atopic diseases and Th2 differentiation of T cells. The Th2 cytokine cluster is a susceptibility locus for atopic diseases (1997; Kawashima, Noguchi et al. 1998; Forrest, Dunn et al. 1999). Coordination of the effector cells in atopic diseases is controlled primarily by the cytokines secreted by Th2 T cells (Abbas, Murphy et al. 1996; O'Garra 1998). Furthermore, over expression of Th2 cytokines by even threefold upon antigen encounter leads to spontaneous atopic dermatitis and lung infiltrations similar to those seen in asthmatic patients (Lee and Flavell 2004).

1.B.2 The Generation of Th2 effector T cells:

The signals that determine T helper cell differentiation and function are not yet completely understood. It is currently thought that several potentially unrelated factors contribute to a cell's lineage commitment choice. Experimental evidence suggests that Th1/Th2 differentiation is influenced by factors such as the strength of the TCR signal,

the-costimulatory ligand engaged, the cytokine environment, and cell cycle progression. It is important to note that the phenotype of helper cells becomes increasingly stable over time and independent of extrinsic factors such as cytokines (Huang, Hu-Li et al. 1997).

1.B.3 Instructive versus Selective signals for T helper differentiation:

There are currently two models for how cyotkines may affect T helper differentiation **(Figure 1-3)**. The Instructive model states that cytokines directly lead to epigenetic modifications of T helper specific loci, increasing the expression of either Th1 or Th2 specific genes. In contrast, the Selective model argues that Th1 or Th2 skewing cytokines favor the growth of T helper cells that stochastically express the respective mRNA. In the case of Th2 differentiation, data exist to support both models (Farrar, Ouyang et al. 2001; Zhu, Guo et al. 2002).

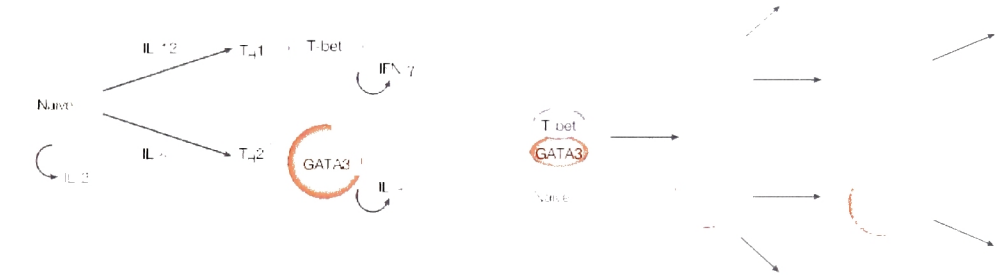


Figure 1-3. Instructive versus selective models of T helper differentiation. **a**. Instructive model. Cytokines provide instructive signals to naïve cells to differentiate along given pathways. **b**. Selective model. Cytokines support the outgrowth of T helper cells that stochastically differentiate along a given pathway. Figure taken from Murphy et al. Nature Reviews Immunology 2002.

1.B.4 The role of Cytokines in T helper differentiation:

It is well established that cytokines play a dominant role in Th1 versus Th2 differentiation under most experimental conditions. The importance of IL-12 and IL-4 in the development of Th1 and Th2 helper cells, respectively, has been established for over a decade (reviwed in (Abbas, Murphy et al. 1996)). The importance of cytokines has been demonstrated in several ways. Addition of exogenous IFN-γ and IL-12 leads to Th1 differentiation (Gajewski and Fitch 1988; Manetti, Parronchi et al. 1993), whereas exogenous IL-4 leads to Th2 differentiation. Conversly, mice deficient in IL-4 or IL-4 Rα have markedly diminished Th2 responses (Kopf, Le Gros et al. 1993; Noben-Trauth, Shultz et al. 1997). The critical role of IL-2 in Th2 differentiation has been demonstrated in similar studies (Ben-Sasson, Le Gros et al. 1990; Le Gros, Ben-Sasson et al. 1990). The role of cytokines in T helper differentiation has also been supported by studies of cytokine signaling pathways. For example, SOCS5, which binds the IL-4R and inhibits it function, leads to Th1 skewing (Seki, Hayashi et al. 2002).

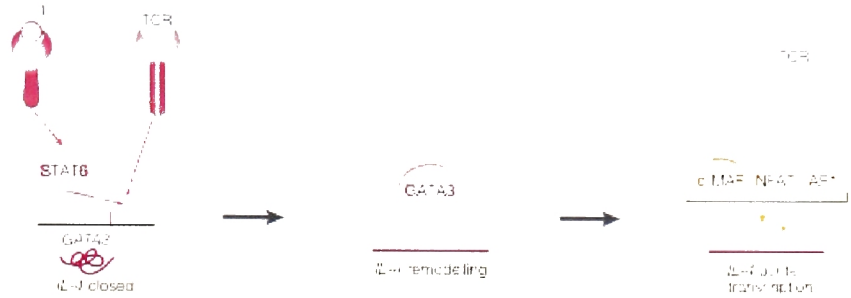
1.B.5 Transcriptional regulation of Th1 versus Th2 differentiation:

T helper differentiation is influenced by cytokines through both autocrine and paracrine mechanisms. The autocrine feedback loop is considered critical for T helper differentiation. As a consequence, the transcriptional regulation of signature cytokines within a differentiating cell plays an important role in determining its eventual fate. Recent work has shown that chromatin remodeling is necessary during the differentiation of both Th1 and Th2 cells (Agarwal and Rao 1998). In addition, specific transcription factors have been shown to support either Th1 or Th2 differentiation. Although Th1 differentiation is mediated primarily by the master regulator T-bet (Szabo, Sullivan et al. 2002), multiple transcription factors are involved in Th2 differentiation **(Figure 1-4)**.

1.B.5.1 NFAT:

The NFAT (nuclear factor of activated T cells) family of transcription factors is involved in regulting T helper differentiation. NFATc and NFATp can both bind to the IL-4 promoter NFAT sites (Timmerman, Healy et al. 1997). It is thought that the two NFAT factors antagonize each other, as NFATp deficient mice have a dysregulated Th2 immune response whereas NFATc deficient mice have a dysregulated Th1 immune response (Hodge, Chun et al. 1996; Xanthoudakis, Viola et al. 1996).

a



b

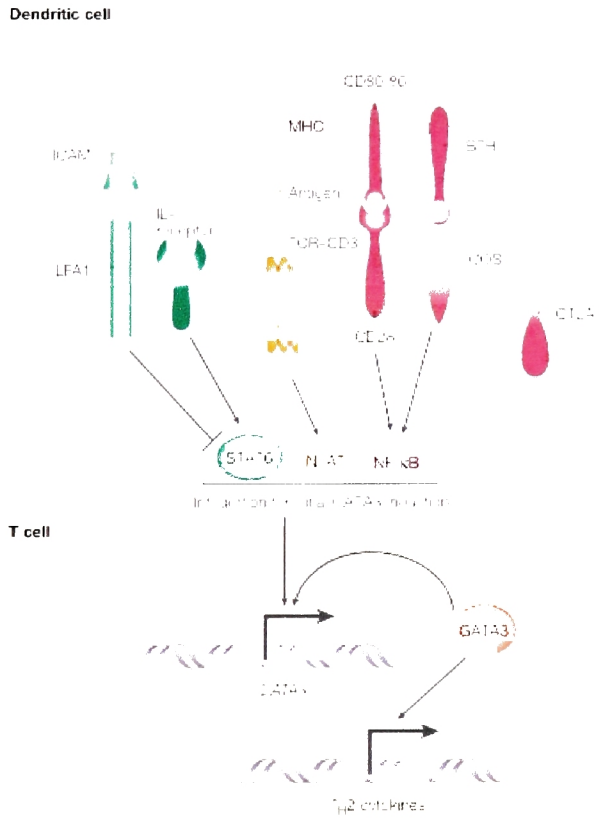


Figure 1-4. Transcriptional regulation of Th2 differentiation. **a.** Pathways for the induction of IL-4 expression. Signals through the T Cell Receptor (TCR) and through the IL-4 receptor are important for the initial induction of GATA-3 and remodeling of the IL-4 locus. NFAT, AP1, and c-Maf are critical for initial IL-4 transcription. **b.** Pathways for the induction of Th2 cytokines. Signals from the TCR, co-stimulatory ligands, and additional ligands synergistically increase GATA-3 expression. Figure taken from Murphy et al., Nature Reviews Immunology, 2002.

1.B.5.2 JunB and c-Maf:

Two other transcription factors that influence Th2 differentiation include Jun B and c-Maf. JunB positively regulates IL-4 gene expression by binding specifically to the AP-1 site in the promoter of IL-4 (Li, Tournier et al. 1999). It does this in combination with c-Maf, a basic region/leucine zipper transcription factor that is specifically upregulated in Th2 cells and also binds to a site in the proximal IL-4 promoter (Ho, Hodge et al. 1996). In transgenic mice overexpressing c-Maf, there is an increased bias towards Th2 differentiation(Ho, Lo et al. 1998). Analysis of c-Maf deficient mice has demonstrated that c-Maf is critical for IL-4 production, as c-Maf deficient T cells are markedly deficient in IL-4 production (Kim, Ho et al. 1999). Similarly, overexpression of JunB in transgenic mice leads to Th2 differentiation of T cells even under Th1 polarizing conditions (Li, Tournier et al. 1999).

1.B.5.3 Stat6:

The transcription factor Stat6 is necessary and sufficient for IL-4's role in Th2 differentiation (Zhu, Guo et al. 2001). Stat6 is phosphorylated and activated in response to IL-4 and IL-13 (Kohler, Alliger et al. 1994), two major cytokines involved in Th2 differentiation. Although IL-4 transcription can occur in the absence of Stat6, IL-4 mediated signaling is completely abrogated (Kaplan, Wurster et al. 1999). As a result, T cells from Stat 6 deficient mice are defective in differentiating into Th2 cells *in vitro* (Kaplan, Schindler et al. 1996). Stat6 deficient mice produce very reduced levels of Th2 cytokines in response to infection with the nematode *Nippostrongylus brazilinesis* and the parasite worm *Schistosoma mansonii* (Takeda, Tanaka et al. 1996; Kaplan, Whitfield et al. 1998). In addition, Stat6 deficient mice show a complete abrogation of antigen induced airway hyperresponsiveness, which is driven by a Th2 T cell response (Akimoto,

Numata et al. 1998; Kuperman, Schofield et al. 1998). Conversely, ectopic expression of Stat6 in cells undergoing TH1 differentiation leads to the development of Th2 cells and the upregulation of GATA-3 and c-Maf, demonstrating that Stat6 regulates Th2 differentiation upstream of these two transcription factors (Kurata, Lee et al. 1999). It should be noted that in some model systems, Th2 differentiation can be induced in the absence of Stat 6 (Ouyang, Lohning et al. 2000) and Th2 driven immune responses are not significantly affected (Finkelman, Morris et al. 2000; Jankovic, Kullberg et al. 2000). These results suggest that Stat6 independent pathways for Th2 differentiation may also exist, though analysis of *in vivo* data is complicated by the fact that IL-4 production from NK and NKT cells is Stat6 independent (Kaplan, Wurster et al. 1999; Stetson, Mohrs et al. 2003).

1.B.5.4 GATA-3:

Of the transcription factors that influence Th2 differentiation, GATA-3 is perhaps the most powerful. GATA-3 is rapidly induced in Th2 differentiating cells in a partially IL-4 and Stat6 dependent manner (Ouyang, Ranganath et al. 1998; Ouyang, Lohning et al. 2000), and it remains expressed in effector Th2 cells (Zheng and Flavell 1997). Transgenic mice in which GATA-3 is overexpressed produce Th2 cytokines (IL-4, IL-5, IL-6, IL-10) even under Th1 polarizing conditions where no exogenous IL-4 is present (Zhang, Cohn et al. 1997; Zheng and Flavell 1997). Antisense mediated reduction of GATA-3 blocks Th2 cytokine production in Th2 clones and allergic airway inflammation *in vivo* (Zheng and Flavell 1997; Finotto, De Sanctis et al. 2001). Expression of a dominant negative GATA-3 also decreases Th2 mediated pulmonary allergic responses in mice (Zhang, Yang et al. 1999). Furthermore, conditional deletion of GATA-3 leads to

impaired Th2 differentiation *in vitro*, even in the presence of exogenous IL-4. In addition, it abolishes Th2 immune responses *in vivo* (Zhu, Min et al. 2004). Deletion of GATA-3 in fully differentiated Th2 cells abolishes IL-5 and IL-13 production, although IL-4 production is only diminished (Zhu, Min et al. 2004).

Although GATA-3 is critically important for Th2 differentiation, it alone cannot account for all aspects of Th2 differentiation. GATA-3 expressing T cells polarized under Th1 differentiation conditions, though they generate Th2 cytokines, do not expand as well as conventional Th2 cells. This growth defect can be rescued by the expression of the Stat6-inducible gene growth factor inhibitor 1 (Gfi-1) (Zhu, Guo et al. 2002).

1.B.5.5 Stat5:

The Stat5 transcription factors regulate Th2 differentiation in a GATA-3 independent fashion. Four isoforms of Stat5 exist, encoded by two genes (Stat5a and Stat5b) that each give rise to two isoforms based upon post-translational modifications (Azam, Erdjument-Bromage et al. 1995; Azam, Lee et al. 1997). Stat5 proteins are activated downstream of the IL-2R, IL-7R, IL-9R, and IL-15R and have been implicated in IL-2 induced cell cycle progression (Hoey and Grusby 1999; Moriggl, Topham et al. 1999). Stat5a-deficient mice or cells in which Stat5 activity is blocked by treatment with cell permeable phospho-Stat5 peptide are defective in Th2 differentiation (Yamashita, Katsumata et al. 2000; Kagami, Nakajima et al. 2001). Furthermore, expression of a constitutively active Stat5a protein removes the requirement for IL-2, IL-4, and Stat6 in Th2 differentiation (Zhu, Cote-Sierra et al. 2003). The constitutively active Stat5a protein does not lead to enhanced GATA-3 expression. Rather, Stat5a and GATA-3

collaborate in Th2 differentiation by "opening" the IL-4 gene locus at different regions (Zhu, Cote-Sierra et al. 2003).

1.B.6 Cell cycle progression and T helper differentiation:

Cell cycle progression is critical for both Th1 and Th2 differentiation. Instructional signals for promoting Th1 or Th2 differentiation can neither induce effector cytokine expression in the absence of cell cycle progression nor alter the pattern of division-dependent expression of effector cytokines, suggesting that epigenetic modifications are critical for T helper differentiation. More cell divisions are required for Th2 differentiation than Th1 differentiation (Bird, Brown et al. 1998).

1.B.7 Co-stimulatory ligands and Th1 versus Th2 differentiation:

The role of antigen presenting cells (APCs) in directing Th1 versus Th2 differentiation is substantiated by numerous studies (reviewed by (Eisenbarth, Piggott et al. 2003; Kapsenberg 2003). APCs influence T helper differentiation through both cytokines and cell surface expressed ligands. Though not all modes of influence are as yet determined, the role of co-stimulatory ligands is clear.

CD28 co-stimulation is particularly important for Th2 differentiation (Seder, Germain et al. 1994; Schweitzer and Sharpe 1998). Stimulation of CD4+ T cells with APCs in the presence of blocking antibodies against CD28 or stimulation with fibroblasts lacking CD28 leads to IFNγ producing Th1 cells but not IL-4 producing Th2 cells. CTLA-4, a member of the CD28 family that acts as a negative regulator of T cell activation, negatively regulates Th2 differentiation. As a consequence, CTLA-4 deficient DO11.10 transgenic mice show increased Th2 differentiation upon stimulation (Oosterwegel, Mandelbrot et al. 1999). Furthermore, Th2 differentiation in the absence

of CTLA-4 occurs in the absence of Stat6 signaling (Bour-Jordan, Grogan et al. 2003). Stimulation with B7.1 or B7.2 leads to increased Th1 or Th2 differentiation, respectively. The differential roles of B7.1 and B7.2 in modulating T helper differentiation may be explained by their selective recruitment of either CTLA-4 or CD28 to the immune synapse (Kuchroo, Das et al. 1995; Pentcheva-Hoang, Egen et al. 2004). Another member of the CD28 family, ICOS (Inducible CO-Stimulator) is highly expressed on Th2 cells and seems to specifically regulate Th2 effector function. In contrast, ICOS inhibits differentiation of naïve cells into either Th1 or Th2 effector cells (Coyle, Lehar et al. 2000). Another co-stimulatory ligand, OX40, participates in Th1 cell differentiation. Mice deficient in OX40 have reduced numbers of CD4+ IFN-γproducing T cell in response to viral infection (Kopf, Ruedl et al. 1999). DR6, a member of the TNF family of receptors, also modulates T helper differentiation, as DR6 deficient CD4+ T cells show increased Th2 cytokine production (Liu, Na et al. 2001).

Co-stimulatory ligands can also indirectly affect a T cell's differentiation fate. For example, expression of CD40L on a T cell leads to increased IL-12 expression in the APC to which it binds (Grewal and Flavell 1998). TRANCE/TRANCE-R ligation on dendritic cells also leads to IL-12 expression *in vitro* and enhanced Th1 mediated IFNγresponses *in vivo* (Bachmann, Wong et al. 1999; Green and Flavell 1999). Although not technically co-stimulatory signals, the LFA (leukocyte function-associated antigen 1)-ICAM (intercellular adhesion molecule 1) binding biases T helper differentiation in favor of Th1 (Salomon and Bluestone 1998; Luksch, Winqvist et al. 1999; Camacho, Heath et al. 2001; Smits, de Jong et al. 2002).

Recently, the Notch pathway has been implicated in regulating T helper differentiation. *In vitro* stimulation with Delta ligand leads to increased Th1 differentiation (Maekawa, Tsukumo et al. 2003). Furthermore, dendritic cells that are primed to stimulate Th1 immune responses express the Delta ligand, whereas dendritic cells, which are primed for Th2 immune responses, express Jagged (Amsen, Blander et al. 2004). Finally, expression of the constitutively active intracellular domain of Notch1 leads to increased Th2 differentiation, even in the absence of Stat6 (Amsen, Blander et al. 2004). These data suggest that the Notch pathway can regulate T helper differentiation independently of cytokines.

1.B.8 TCR signaling and Th1 versus Th2 differentiation:

T cell activation is strictly controlled by signals emanating from the T Cell Receptor (TCR) and co-stimulatory ligands. The biochemical pathways coupling TCR engagement with downstream signaling events include a tyrosine kinase cascade, serine threonine kinases, G proteins, and Ca^{2+} mobilization. In addition, actin polymerization and receptor turnover critically regulate the duration of an antigenic stimulus by affecting the stability of the immune synapse.

The strength of the stimulation through the TCR has also been shown to modulate the differentiation decision of helper T cells. T cells need prolonged stimulation (> 72 hours) in order to differentiate into either Th1 or Th2 cells in the absence of exogenous cytokines (Iezzi, Scotet et al. 1999). Th2 differentiation seems to be favored at the extremes of antigenic stimulation, with both low dose and low affinity ligand stimulation (Constant, Pfeiffer et al. 1995; Pfeiffer, Stein et al. 1995) as well as high antigenic dose stimulation (Hosken, Shibuya et al. 1995) favoring Th2 differentiation. In addition T cells

require simultaneous stimulation through the TCR and cytokine receptor in order to differentiate into Th2 cells, although Th1 differentiation can occur if both signals are provided separately (Iezzi, Scotet et al. 1999).

Differences in signaling downstream of the TCR have been described for Th1 and Th2 cells, but these initial differences were noted between differentiated effector Th1 and Th2 cells (Fitch, McKisic et al. 1993; Fallon, Smith et al. 1998; Balamuth, Leitenberg et al. 2001). The pathways downstream of the TCR that are involved in T helper cell differentiation are currently being elucidated. These pathways can either be involved in directly promoting T helper differentiation along a given pathway, or can influence differentiation by inhibiting differentiation along the alternate pathway. An example of the second, indirect mode of action is the Tek family member Itk, which promotes Th2 differentiation by negatively regulating T-bet (Miller, Wilcox et al. 2004).

One of the most proximal events after TCR engagement that has been linked to the differentiation process is ZAP-70 signaling. Inhibition of ZAP-70 signaling leads to a bias towards Th2 differentiation (Tanaka, Bi et al. 2003). In Th2 differentiating T cells, this inhibition is mediated by the SWAP-70 like adaptor of T cells (SLAT) (Tanaka, Bi et al. 2003). Recently, a role for SAP (SLAM associated protein) in Th2 differentiation has been described. Stimulation of SAP deficient CD4+ T cells *in vitro* reveals reduced Th2 cytokine production (Czar, Kersh et al. 2001; Wu, Nguyen et al. 2001). SAP functions as an adaptor protein that recruits FynT to SLAM family receptors. The critical role of Fyn phosphorylation of SLAM in Th2 differentiation has been established using Fyn-binding deficient SAP knock-in mice (Davidson, Shi et al. 2004). Furthermore, the downstream pathways affected by SAP deficiency, including PKC-θ recruitment, Bcl-10

phosphorylation, IκB-α degradation, and nuclear accumulation of NF-κB1 (Cannons, Yu et al. 2004), may also be involved with Th2 differentiation. Consistent with this idea is the finding that mice containing a point mutation in the Carma protein, which acts downstream of SAP and Fyn, also show a skewed Th2 bias (Jun, Wilson et al. 2003).

Further downstream of the TCR, JNK signaling has been linked to T helper differentiation. Mice deficient for JNK1, JNK1 and JNK2, or the JNK activating kinase MKK7 have exaggerated Th2 responses, although these T cells are capable of undergoing Th1 differentiation under Th1 polarizing conditions (Dong, Yang et al. 1998; Dong, Yang et al. 2000). Conversely, mice deficient in JNK2 show deficient Th1 immune responses (Yang, Conze et al. 1998). Moderate increases in the levels of nuclear Jun B and significant increases in nuclear NFATc were seen in anti-CD3 and anti-CD28 activated T cells from JNK1 deficient mice. The accumulation of NFATc is presumably due to the absence of JNK1 mediated phosphorylation of NFATc, which is involved in inhibiting calcineurin binding and hence NFATc translocation to the nucleus (Chow, Dong et al. 2000).

1.C. CD8+ Memory T cells Maintenance and Generation:

During a primary infection, one critical aspect of the adaptive immune system is the generation of immunological memory against the invading pathogen. The basis of immunological memory lies in a specialized subgroup of cells called memory cells, which have a number of characteristics that distinguish them from naïve cells (Ahmed and Gray 1996; Gray 2000). Memory cells survive longer compared to naïve cells and respond more rapidly to antigen in terms of entry into cell cycle and conversion into cytokine secreting effector cells (Pihlgren, Dubois et al. 1996; Rogers, Dubey et al.

2000). Because of the cytotoxic abilities of CD8+ effector cells, memory CD8+ cells are critical mediators of immunity against a broad range of bacterial, viral, and protozoal intracellular pathogens during secondary infections. Understanding the generation and maintenance of CD8+ memory cells is thus critical for further improvements in vaccination against infectious agents.

Although small and quiescent like naïve cells, CD8+ memory cells differ from their naïve CD8+ counterparts in several aspects. CD8+ memory cells constitutively express genes that are involved in the effector functions of CD8+ cells, such as IFNγ, perforin, and granzyme B. Though the proteins are synthesized only upon antigen encounter, the elevated mRNA levels of these genes allows for the synthesis of increased amounts of proteins in a shorter amount of time (Bachmann, Barner et al. 1999; Veiga-Fernandes, Walter et al. 2000; Swanson, Murakami et al. 2002). Memory CD8+ cells also express different surface proteins involved in cell adhesion and chemo taxis. These molecules allow memory cells to extravasate into non-lymphoid tissue and mucosal sites. Finally, as secondary infections can occur years after the initial infection, memory CD8+ cells maintain themselves over an extended period of time through slow, homeostatic proliferation (Homann, Teyton et al. 2001). Because of these fundamental differences, differentiation of naïve CD8+ cells into effector and/or memory cells involves structural reorganization of the membrane and cytoskeleton, chromatin remodeling, expression of new genes, and induction of cell division (Kaech, Hemby et al. 2002).

1.C.1 Central versus Effector Memory CD8+ T cells:

1.C.1.1 Phenotypic characterization:

All CD8+ memory cells can be distinguished by their surface expression of canonical memory cell markers such as CD44, CD122, Ly6C, CD11a, and CD132. In addition, CD8+ memory cells do not express the activation markers CD25 and CD69 (Wherry, Teichgraber et al. 2003). Analysis of these cell surface markers allows for the identification of potential memory CD8+ cells in polyclonal populations *in vivo*, where the specific antigen to which a memory CD8+ cell was formed is unknown.

CD8+ memory cells can be further subdivided into central and effector memory cells based on location and expression of specific cell surface markers (Sallusto, Lenig et al. 1999). CD62Lhi CCR7+ cells are deemed central memory cells, as expression of these ligands allows for their efficient homing to lymph nodes via interactions with the chemokines CCL19 and CCL21 (Forster, Schubel et al. 1999; Mori, Nakano et al. 2001). Upon restimulation, these cells can proliferate and differentiate into effector cells (Zinkernagel, Bachmann et al. 1996). Conversely, long-lived CD62Llo CCR7- cells cannot home to lymph nodes but exist in the periphery and are termed effector memory cells. Effector memory cells have been found in the bone marrow in response to LCMV (Slifka, Whitmire et al. 1997), in the lung in response to intranasal influenza (Flynn, Belz et al. 1998), respiratory viruses (Hogan, Usherwood et al. 2001) or Sendai virus (Usherwood, Hogan et al. 1999), in the liver in response to oral *Listeria monocytogenes* infection (Pope, Kim et al. 2001), in gut mucosal tissue in response to vesicular stomatis virus, vaccinia virus, and Listeria (Masopust, Jiang et al. 2001) and in the brain in response to dengue virus (van der Most, Murali-Krishna et al. 2003). Both central and effector memory populations are found in the blood and spleen.

1.C.1.2 Functional characterization:

The distinct roles central and effector memory subsets in providing protective immunity for the host are presently unclear (Masopust and Lefrancois 2003; Masopust, Kaech et al. 2004). Contradictory data exist regarding the speed with which effector memory and central memory cells acquire effector functions such as cytokine secretion and cytotoxic activity. Experiments involving polyclonal stimulation of effector and central memory cells have shown that effector memory responses are faster (Sallusto, Lenig et al. 1999; Tussey, Speller et al. 2000; Masopust, Vezys et al. 2001); however, experiments involving stimulation with cognate antigen have demonstrated equivalent responses between the two subpopulations (Unsoeld, Krautwald et al. 2002; Wherry, Teichgraber et al. 2003). In addition, central memory cells have a proliferative advantage over effector memory cells in response to antigenic stimulation, suggesting that central memory cells may provide better immunological protection over time (Wherry, Teichgraber et al. 2003)

1.C.1.3 Generation of CD8+ Central and Effector Memory Cells:

The lineage relationship between central and effector memory is also presently unclear. Two opposing models exist for the relationship between these two subtypes, with experimental data existing to support both. In one model, central memory and effector memory cells are two independent potential pathways for CD8+ memory cell differentiation (Sallusto and Lanzavecchia 2001). Data consistent with this theory comes from *in vitro* studies showing that the cytokine environment can influence the phenotypic generation of central or effector memory cells (Manjunath, Shankar et al. 2001). Furthermore, the TCR repertoire in human central and effector memory cells is distinct,

supporting the argument that these two subpopulations are distinct (Baron, Bouneaud et al. 2003).

In sharp contrast, *in vivo* data from mice using both a viral and bacterial model of infection suggest that there is a linear progression from effector memory cells to central memory cells (Wherry, Teichgraber et al. 2003). Furthermore, the conversion rate of effector memory cells to central memory is linked to the initial antigenic challenge and programmed during the initial antigen encounter. Low dose antigen exposure leads to faster conversion rates from effector memory to central memory cells, whereas high dose antigen exposure leads to slower rates. Collectively, these findings provide a model for linear CD8+ memory T cell differentiation from effector memory to central memory in the case of acute infections.

The relationship between central and effector memory cells in the case of chronic infections is also unclear. Recent experimental data has shown that persistent viral antigen leads to the accumulation of distinct populations of memory cells or different stages of memory cells, depending on the viral infection (Champagne, Ogg et al. 2001; Appay, Dunbar et al. 2002). The lineage relationship between these different populations has been difficult to ascertain *in vivo*.

1.C.2 Progressive versus Linear models for CD8+ Memory Cell Generation:

The generation of memory cells from naïve cells is a poorly understood process. There are currently two models for how this differentiation occurs **(Figure 1-5)**. The progressive model of differentiation argues that a naïve cell commits to either memory cell formation or effector cell generation based on the strength of the TCR signal during priming, with stronger signals leading to effector cells and weaker signals leading to the

generation of memory cells (Sallusto and Lanzavecchia 2001). In contrast, the linear differentiation model argues that memory cell precursors are a subset of effector cells that survive the contraction phase of the memory cell response. There is data to support both these viewpoints.

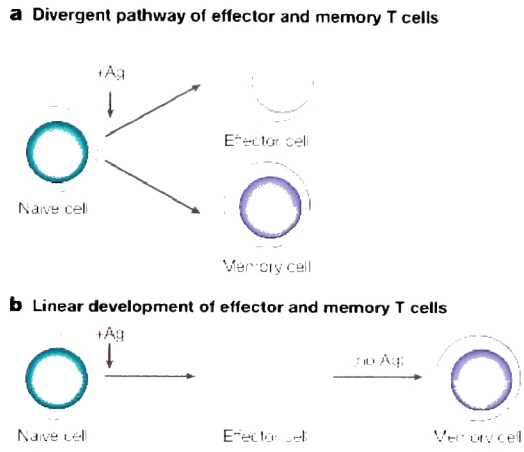


Figure 1-5. Progressive and linear models for memory CD8+ cell generation. **a**. Progressive model. CD8+ T cells can bypass the effector stage and directly become memory cells. **b**. Linear model. Memory CD8+ cells are direct descendents of effector CD8+ cells. Figure taken from Kaech et al., Nature Reviews Immunology, 2002.

The data in favor of the linear progression model is three fold. Experiments utilizing Cre-mediated genetic marking of activated CD8+ cells have shown that memory cells can arise from activated CD8+ cells (Jacob and Baltimore 1999; Opferman, Ober et al. 1999). In addition, the signals that mediate naïve CD8+ cell differentiation into effector cells are also sufficient for CD8+ memory cell formation. Cells stimulated with antigen for a brief period not only undergo programmed division and gain effector function but also generate long-lived memory cells (Mercado, Vijh et al. 2000; Kaech and Ahmed 2001). Finally, identification of CD8+ memory cell precursors during an

immune response has shown that precursors upregulate expression of genes required for effector functions (Kaech, Tan et al. 2003).

Data also exist that are more consistent with the progressive model of differentiation, which states that CD8+ memory cell formation and CD8+ effector cell generation are two independent pathways. It has been shown both *in vitro* and *in vivo* that it is not necessary for CD8+ cells to pass through an effector phase for memory cell generation (Lauvau, Vijh et al. 2001; Manjunath, Shankar et al. 2001). Furthermore, CD8+ cell proliferation, which is critical for memory cell generation, can be uncoupled from gain of effector function (Hernandez, Aung et al. 2002). The discrepancies between this data and the data in support of the linear differentiation model may be resolved once central and effector memory cell generation are considered separately.

1.C.3 The impact of Proliferation on CD8+ Memory Cell Development:

Upon antigen encounter in lymphoid tissues, CD8+ T cells undergo a programmed proliferative response. This proliferation leads to a 10,000-100,000 -fold increase in the number of antigen specific cells (Murali-Krishna, Altman et al. 1998; Blattman, Antia et al. 2002). The extent of proliferation is important because the size of the memory pool is directly correlated with the size of the effector-cell population (Hou, Hyland et al. 1994; Busch, Pilip et al. 1998). Memory cell numbers after antigen encounter are always roughly 10% of cell numbers seen at the peak of the effector response, regardless of whether the number of antigen specific cells recruited initially is only 1-2% of total CD8+ cells, as in the case for *Listeria*, or is over 50%, as is the case for LCMV (Busch, Pilip et al. 1998; Butz and Bevan 1998). Thus mechanisms that

modify CD8+ effector cell generation will invariably effect CD8+ memory cell generation as well.

1.C.4 The effect of Antigen Dose on CD8+ Memory cell Generation:

Both *in vitro* and *in vivo* data demonstrate that only a brief antigenic exposure (2-24 hours) is required for commitment to 7-10 rounds of programmed division (Mercado, Vijh et al. 2000; Kaech and Ahmed 2001; van Stipdonk, Lemmens et al. 2001; Wong and Pamer 2001; van Stipdonk, Hardenberg et al. 2003). The daughter cells derived during this proliferation need no further antigenic stimulation for proliferation to proceed. Furthermore, weak signaling through the peptide-MHC complex, which does not induce calcium fluxes or phosphorylation of MAPK, can none-the-less lead to proliferation (Rosette, Werlen et al. 2001).

Although a proliferative response can occur in response to minimal antigen exposure, the amount of antigen available plays a critical role in the extent of proliferation *in vivo*. Infection of mice with recombinant vaccinia strains expressing different levels of an ovalbumin epitope demonstrate that the magnitude of the CD8+ T cell response varies with the levels of epitope presented (Wherry, Puorro et al. 1999). Similar results have been obtained by titrating peptide and peptide loaded dendritic cells (Ludewig, Ehl et al. 1998; Kedl, Rees et al. 2000; Kaech and Ahmed 2001). These data show that upon minimal antigen exposure, CD8+ cells proliferate 7-10 times, but that both the recruitment of naïve precursors and the rate of expansion of the recruited cells can be increased by further antigen exposure, leading to an increase of the magnitude of the expansion (Badovinac, Porter et al. 2002). In this way, the strength of antigenic

stimulation plays a role in the development of effector and memory CD8+ cells upon acute antigen exposure.

The role of antigen in CD8+ memory cell formation is more complicated in persistent infections. Antigen dependent proliferation does not continue indefinitely, and expansion ceases in the case of chronic persisting antigen (Badovinac, Porter et al. 2002). Furthermore, persisting antigen can compromise CD8+ memory cell development (Wherry, Teichgraber et al. 2003).

1.C.5 The role of co-stimulatory ligands in CD8+ memory cell generation:

There are two ways in which co-stimulatory ligands can effect CD8+ memory cell generation. The first is by modulating the strength of the proliferative response. The second is by providing signals necessary for CD8+ memory cell generation. Data exist to support a role for both of these pathways.

The strength of the T cell receptor (TCR) signal is determined not only by the presence of antigen but also by the co-stimulatory signals present. Studying the role of co-stimulatory ligands in CD8+ effector cell generation can be confounded by the fact that CD4+ cells are at times necessary for CD8+ effector cell generation (Kaech, Wherry et al. 2002). Fortunately, some viral infections in mice induce CD8+ effector cells in the absence of CD4+ T cells. Studies performed with these viral infections in mice deficient in specific co-stimulatory ligands has shed light on which ligands are important for a robust CD8+ effector cell response. The co-stimulatory ligands CD40L and OX40 are not critical for CD8+ effector cell function. (Kopf, Ruedl et al. 1999; Whitmire and Ahmed 2000). The role of the co-stimulatory ligand CD28 seems to be virus dependent, as generation of CD8+ effector cells in response to LCMV is not affected (Shahinian,

Pfeffer et al. 1993) but responses to VSV (McAdam, Farkash et al. 2000), vaccinia (Sigal, Reiser et al. 1998), and influenza (Wu and Liu 1994) are reduced. 4-1BBL is critical for CD8+ effector function. (DeBenedette, Wen et al. 1999; Tan, Whitmire et al. 1999; Tan, Whitmire et al. 2000). Negative regulators, such as CTLA-4, BTLA, and PD-1 also play a role in attenuating clonal expansion (Greenwald, Latchman et al. 2002; Watanabe, Gavrieli et al. 2003). Thus, modulation of co-stimulatory pathway can effect CD8+ memory cell generation indirectly via expansion of CD8+ effector cells.

A role for co-stimulatory ligands in regulating memory CD8+ cell generation also has experimental support. Although CD4+ cells can be dispensable for CD8+ effector cell generation, recent data suggest that CD4+ cells are crucial for CD8+ memory cell formation. The first evidence of this phenomenon came from analysis of female HY TCR transgenic mice exposed to male HY antigen. Although CD8+ effector generation and expansion in response to HY occurred normally, CD8+ cells showed poor proliferative capacity and effector function upon restimulation in *vitro* (Bourgeois, Rocha et al. 2002). Upregulation of CD40 on CD8+ cells during activation led to the hypothesis that CD40-CD40L interactions between CD4+ and CD8+ cells is critical for memory cell formation. Additional support for a role for CD4+ cells in CD8+ memory cell generation came from a model involving cross-priming-induced CD8+ T cell activation. Depletion of CD4+ cells as short as 3 days after the infection revealed that CD4+ cell help was required only during the initial antigen exposure (Janssen, Lemmens et al. 2003). Finally, the importance of CD4+ cells in CD8+ memory cell generation has been extended to include CD8+ memory cell generation after acute infections (Shedlock and Shen 2003; Sun and Bevan 2003). Collectively, these data support the uncoupling of

CD8+ memory cell formation from CD8+ effector generation in the absence of CD4+ cells. Furthermore, robust secondary responses even in the absence of CD4+ helper cells suggest that an instructive signal for CD8+ memory cell generation is provided by CD4+ cells during the initial CD8+ cell priming (Masopust, Kaech et al. 2004).

1.C.6 Proliferation *per se* and CD8+ memory cell generation:

The process of proliferation is critical for memory cell development. Experiments addressing the role of cell division in CD8+ memory cell generation used *in vitro* proliferation of CD8+ cells labeled with CFSE (carboxyfluoroscein diacetate succinimidyl ester) to allow for the isolation of activated CD8+ cells having undergone a specific number of divisions. Seventy days after transfer, cytotoxic T lymphocyte (CTL) precursor frequencies from mice adoptively transferred with activated CD8+ cells that had undergone less than five cell divisions were similar to those of mice transferred with naïve cells. In contrast, transfer of activated CD8+ cells that had undergone more than nine divisions lead to a significant increase in CTL precursor frequency in the transferred mice, suggesting that memory cells had been transferred (Opferman, Ober et al. 1999).

Proliferation is more important than even cognate antigen stimulation, as memory-like cells also develop after homeostatic proliferation in a lymphopenic environment (Cho, Rao et al. 2000; Goldrath, Bogatzki et al. 2000; Murali-Krishna and Ahmed 2000; Goldrath, Sivakumar et al. 2002). Homeostatically proliferating cells not only express memory cell-surface markers but also have cytolytic activity and secrete IFNγ faster in response to antigen (Cho, Rao et al. 2000; Goldrath, Bogatzki et al. 2000). These memory-like cells do not behave exactly as memory cells, however. In contrast to true memory cells, memory-like cells revert to a naïve phenotype upon removal from a

lymphopenic environment (Cho, Rao et al. 2000). Furthermore naïve CD8+ cells undergoing homeostatic expansion do not survive in MHC I deficient hosts (Murali-Krishna JI 2000), in direct contrast to memory CD8+ cells (Murali-Krishna, Lau et al. 1999).

1.C.7 Cell Survival Factors and CD8+ Memory Cell Development:

After antigen encounter, the proliferative phase of the immune response is followed by a contraction phase, where 90-95% of the CD8+ effector cells undergo programmed cell death (Ahmed and Gray 1996). This contraction phase is programmed during the initial antigen encounter. Treatment of mice infected with *Listeria monocytogenes* with antibiotics 24 hours after infection reduces the magnitude of expansion and the course of infection, but has no effects on the kinetics and extent of contraction (Badovinac, Porter et al. 2002). Conversely, treatment with a higher dose of *Listeria* prolongs the infection but does not change the magnitude or kinetics of the contraction phase, even when there is a persistent infection at the beginning of the contraction phase (Badovinac, Porter et al. 2002). This contraction in the face of persisting antigen may be important for preventing lethal autoimmune pathologies.

The factors that determine the kinetics of the contraction phase as well as which cells will survive to become memory cells are currently being elucidated. IFNγ has been implicated in the timing of the contraction phase, as the delayed kinetics of contraction seen in a secondary immune response are also seen in the primary immune response of IFNγ deficient mice (Badovinac, Tvinnereim et al. 2000). Furthermore, inhibiting inflammation and IFNγ production by giving antibiotics prior to an infection limits the extent of contraction but does not effect memory cell formation (Badovinac, Porter et al.

2004). These data suggest that memory cells do not form by a default pathway after surviving the contraction phase.

1.C.7.1 IL-7:

Memory cell precursors exist in the effector cell population at the peak of the immune response, but at this time they do not show the functional hallmarks of memory T cells (Kaech, Hemby et al. 2002; Wherry, Teichgraber et al. 2003). These properties are gained over the course of several weeks, but the signals that drive this differentiation are unknown. What is clear is that survival is a prerequisite for memory cell generation, but the mechanisms that lead to the selective survival of precursor memory cells are still under investigation.

The clearest case for a survival factor being involved in memory cell generation is IL-7. IL-7 is required for the survival of naïve T cells (Tan, Dudl et al. 2001). In addition, it is required for naïve CD8+ cell proliferation in a lymphopenic environment (Goldrath, Sivakumar et al. 2002). Recently, it has been shown that CD8+ memory cell precursors can be identified by their expression of IL-7Rα (CD127) during the peak effector response after antigen encounter (Kaech, Tan et al. 2003). IL-7Rαhi cells are identified as memory cell precursors by several lines of evidence. Experiments involving transfer of IL-7Rαhi or IL-7Rαlo cells into naïve mice and challenge three weeks later show that IL-7Rαhi cells but not IL-7Rαlo provided immunological protection. Although IL-7Rαhi memory precursor cells express granzyme B like other effector cells, they differ in their expression of tumor necrosis factor receptor superfamily member 7 (CD27) and killer cell-lectin like receptor G1 (KLRG-1), which have been linked to senescent effector cells (Hamann, Baars et al. 1997; Voehringer, Blaser et al. 2001).

Furthermore, IL-7Rαhi cells undergo homeostatic proliferation *in vivo*, an important characteristic for potential memory cells.

Since IL-7 is a survival factor for T cells, it is thought that expression of IL-7Rα leads to the selective survival of the 10-15% of effector cells that express it. Indeed, memory precursor survival depends on the presence of IL-7. How IL-7 increases survival is presently unclear. Signals that modify a cells ability to survive do not necessarily effect CD8+ memory cell formation. For example, p53 deficiency does not lead to an increase in the generation of memory cells (Grayson, Lanier et al. 2001). Although Bcl-2 is upregulated in response to IL-7 signaling and in memory cells, transgenic Bcl-2 and Bcl-XL mice also show no decrease in the contraction phase or increase the size of the memory cell pool after infection (Razvi and Welsh 1993; Petschner, Zimmerman et al. 1998). Thus the mechanisms that allow a minority of cells expressing IL-7Rα to survive and become long-lived memory cells remain to be elucidated.

Recently, a role for CD8αα expression in defining memory precursor cells and causing IL-7Rα upregulation has been demonstrated (Madakamutil, Christen et al. 2004). CD8αα binds the nonclassical MHC I molecule Thymus Leukemia antigen (TL) and is thought to modulate TCR signaling (Leishman, Naidenko et al. 2001). Expression of CD8αα has been linked to thymocyte survival (Barnden, Heath et al. 1997; Chidgey and Boyd 1997). Why CD8αα is upregulated on a subset of effector cells, or how its upregulation regulates IL7Rα upregulation and survival remain unclear.

1.C.7.2 Bcl-6:

Data exist that implicate the survival factor Bcl-6 in CD8+ memory cell generation. Bcl-6 deficient and Bcl-6 transgenic mice have a decrease and an increase in memory phenotype CD8+ cells, respectively (Ichii, Sakamoto et al. 2002). Surprisingly, there is no change in the rate of apoptosis of CD8+ cells in Bcl 6-/- mice; rather, Bcl 6 seems to be involved in maintaining cells in a CD44hi Ly6C+ state and in generating central memory cells (Ichii, Sakamoto et al. 2004). Bcl6 may play a similar role in CD8+ T cells as it does in B cells, where it is involved in inhibiting terminal differentiation and maintaining stem-cell like proliferative qualities (Fearon, Manders et al. 2001).

1.C.8 Homeostatic proliferation of memory CD8+ cells:

One of the hallmarks of memory CD8+ cells is their ability to undergo slow homeostatic proliferation (Tough and Sprent 1994; Sprent 2003). The rate of homeostatic proliferation can vary between CD8+ memory cells, as 40-50% of memory cells remain in interphase and do not incorporate BrdU even after five weeks of labeling (Tough and Sprent 1994). Survival and homeostatic proliferation are both independent of TCR signaling, as memory-phenotype cells persist and proliferate in MHC I deficient hosts (Murali-Krishna, Lau et al. 1999). Instead, cyotokines play a key role in homeostatic maintenance.

1.C.8.1 The role of IL-15 in CD8+ memory cell homeostasis:

Interleukin 15 (IL-15) is synthesized by a wide variety of cells, especially macrophages, but not by T cells (Kennedy and Park 1996). Memory CD8+ cells are uniquely sensitive to IL-15, as demonstrated by several lines of evidence. Treatment of CD44hi CD8+ memory-like cells with low levels of IL-15 *in vitro* leads to their proliferation, in direct contrast to CD44lo naïve CD8+ cells or CD44hi CD4+ cells

(Zhang, Sun et al. 1998). Injection of IL-15 *in vivo* also causes a proliferative increase in the CD44hi CD8+ population (Zhang, Sun et al. 1998). IL-15 transgenic mice have a selective accumulation of memory phenotype CD8+ cells (Marks-Konczalik, Dubois et al. 2000; Yajima, Nishimura et al. 2001). Conversely, studies *in vivo* using blocking antibodies have revealed that CD8+ memory cell division requires IL-15 but is inhibited by IL-2 (Dai, Konieczny et al. 2000; Ku, Murakami et al. 2000). Further evidence for the critical role of IL-15 *in vivo* comes from the selective decrease in CD8+ memory cells in IL-15 deficient mice (Lodolce, Boone et al. 1998; Kennedy, Glaccum et al. 2000). Transfer of memory CD8+ cells into IL-15 deficient hosts leads to the loss of memory cells over time due to a lack of homeostatic proliferation (Goldrath, Sivakumar et al. 2002). Surprisingly, transfer of IL-15Rα deficient CD8+ cells into normal hosts followed by immunization revealed that IL-15Rα expression by CD8+ cells is not required for memory cell generation (Burkett, Koka et al. 2003). This finding was reconciled with existing data by showing that IL-15Rα can bind and present IL-15 *in trans* to the common βγ chains. Finally, a role for IL-15 in the homeostatic proliferation of antigen induced bona-fide memory cells has also been demonstrated (Wherry, Becker et al. 2002). The sensitivity of memory CD8+ cells to IL-15 may be due to the selective upregulation of the shared IL-2 and IL-15Rβ chain (Giri, Ahdieh et al. 1994) on memory-like CD8+ cells.

1.C.8.2 The role of IL-7 in CD8+ memory cell homeostasis:

The role of IL-7 in homeostatic maintenance of memory CD8+ cells is less clear, presumably because of the partially overlapping functions of IL-15 and IL-7. Transfer of CD8+ memory cells into hosts given IL-7Rα blocking antibody decreases the extent of

homeostatic proliferation but does not completely block it (Goldrath, Sivakumar et al. 2002). Further evidence for the functional redundancy between IL-15 and IL-7 comes from transfer experiments into lymphopenic hosts. Unlike CD8+ memory cells transferred into complete hosts, which are completely dependent on IL-15 for their basal homeostatic proliferation, CD8+ memory cells transferred into either IL-15 or IL-7 deficient lymphopenic hosts have only a slight impairment in acute homeostatic proliferation. Acute homeostatic proliferation is blocked only in the absence of both of these cytokines (Schluns, Kieper et al. 2000; Goldrath, Sivakumar et al. 2002). Finally, overexpression of IL-7 in IL-7 transgenic animals leads to IL-15 independent maintenance of memory CD8+ cells (Kieper, Tan et al. 2002). These data taken together suggest that both IL-7 and IL-15 are involved in regulating homeostatic proliferation of CD8+ memory cells, but that in a normal host, competition for IL-7 with other cell types makes the role of IL-15 more critical in CD8+ memory cell maintenance.

1.C.9 Biochemical pathways involved in CD8+ memory cell generation and maintenance:

Although the requirements for CD8+ memory cell generation and maintenance have been defined at the cellular and even cytokine level, the biochemical pathways governing a cell's decision to commit to the effector and memory lineage remain to be determined. Insight into the potentially important signaling molecules has been provided by analysis of expression levels of genes in naïve, memory, and activated CD8+ cells (Kaech, Wherry et al. 2002), but the definitive importance of these candidates awaits confirmation from *in vivo* analysis.

Genetic proof exists for the role of SOCS1 in CD8+ memory cell generation. SOCS1 fl/fl Lck-Cre mice, which under go SOCS1 deletion specifically in the thymus

and T cell compartments, shows an increase in the memory-like compartment of CD8+ cells in the periphery (Chong, Cornish et al. 2003). Thymocytes from these mice are hypersensitive to IL-7 induced Stat5 phosphorylation. SOCS1-/- IFNγ-/- mice, which do not die from the lethal IFNγ induced inflammation seen in SOCS1 -/- mice, also accumulate memory phenotype CD8+ cells because of an increased sensitivity to IL-15 induced Stat5 phosphorylation (Ilangumaran, Ramanathan et al. 2003).

Stat5 has independently been linked to memory CD8+ cell generation and maintenance. Stat5a-/- and Stat5b-/- mice have decreased CD8+ memory cells, whereas Stat5 transgenic mice have markedly increased CD8+ memory cell numbers (Kelly, Spolski et al. 2003). Stat5 overexpression also rescues the CD8+ memory cell deficiency seen in common gamma chain deficient mice (Kelly, Spolski et al. 2003). The precise role that Stat5 plays either in the generation or maintenance of memory CD8+ cells remains unclear at this time.

1.D. Ubiquitination:

Ubiquitination of a protein refers to the covalent attachment of the 76 amino acid, ubiquitously expressed globular protein ubiquitin by the stepwise action of three enzymes. It is a commonly occurring phenomenon in eukaryotes, second only to phosphorylation as a means of post-translational modification. The universality of this form of regulation is highlighted by the fact that there is only a three amino acid difference in the ubiquitin sequence between yeast and humans (Weissman 2001).

1.D.1 Types of ubiquitination:

The ubiquitination of proteins is not a homogenous modification. Proteins can be ubiquitinated on either internal lysine residues or on the α-NH2 at their N-termini (Breitschopf, Bengal et al. 1998). Some proteins are ubiquitinated on only specific lysine (K) residues (Chau, Tobias et al. 1989; Baldi, Brown et al. 1996), while other proteins show little specificity regarding which lysine residue is ubquitinated (Hou, Cenciarelli et al. 1994; Treier, Staszewski et al. 1994). The importance of which residues are ubiquitinated is unclear at present.

In addition to the location of the ubiquitin moiety, the length of the ubiquitin chain can also vary. Proteins can be mono- or poly- ubquitinated on a given lysine residue, and different lysine residues within the same protein may be targeted for mono- or poly- ubiquitination. Mono-ubiquitination serves as a signal for chromatin modification and regulates various trafficking events (Hochstrasser 2004). Polyubiquitination is important for regulating a protein's abundance, as a chain of four or more ubiquitin tags is required to target a protein for proteosomal degradation (Thrower, Hoffman et al. 2000).

The branching of the ubiquitin chain is also important. Ubiquitin contains seven conserved lysine residues, all of which could potentially be used for iso-peptide linkage. Thus far, chain linkage sites at ubiquitin K11, K29, K48, and K63 have been described *in vivo*. K48 linked polyubiquitin chains historically have been defined as signals for proteosomal degradation (Chau, Tobias et al. 1989; Thrower, Hoffman et al. 2000); however, emerging data suggests that they may also inhibit a protein's activity without leading to its degradation (Elsasser, Gali et al. 2002; Bai, Yang et al. 2004; Flick, Ouni et al. 2004). In contrast, polyubquitin chains formed by attachment at K63 have been linked

to protein activation (Deng, Wang et al. 2000) and endocytosis (Bonifacino and Weissman 1998).

1.D.2 The roles of ubiquitination:

Ubiquitination has been linked to myriads of cellular processes, including bulk-protein degradation, cell-cycle control, DNA repair, stress response, vesicular trafficking transcriptional regulation, as well as signal transduction (Hershko and Ciechanover 1998). Protein ubiquitination regulates the participation of a protein in a particular biological process by changing its abundance, activity, or subcellular localization (Mueller 2004). The way in which a ubiquitin moiety can regulate a protein's activity in so many diverse ways is only beginning to be understood, and there remain many unanswered questions.

1.D.3 The enzymatic reactions of ubiquitination:

Ubiquitination consists of three steps **(Figure 1-6)**. First, the ubiquitin-activating enzyme E1 activates ubiquitin by forming a thioester bond between the c-terminus glycine residue of ubiquitin and the active cysteine residue in E1. The universality of this step is underscored by the fact that there is a single E1 gene in humans that gives rise to two isoforms of E1 (Grenfell, Trausch-Azar et al. 1994). The activated ubiquitin is then transferred via *trans*-acylation to the active cysteine residue of one of at least 25 human ubiquitin conjugating enzymes (E2). Ubiquitin is then transferred to target proteins with the help of E3 ligases, which confer substrate specificity of ubiquitination. There are four families of E3 ligases, namely U box (Ufd2 related), RING (Really Interesting New Gene), SOCS (suppressor of cytokine signaling), and HECT (homologous to E6-AP C-terminus) ligases. The distinction between these families is based on structural as well as

functional features (Davis and Ben-Neriah 2004). Finally, an additional factor (E4) can be necessary for some ubiquitin chain-assembly reactions (Koegl, Hoppe et al. 1999)

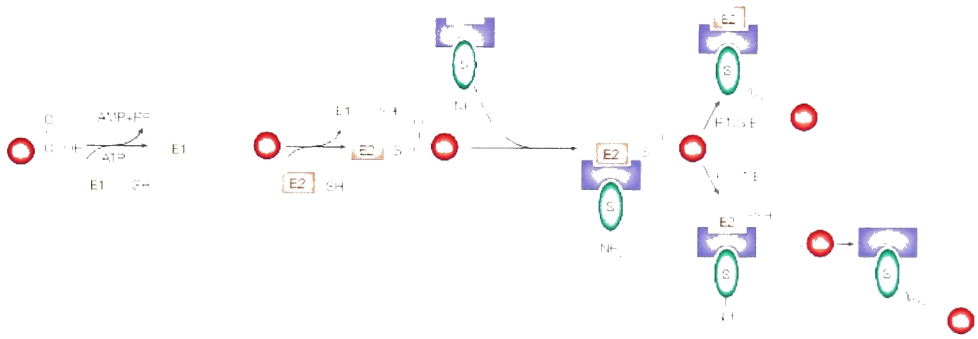


Figure 1-6. The ubiquitination pathway. The C-terminus of ubiquitin is linked by a thiol-ester bond to E1 in an ATP dependent reaction. Ubiquitin is transferred to E2s. E2s associate with E3s. In the case of HECT E3 ligases, ubiquitin is transferred to the active cysteine of the HECT domain. Figure taken from Weissman, Nature Reviews Molecular Cell Biology, 2001.

1.D.4 U box E3 ligases:

The U box domain was initially identified in the yeast protein Ufd2, which possesses E4 activity. Homologs of this protein contain U box domains and comprise the U box family of E3 ligases **(Figure 1-7)**. These proteins function as E3 ligases in an E2 dependent fashion, because they cannot directly ubiquitinate target proteins. None of the murine or human family members has been shown to have E4 activity (Hatakeyama and Nakayama 2003).

1.D.5 RING finger E3 ligases:

The RING finger domain contains an octet of cysteine and histidine residues that constitute a zinc-binding domain **(Fig 1-7)**. Most RING-finger domain proteins function as E3 ligases by binding both target substrates and E2 ligases. RING-finger E3 ligases do not directly ubiquitinate target proteins but rather function to specify the targets of E2 ligases. Members of the RING-finger E3 ligase family are further divided into four subfamilies based on structural and functional differences. These subgroups are the multi-protein subunit families SCF(SKP1,Cul1, F-Box), CBC (Cul 2-Elongin, B Elongin, C), APC/C (Anaphase Promoting Complex/cyclosome), and the single polypeptide RING-finger E3 ligase subfamily.

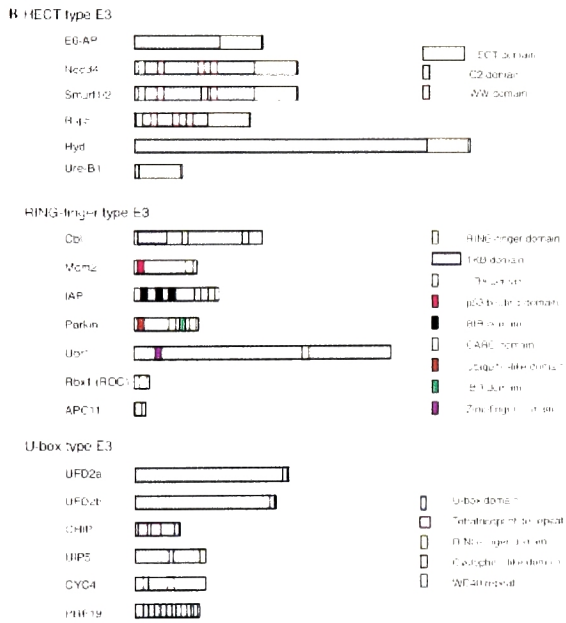
1.D.6 SOCS E3 ligases:

The eight SOCS proteins contain a central src-homology 2 (SH2) domain and a C-terminal 40 amino acid SOCS box domain **(Figure 1-7)**. SOCS proteins are negative regulators of cytokine signaling cascades, but they use a variety of mechanisms to achieve this end. One mechanism is through protein ubiquitination, in which the SOCS-box domain binds elongins B and C and forms part of an E3 ligase ubiquitin complex (Alexander 2002).

1.D.7 The HECT family of E3 ligases:

The HECT (homology to E6-AP C terminus) family members are defined by their conserved catalytic C-terminal domain, first identified in the E6- associated protein (E6-AP) **(Figure 1-7)**. This protein, together with the papillomavirus oncoprotein E6, leads to the ubiquitination and degradation of p53 (Scheffner, Huibregtse et al. 1993). The conserved C-terminal in the HECT E3 ligases forms a covalent bond with ubiquitin, allowing these E3 ligases to directly ubiquitinate substrates. This is contrast to other E3

ligases, which bind both E2 ligases and target substrates, bringing the E2 ligase in close enough proximity to ubiquitinate the substrate. The N-termini of HECT E3 ligases are less conserved and contain protein-protein interaction domains or intracellular localization domains. Several HECT E3 ligases contain WW domains in groups of two or four in the N-termini. These WW domains form hydrophobic pockets for binding proline rich sequences. Most HECT E3 ligases that contain WW domains also contain a C2 domain that mediates translocation to the membrane in response to increases in intracellular calcium.



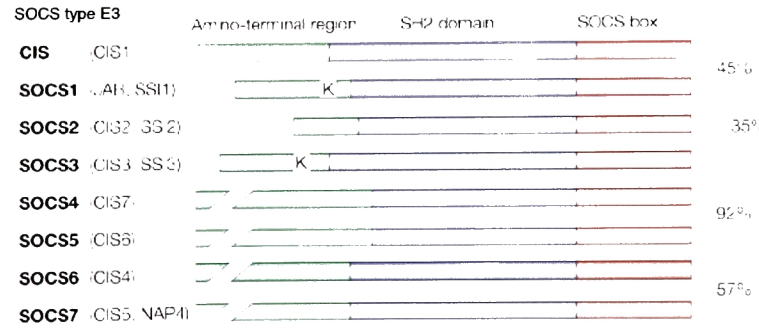


Figure 1-7. The structural domains of HECT, RING, Ubox and SOCS family E3 ligases. Figures taken from Hatakeyama et al., Biochem Biophys Res Com, 2003 and Alexander, Nature Reviews Immunology 2002.

1.D.7.1 The HECT E3 ligase Itch

Itch belongs to the HECT class of E3 ligases. It contains an N-terminal C2 domain, 4 WW protein interaction domains, and a C-terminal HECT ubiquitin ligase domain. It is a highly conserved protein, with homologs in yeast (Rsp5p) (Hicke and Dunn 2003), C. *elegans* (CelWWP1) (Courbard, Fiore et al. 2002), and Drosophila (Su(dx)) (Cornell, Evans et al. 1999). Biochemically, several substrates have been identified for Itch. Studies exist implicating Itch in ubiquitinating Notch (Qiu, Joazeiro et al. 2000), Bcl-10 (Scharschmidt, Wegener et al. 2004), CXCR4 (Marchese, Raiborg et al. 2003), human enhancer of filamintin (Feng, Guedes et al. 2004), endophilin (Angers, Ramjaun et al. 2004), Smad 2 (Bai, Yang et al. 2004), Cbl (Magnifico, Ettenberg et al. 2003), LMP2A (Ikeda, Caldwell et al. 2003), occludin (Traweger, Fang et al. 2002), NF-E2 (Chen, Frank et al. 2001), phospholipase C gamma (PLCγ) (Heissmeyer, Macian et al. 2004), and Jun-B (Fang, Elly et al. 2002). The physiological relevance of these findings, especially with regard to the immune system, is still unclear in many cases.

1.D.8 Roles for Ubiquitin in the Immune System:

Since ubquitination is such a universal and fundamental cellular process, it is not surprising that cells of the immune system utilize it as well; however, understanding of ubiquitination-regulated processes in the immune system is still in its infancy. One of the best-described examples is the activation of NF-κB. NF-κB translocation to the nucleus requires the IKK-regulated, ubiquitin-mediated degradation of IκB (Karin and Ben-Neriah 2000). The RING domain containing multimeric structure $SCF^{\beta\text{-}TrCP}$ is responsible for this ubiquitination.

The Cbl-RING and SOCS family E3 ligases are implicated in the termination of activation signals. c-Cbl downregulates receptor tyrosine kinases via targeting to the

endocytic pathway (Levkowitz, Waterman et al. 1998; Joazeiro, Wing et al. 1999; Thien and Langdon 2001). In T cells, c-Cbl ubiquitinates the TCRζ chain in a Zap-70 dependent fashion, leading to TCR downmodulation upon antigen stimulation (Wang, Altman et al. 2001; Naramura, Jang et al. 2002). Ubiquitin also regulates immune cell signaling cascades in a degradation-independent manner. For example,TRAF6 uibiquitination in response to IL-1β leads to its activation (Deng, Wang et al. 2000; Wang, Deng et al. 2001). In addition, Cbl-b ubiquitinates the phosphotidyl inositol 3 kinase (PI3K) subunit p85, preventing its association with CD28 and TCRζ (Fang, Wang et al. 2001). This ubiquitination is critical for inhibiting a cell's response in the absence of co-stimulation. The importance of Cbl-b mediated regulation is underscored by the occurrence of spontaneous autoimmune disease in Cbl-b deficient mice and the inability of Cbl-b deficient T cells to be anergized (Bachmair, Finley et al. 1986; Jeon, Atfield et al. 2004).

1.D.9 The role of Itch in the immune system:

The relevance of Itch to the immune system was initially discovered with the cloning of the inversion breakpoints in the murine agouti mutant strain a^{18H}, which along with a darker coat color displayed an inflammatory disease consisting of interstitial inflammation of the lungs, splenomegaly, lymphadenopathy, and dermatitis in the mice. The inversion resulted not only in a break in the agouti locus, but also disrupted the gene for the E3 ligase Itch (Perry, Hustad et al. 1998). Subsequent analysis of the naturally occurring Itch deficient mice revealed increased serum IgG1 and IgE titres, prompting an analysis of Th differentiation in Itch deficient mice (Fang, Elly et al. 2002). It was found that CD4+ T cells from Itch deficient mice display a bias towards Th2 differentitation *in*

vitro, and further biochemical analysis demonstrated that this bias was due to increased JunB levels (Fang, Elly et al. 2002).

Although the inversion clearly affected the Itch protein, it was possible that the regulation of other genes within the inverted region was affected. Furthermore, although the inflammatory disease in Itch deficient mice was consistent with a bias in Th2 differentiation, the experiments performed could not address whether the changes in CD4+ T cell differentiation were secondary to the inflammatory pathology in these mice or a cause for it. Since all cells were deficient for Itch, it was difficult to ascertain which lymphocyte subpopulation was responsible for initiating the disease.

1.D.10 Generation of mice with cell specific inactivation of Itch:

The limitations inherent in the analysis of Itch deficient mice led Kristina Jönsson to generate mice in which it would be possible to inactivate Itch in a cell specific manner (Jonsson 2002) **(Figure 1-8)**. These mice were generated by flanking the last exon of Itch containing the catalytic cysteine of the HECT domain with loxP sites (Itch fl/fl mice). Breeding of Itch fl/fl mice to transgenic mice expressing Cre in specific cell types allowed for the analysis of Itch inactivation in a cell specific manner. Because Cre mediated deletion leads to the loss of only the last 32 amino acids of the 864 amino acid protein, a truncated Itch protein may still be present in cells in which Cre is expressed. The E3 ligase activity of this protein is abolished, however, because of the absence of the catalytic cysteine in the mutant protein. Itch fl/fl Deleter Cre mice, in which Itch is deleted in all cells, have the same inflammatory disease as the Itch deficient mice resulting from an inversion in the

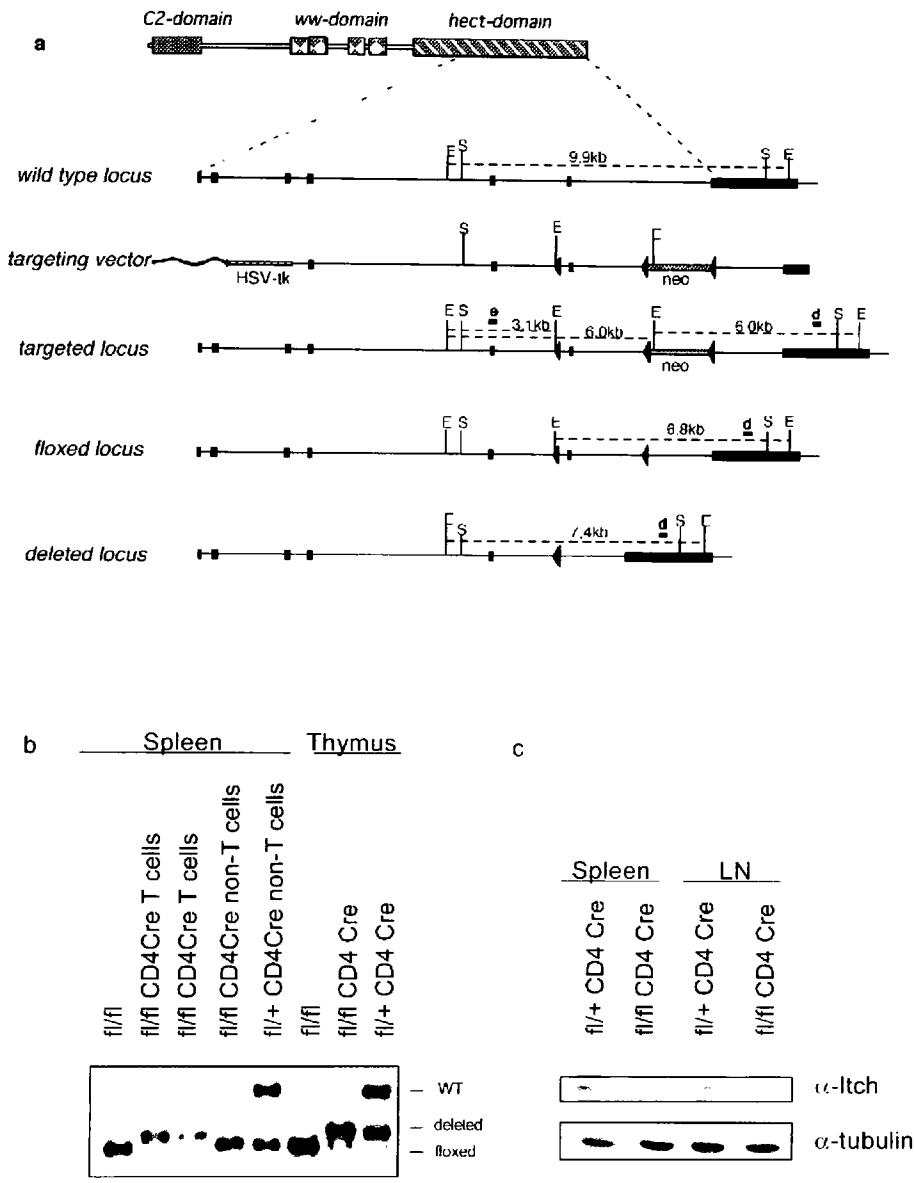


Figure 1-8. Generation of conditional Itch deficient mice: **a.** Strategy for Itch gene targeting. Domain structure of the Itch protein, partial structure of the Itch genomic locus, maps of the targeting vector and the targeted Itch locus before and after Cre mediated recombinations are delineated. Filled rectangles represent exons. Triangles represent loxP sites. The partially filled rectangles represents the selection marker genes. Restriction sites are E: *Eco RV.* Probes d and e used in Southern blots are depicted by filled rectangles in the map and the length of respective restriction fragments detected by these probes is depicted numerically above dashed lines representing the restriction fragments. **b.** Deletion efficiency in Itch fl/fl CD4 Cre mice. Southern blot analysis of genomic DNA from splenocytes or thymocytes digested with EcoRV and visualized with probe d. T cells were purified by depletion and the bound fraction consisting of non-T cells was used to determine CD4 Cre specificity. Genomic DNA was digested using EcoRV and a Southern blot was performed with probe d. The size of wt, deleted, and floxed restriction fragments are indicated. **c.** Western blot anlaysis of the wild-type Itch protein in CD4+ cells purified from the spleen and lymph nodes of Itch fl/fl CD4 Cre mice and littermate controls. Expression of tubulin was measured to control for the accuracy of protein loading.

agouti locus. This data supports the role of Itch in the pathology described in the a^{18H} inversion mutant.

1.E Overall aims of this study:

The experiments described in this thesis present an attempt to define which Itch deficient lymphocyte population is sufficient for generating the previously described Itch pathology, which regulatory networks of the immune system are disrupted as a consequence of Itch deficiency, and which signaling pathways are affected due to Itch deficiency. The findings are discussed in three chapters, based on the immune responses analyzed.

Chapter 2: Materials and methods:

2.A Reagents

2.A.1 Antibodies used for flow cytometric analysis and coating:

All antibodies were purchased from BD Pharmingen unless otherwise noted.

anti-mouse CD11b (clone M/170) biotin 1:100

anti-mouse CD122 (clone TM-B1) biotin 1:100

anti-mouse CD127 (clone SB/199) PE 1:50

anti-mouse CD19 (clone ID3) biotin 1:100

anti-mouse CD19 (clone 1D3) biotin 1:100

anti-mouse CD24 (clone M1/69) biotin 1:400

anti-mouse CD25 (clone 7D4) PE 1:100 (Southern biotech)

anti-mouse CD25 (clone 7D4) biotin 1:100

anti-mouse CD28 (clone 37.51) purified 1:100

anti-mouse CD3ε (clone 145-2C11) purified 1:400

anti-mouse CD4 (clone GK1.5) PE 1:200

anti-mouse CD4 APC 1:100

anti-mouse CD44 (clone IM7) FITC, PE, and biotin 1:100

anti-mouse CD45R/B220 (clone RA3-6B2) cychrome 1:75

anti-mouse CD5 (clone 53-7.3) biotin 1:100

anti-mouse CD62L (clone MEL-14) 1:100

anti-mouse CD69 (clone H1.2F3) 1:100

anti-mouse CD86(B7.2) (clone GL1) biotin 1:100

anti-mouse CD8 (clone 53-6.7) FITC 1:100

anti-mouse CD90.2 (clone 53-2.1) PE 1:400

anti-mouse gamma delta TCR (clone GL3) biotin 1:100

anti-mouse LY-6G (GR-1) (clone RB6-8C5) FITC and biotin 1:100

anti mouse CD49b (clone DX5) biotin 1:100

anti-mouse TCR beta chain (H57-597) biotin 1:100

anti-mouse TER-119 biotin 1:100

anti-mouse NK1.1 (clone PK136) biotin and PE 1:100

streptavidin-cy7 PE 1:100

anti-mouse IFNγ (clone XMG1.2) FITC 1:100

anti-mouse IL-4 (clone 11B11) PE 1: 75

anti-mouse TCRVα11.1 biotin 1:100

anti-mouse TCRVβ11 biotin 1:100

anti-mouse TCRVβ8 (clone F23.1) bitoin 1:100

anti-mouse TCRVβ3 (clone KJ25) biotin and PE 1:100

anti-BrdU FITC 1:50

2.A.2 Antibodies used for Western blots:

Rabbit α-Itch (1383, recognizing the C-terminus of Itch) 1:1000

Rabbit α -JunB (SantaCruz) 1:500

Mouse α -tubulin (Sigma) 1:1000

Mouse α -actin (Santa Cruz) 1:20,000

α -mouse IgGHRP (Amersham) 1:10,000

α -rabbit IgG HRP (Amersham) 1:10,000

α -mouse IgM HRP (Amersham) 1:20,000

2.A.3 Cytokines used for cell culture:

IL-2 (laboratory made; supernatant of X63-Ag8-653 plasmacytoma cell lines transfected with IL-2 expression vector. 1:1000 dilution equivalent to 50 U/ml recombinant IL-2 based on ELISA)

IL-4 (R&D)

IL-12(R&D)

IL-15 (recombinant human IL-15, RDI)

2.A.4 Synthetic oligonucleotides:

Oligonucleotides for PCR and sequencing were purchased lyophilized from Fisher and Invitrogen and reconstituted at a concentration of 200μM in water.

2.A.5. Primers and PCRs used for gentoyping:

Table 2.1 Primers

PCR for	**Primer sequences**	Primer names	Expected prouct sizes	PCR program description
β2M	5'-TCT ggA CgA AgA gCA TCA ggg-3' 5'-TAT CAg TCT CAg Tgg ggg Tg-3' 5'-CTg AgC TCT gTT TTC gTC Tg-3'	Neo160 184WT 185 WT	+/+ 280 bp +/- 280bp 400bp -/- 400bp	1
Itch	5'-ACA AgA ggTAgg AgA CAA gCA TT-3' 5'-TgC AgC TTA TTT ATC ATT CCT TA-3' 5'-Tgg TTA CTT TAT TTg gCT TTg ACA A-3'	Itch A Itch B Itch C'	Wt 237 bp Fl 270 bp Del 497 bp	2 (C57Bl/6) 3 (mixed)
AND	5'-gAC TTg gAg ATT gCC AAC CCA TAT CTA AgT-3' 5'-TgA gC gAA ggT gTA gTC ggA gTT TgC ATT	TgN101 TgN 102	400bp	2
OVA	5'- CAg gAg ggA TCC AgT gCC AgC-3' 5'- Tgg CTC TAC AgT gAg TTT ggT-3'	OVA F OVA R	300bp	2
Stat 6	5'-CTg gAC CTC ACC AAA CgC-3' 5'- CCC ggA TgA CgT gTG C-3' 5'-CTg AAT gAA CTg CAg gAC gA-3' 5'-ATA CTT TCT Cgg CAg gAg CA-3'	Stat6F Stat6R 158NeoF 158NeoR	+/+ 275 bp +/- 275 bp 172 bp -/- 172 bp	4
Rag	5'- AgA CAC ACC ggC TTg CAA CAC Ag -3' 5'- TgC CgA gAA AgT CCT TCT gCC Ag-3' 5'- gTg gAA TgA gTg CgA ggC CAg A-3'	Rag4 Rag5 Rag6	WT 450 bp Mut 344 bp	2
Cre	5'-TAA TCg CCA TCT TCC AgC Ag-3' 5'-CAA TTT ACT gAC CgT ACA C-3'	Cre 1 Cre 2	1025 bp	2
CD4	5' CAA CCA ACA AgA gCT CAA gg-3'	CD4tgF	390 bp	5

Cre	5'-CgC ATA ACC AgT gAA ACA gCA T-3'	MxCreR		
Deleter Cre	5'-gAA gAC ACC ggg ACC gAT CCA g-3' 5'-CgC ATA ACC AgT gAA ACA gCA T-3'	DelCreF MxCreR	474 bp	2
JHT	5'- CAg TgA ATgACA gAT ggA CCT CC-3' 5'-gCA gAA gCC ACA ACC ATA CAT TC-3' 5'-CAC AgT AAC TCg TTC TTC TCT gC-3'	JHT1 JHT2 JHT3	WT 400 bp JHT 650 bp	2
Haplotyping	5' CTC CTT CAT CCA CCA AgA CTA ACA CA-3' 5'- CAT ggC ACA ACT CAg gCA ACT AgA-3' 5'- ggT ACC TgC AgT TCg CCT ATg A-3' 5'-Tgg ggC AAC CAg gCT Agt gTA TA-3'	H-2Dd1F H-2Dd1R H-2Db8F H-2Db8R	b 640 bp d 444 bp	6
d2 probe	5'- AgC ACT gTG gTT CCT TC-3' 5'- Agg TCC CCA TTA gAg AA-3'	D2F D2R	571 bp	1
Foxp3	5'-CAG CTG CCT ACA GTG CCC CTA G-3' 5'-CAT TTG CCA GCA GTG GGT AG-3'	Foxp3F Foxp3R	384 bp	
Foxp3 hyb oligo	5'-ATC CTA CCC ACT GCT GGC AAA TGG AGT C-3'	Foxp3hyb oligo		3
HPRT	5'-GCT GGT GAA AAG GAC CTC T-3' 5'-CAC AGG ACT AGA ACA CCT GC-3'	HPRT-1 HPRT-2	250 bp	1
HPRT hyb oligo	5'-CAC AGG ACT AGA ACA CCT GC-3'	HPRT-2		

Table 2.2 PCR program cycles

PCR program number	Annealing temperature	Annealing time	Extension time	cycles
1	64°C, -0.5°C per cycle 58°C	30 seconds 30 seconds	35 seconds 35 seconds	1-12 13-37
2	62°C	45 seconds	90 seconds	35
3	58°C	45 seconds	90 seconds	35
4	54°C	30 seconds	30 seconds	35
5	62°C	45 seconds	90 seconds	25
6	68°C	30 seconds	45 seconds	30

2.B Molecular Biology Methods:

2.B.1 Bacterial transformation:

Heat shock competent E. coli DH5α were used for transformation. Bacteria were thawed and incubated with plasmid DNA for 20 minutes on ice, heat-shocked at 42°C for 45 seconds, and kept on ice for 2 minutes. Bacterial cells were incubated in 1 ml LB medium at 37°C for 30 minutes prior to plating on LB agar plates containing appropriate selection antibiotics. Colonies were picked 12-16 hours after incubation at 37°C.

2.B.2 Purification of DNA:

Plasmid DNA was isolated by mini, midi, and maxi-prep kits from Qiagen according to the manufacturer's protocol. DNA fragments were excised from agarose gels using a scalpel and purified using Qiaquick gel extraction kit (Qiagen) according to the manufacturer's protocol

2.B.3 Restriction enzyme analysis:

Restriction enzymes (Boeringer, Gibco, Takara, New England Biolabs) were used according the manufacturer's instructions.

2.B.4 DNA sequencing:

DNA sequencing was performed by the DNA sequencing core facility at the Rockefeller University.

2.B.5. Generation of Itch expression vectors:

The murine Itch gene was PCR amplified from genomic DNA and subcloned into the Bluescript plasmid. Itch was further subcloned into the EGFP_C1 expression vector. The Itch gene with the N terminus C2 region deleted was also PCR amplified from genomic DNA and cloned into the EGFP_C1 expression vector. In the Itch-EGFP_C1

expression vector, the c-terminus cysteine required for the catalytic function of the HECT domain of Itch was mutated to an alanine using the ExSite PCR based site directed mutagenesis kit (Stratagene) according to the manufacturor's protocol.

2.B.6 Amplification and purification of probes used for Southern blot analysis:

The probe d and probe e were amplified from genomic DNA by PCR using primers d2 forward and d2 reverse and e2 forward and e2 reverse, respectively. The probes d and e fragments were subcloned into Bluescript BSKS II (+). Probes were amplified from the subcloned DNA for Southern blot analysis.

2.B.7 Radioactive labeling of probes:

Probes were labled with radioactive dCTP ("ready to go DNA Labelling beads, Amersham Pharmacia) according to the manufacturer's protocol.

2.B.8 Southern blot analysis:

Southern blot analysis was performed by standard procedures. Briefly, genomic fragments or PCR products were resolved on 0.8% or 1.2% agarose gels, respectively. Gels were stained in ethhidium bromide-containing running buffer (1x TAE) and photographed with ultraviolet light to control for resolution of marker fragments. After equilibrating the gel in transfer buffer (0.4M NaOH, 0.6M NaCl) for 20 minutes, DNA was transferred onto a nitrocellulose membrane (Hybond N+, Amersham Biosciences) by capillary blotting. Following overnight transfer, the membrane was rinsed in neutralization solution (0.5 M Tris-HCl pH 7.0, 1M NaCl) and DNA was permanently fixed to the membrane by UV cross-linking in a photo-crosslinker (UV Stratalinker, Stratagene). The membrane was blocked overnight at 65 C in hybridization buffer (50mM Tris-HCl pH7.5, 1M NaCl, 1% SDS, 10% dextran sulfate, 300 μg/ml sonicated

salmon sperm DNA). The labeled probe was added to the hybridization buffer and incubated at 65°C overnight. Finally, the membrane was washed with 0.5-2x SSC (150mM NaCl, 15mM tri-sodium citrate) and 0.1-1% SDS, and exposed for autoradiography.

2.B.9 Polymerase chain reactions (PCRs):

Unless otherwise noted, PCRs were performed using Klentherm buffer (67 mM Tris HCl, pH 9.2, 16mM $(NH3)_2SO_4$, 0.15 mg/ml BSA, and 3.5 mM $MgCl_2$), 25μM dNTPs, and 4pmol primers, and 1 ul Taq polymerase (Invitrogen) in a 20-25 μl reaction volume.

2.B.10 Semiquantitative RT-PCR:

Semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) was performed to assess mRNA levels of target genes. Total RNA was isolated from $5x10^5$-$1x10^6$ cells using Trizol (GibcoBRL) and the concentration was measured by spectophotometry. Equal concentrations of RNA were reverse transcribed into cDNA using oligo(dT) primers and the Superscript reverse transcription kit (Invitrogen) according to the manufacturer's protocol. A control reaction was performed without the addition of reverse transcriptase to control for DNA contamination. Three fold serial dilutions of cDNA were used for PCR reactions. Foxp3 PCR reactions were performed with 1.5 mM $MgCl_2$.The PCR reactions were visualized by Southern blotting with HPRT and Foxp3 specific probes.

2.B.11 Isolation of genomic DNA:

Genomic DNA was isolated from 1/4 inch mouse tail clips following a standard protocol (Laird, Zijderveld et al. 1991) Briefly, tails were digested in 500 µl tail lysis buffer (10 mM Tris-HCl pH8.5, 5mM EDTA, 0.2% sodium dodecyl sulfate (SDS), 200 mM NaCl, 100 µg/ml proteinase K) at 55°C over night. Cell debris was pelleted at 15000 x g for 8 minutes and the supernatant was transferred to a fresh epindorf tube. DNA was precipitated with the addition of 1 ml 100% ethanol and vigorous shaking. The precipitate was pelleted at 15000 x g for 2 minutes, washed in 70% ethanol, air dried for 5 minutes, and resuspended in 200-400 µl TE (10 mM Tris-HCl pH 8.0, 1mM EDTA). DNA was resolved at 55°C for 30 minutes. 1 µl of the resolved DNA solution was used per PCR reaction.

2.B.12 Preparation of cell lysates:

Cells were washed one time in plain RPMI medium prior to lysis. If the cell number was less than $5x10^6$ cells, the tubes into which the cells were placed were precoated with fetal calf serum for 5 minutes at room temperature. Cells were lysed in n-octylglucoside (ODG) lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 20 mM NaF, 1mM EDTA, 1mM EGTA, 2% ODG, 10% Glycerol) with fresh protease inhibitors (Sigma) and 2mM Na orthovanadate added prior to lysing. Cells were lysed on ice for 30 minutes and nuclei were pelleted by centrifugation at 15000 x g for 10 minutes at 4°C. If not used immediately, the supernatants containing cellular protein extracts were frozen at -80°C.

2.B.13 Western blots:

The total protein concentration of lysates was determined by a BCA assay (Pierce) against a bovine serum albumin standard according to the manufacturers protocol. Lysates were resolved by 8%-15% SDS polyacrilamide gel electropheresis (SDS-PAGE) and transferred to polyvinylidenediflouride (PVDF) membrane (Immobilon P, Millipore) by semi-dry blotting according to the manufacturor's protocol. Unspecific binding to membranes was blocked by one hour incubation with 5%BSA TBST (100 mM Tris-HCl pH 7.6, 140 mM NaCl, 0.2% Tween). Primary antibodies were incubated overnight in TBST at dilutions indicated elsewhere. Membranes were washed 5 times for 5 minutes in TBST and incubated with secondary HRP-coupled anti-species antibodies (Amersham) for 30-60 minutes at room temperature. Membranes were washed again five times for five minutes and proteins were detected by chemiluminescence (ECL, Amersham) according to the manufacturer's protocol.

2.C Cellular Methods:

2.C.1 Preparation of murine lymphocyte suspensions:

Mice were killed using dry ice (CO_2) and blood was removed from the heart by a 1 ml syringe with a 26 gauge needle. Thymus, spleen, and lymph nodes (superficial cervical, mandibular, axillary, lateral axillary, pancreatic, superficial inguinal, iliac, hypogastric, and mesenteric) were isolated and placed in 5 ml ice cold Hanks Balanced Salt Solution (BSS). Single cell suspensions were obtained by either gentle shearing of organs placed in meshes using a blunt forcep or by incubation or organs in a collagenase enzyme mixture (Blendzyme II, Roche). For preparation of single cell suspension using Blendzyme, a PBS solution containing 130 μg/ml blendzyme and 100 μg/ml DNAase was prepared fresh. A spleen (cut into four pieces) or pooled lymph nodes from one

mouse were placed into one well of a 48 well plate containing 350 µl of solution Blendzyme solution. Cells were incubated at 37 C for 15 minutes and single cell suspensions were made by gentle mechanical disruption using a 1 ml syringe (with no needle). Cells were placed in 5 ml BSS to dilute the suspension, and the cell suspension was filtered through an 0.7 µm mesh and washed one time in BSS. Splenic erythrocytes were lysed by incubating cells for 1 minute at room temperature in 1 ml ACK buffer (0.15M NH_4Cl, 10 mM $KHC0_3$, 0.1 mM EDTA). The reaction was stopped by adding 9 ml BSS. Cells were washed once with BSS and the single cell suspension was filtered through an 0.7 µm mesh. Cells were counted using a hemacytometer. Dead cells were excluded by staining with trypan blue (Gibco) prior to counting.

2.C.2 T cell purification from spleen and lymph node:

Unless otherwise mentioned, T cells were purified by depletion using MACS LS columns. For purification of CD4+ T cells, cells were incubated at $2x10^8$ cells/ml in Hanks Balanced Salt Solution (BSS) with a mixture of biotinylated antibodies against CD8, CD19, CD24, CD11b, GR-1, CD49b, NK1.1, TCR-γδ, and TER-119 followed by incubation with Streptavidin beads. Naïve CD44 low cells were further purified from purified T cells by incubation in BSS at a concentration of $1x10^7$ cells/ml with a mixture biotinylated and FITC coupled CD44 antibodies at 1: 300 dilution. The FITC coupled CD44 antibodies were added for flow cytometric analysis of the purification procedure. CD8 T cells were purified by the same protocol, except that CD4 antibodies were added rather than CD8 antibodies and GR-1(Ly-6C) antibodies were omitted from the depletion antibody cocktail. Cell purity after purification was consistently > 95% as assessed by flow cytometry.

2.C.3 T cell purification from thymus:

Cells were purified by depletion using MACS LS columns. Cells were incubated at $4x10^8$ cells/ml in BSS with a mixture of biotinylated antibodies against CD19, CD11b, GR-1, CD49b, NK1.1, TCR-γδ, TER-119. For CD4 single positive cell purification, CD8 antibodies were added at 1:50 dilution. For CD8 single positive cell purification, CD4 antibodies were added at 1:50 dilution. After washing, cells were incubated with strepavidin beads prior to running on an LS column. Purified cells consisted of double negative (DN) thymocytes and the single positive thymocytes that were purified.

2.C.4 T cell culture:

Unless otherwise noted, T cells were cultured in T cell medium that consisted of RPMI (Gibco), 10% fetal calf serum, 2mM Glutamine (Gibco), 100U/ml penicillin/streptomycin (Gibco), and 55μM 2-mercaptoethanol (Gibco).

2.C.5 Coating of plates for stimulation of purified T cells:

96 well round bottom plates were coated with indicated concentrations of antibodies in 30 μl volume for 1-2 hours at 37°C. 24 well plates were incubated with 200 μl of antibody solution. α-CD3ε (clone 145-2C-11) used for coating was made in the laboratory and α-CD28 (clone 37.51) was bought from Pharmingen. After the incubation time period, the antibody mixture was aspirated, and the wells were washed one time with PBS prior to addition of cells.

2.C.6 Standard flow cytometric analysis:

$2x10^5$-$1x10^6$ cells were placed per well of a 96 well round bottom plate and washed in PBS/1%BSA/0.01%azide (PBA). Cells were incubated with indicated dilutions of antibody (listed elsewhere) in 15 μl PBA for 10 minute at 4°C, washed in 100

μl PBA, and, if necessary, incubated with secondary antibodies in 15 μl. After a secondary wash, cells were resuspended in 50-100 μl PBA containing 2nM TO-PRO-3 (Molecular Probes) to exclude dead cells.

2.C.7 Intracellular stainings for flow cytometric analysis:

Brefeldin A was added at a concentration of 10 μg/ml four-six hour prior to cell staining. Intracellular stainings were performed using the BD Pharmingen Cytofix/Cytoperm kit according to the manufacturer's protocol, with the following variations. Cells were fixed for 20 minutes at RT in 30 μl Cytofix, cytoperm solution. Antibody stainings were performed in 15 μl volume for 15 minutes at room temperature. Antibody dilutions are listed elsewhere.

2.C.8 Assessment of cell proliferation by [^{3}H]-thymidine incorporation:

Eight hours prior to harvest, 1 μCi [^{3}H]-thymidine was added in 50 μl volume to each triplicate well of a 96 well plate. For analysis of [^{3}H]-thymidine incorporation, cells were harvested onto glass fibre filter paper (Printed Filtermat A, Wallac) using a cell harvester. The filter paper was dried using a microwave prior to being immersed in β-scintillation fluid. Scintillation counts were measured by a β-counter.

2.C.9 Th1/Th2 differentiation of CD4+ T cells *in vitro*.

1x10^6 purified naïve CD44lo CD4 cells were incubated in a 24 well plate with plate bound 10 μg/ml α-CD3 and 5 μg/ml α-CD28 antibodies in T cell medium. Th1 or Th2 differentiation of cells was stimulated using a mix of cytokines and antibodies. For Th1 differentiation, cells were placed in T cell medium containing 2 μg/ml anti-IL4 antibody, 3.5 ng/ml IL-12 and 25 U/ml IL-2. For Th2 differentiation, cells were

maintained in medium containing 2 μg/ml anti-IFNγ antibody, 1000U/ml IL-4 and 25 U/ml IL-2. Cells maintained in non-biased differentiation conditions were maintined in 25 U/ml IL-2. In some experiments laboratory made IL-2 supernatant was added at equivalent concentrations. Cells maintained in blocking conditions were stimulated in the presence of 10 μg/ml α-IFNγ and α-IL-4. Cells were stimulated for 2 days in the appropriate differentiation medium in antibody coated wells, split 1:4, and rested for four days in differentiation medium in uncoated wells. On the sixth day, cells were washed two times in T cell medium to remove antibodies and cytokines, and $2x10^5$ cells were restimulated in 96 well round bottom plates with plate-bound α-CD3 and α-CD28 antibodies. Brefeldin A was added at a concentration of 10 μg/ml 1 hour afterwards, and four hours later cells were stained for intracellular IL-4 and IFNγ production using a standard intracellular staining protocol. Flow cytometric analysis of intracellular stainings was performed thereafter.

2.C.10 TGF-β sensitivity assay:

$1x10^5$ purified CD4+ or CD44lo CD4+ T cells were cultured per triplicate well in 96 well round bottom plates coated with 10 μg/ml α-CD3ε and 5 μg/ml α-CD28 antibodies. Cells were cultured in medium alone or in medium containing 1, 2.5, 5, 10, or 25 ng/ml recombinant human TGFβ (R&D). Cells were cultured for 72 hours at 37C, and 1μCi/well of [^{3}H] thymidine was added for the final 8 hours of culture. Incorporated [^{3}H] thymidine levels were measured using a scintillation counter.

2.C.11 CFSE proliferation analysis:

Purified cells were washed in RPMI and then $1x\ 10^7$ cells/ml were incubated with 2.5μM carboxyfluoroscein diacetate succinimidyl ester (CFSE; Molecular Probes) in

RPMI at 37 C for 10 minutes. One volume primary T cell medium was added to stop the labeling reaction. After the indicated days in culture, flow cytometric analysis of proliferation as measured by CFSE dilution was performed. Cells were surface stained for CD4 or CD8 prior to flow cytometric analysis. For all data plots, only live events are displayed.

2.C.12 Proliferation of CD8+ memory cells:

$1x10^5$ purified CD44lo and CD44hi CD8+ cells were cultured in the presence of different amounts of recombinant human IL-15 for three days. 8 hours prior to harvest 1 μCi [^{3}H]-thymidine was added to the cultures. Proliferation was measured by [^{3}H]-thymidine incorporation of flow cytometric analysis of CFSE dilution.

2.C.13 *In vitro* regulatory T cell suppressor assay:

CD4+ T cells were purified by the standard depletion protocol. The cells in the bound fraction were retained for use as antigen presenting cells (APCs) in the suppressor assay. CD25- vs. CD25+ CD4+ cells were further sorted by incubating 1 x 10^8 cells/ml with a 1:8 dilution of α-CD25 (7D4) PE antibody. Cells were incubated for 15-20 minutes at 4C with occasional agitation. Cells were washed, resuspended at 1 x 10^8 cells/ml and incubated with anti-PE microbeads at 1:8 dilution for 15-20 minutes at 4°C. CD25- responder cells were collected as the flow through fraction from the MACS column. CD25 + suppressor cells were collected as the retained fraction after two rounds of column purification. Suppressor assays were set up using $5x10^4$ irradiated (3000rad) APCs, $5x10^4$ CD25- responder cells, 10 μg/ml soluble anti-CD3 antibody, and titrated numbers of suppressor cells ($5x10^4$, $2.5x10^4$, $1.25x10^4$, $6x10^3$, and $3x10^{3)}$ per triplicate well of a 96 well round bottom plate. Control wells included wells with medium alone,

wells with APCs and anti-CD3ε antibody only, wells with responder/suppressor co-culture without anti-CD3 antibody, and wells with APCs, anti-CD3 antibody, and CD25+ suppressor cells only. Cells were cultured for 72 hours at 37C and 1μCi/well of [^{3}H] thymidine was added for the final 8 hours of culture. Incorporated ^{3}H thymidine levels were measured using a scintillation counter.

2.C.14 *In vitro* deletion of Itch using TAT-Cre:

$2x10^7$ purified CD4+ T cells from fl/fl or fl/+ mice were washed four times in plain RPMI medium in epindorf tubes. Cells were incubated with 100μg/ml TAT-Cre (made in the lab) at a concentration of 1 x 10^8 cells/ml for one hour at 37°C in an incubator. Cells were washed two times with T cell culture medium and then set up for culture.

2.D In vivo *assays:*

2.D.1 Mice:

C57Bl/6 and Balb/c mice were maintained at the Labarotory Animal Research Center (LARC) at the Rockefeller University in a pathogen free environment. Conditional Itch mice were generated by Kristina Jönsson in the University of Cologne and maintained on a pure C57Bl/6 background (backcrossed more than 10 generations). C57Bl/6 JHT (Jackson Laboratory), C57Bl/6 β_2M -/-(Jackson Laboratory), C57Bl/6 CD1d -/- (Nussenzweig laboratory), C57Bl/6 MHC II -/- (Taconic Farms), C57Bl/6 Rag -/- (Jackson Laboratory), C57Bl/6 AND transgenic (Jackson Laboratory), Balb/c Stat 6 -/- (Jackson Laboratory), and mixed background D011.10 rag -/-mice were crossed to Itch fl/fl CD4 Cre mice to obtain genotypes discussed in the results section. Genotypes were assessed using PCR as described elsewhere. Unless otherwise noted, littermate controls

were used for all experiments. The genotype of these controls was Itch fl/+, Itch fl/+CD4 Cre, or Itch fl/fl. No phenotypic difference was ever noted between control genotypes. Whenever possible, results were confirmed with mice from independent crosses.

2.D.2 Adoptive transfer of CD8 SP thymocytes:

1 x 10^7 thymocytes enriched for CD8 SP and DN cells by MACS depletion of CD4+ cells were CFSE labeled and retroorbitally injected into unirradiated wild-type recipients in 200 µl PBS. Two Itch fl/fl CD4 Cre or wild-type littermate controls were used per transfer. Recipient mice were sacrificed four weeks later, and $1x10^7$ splenocytes or pooled lymph node lymphocytes were stained for CD8 and CD44. Flow cytometric analysis of CD44 expression was performed on gated CD8+ CFSE labeled cells.

2.D.3 Adoptive transfer of peripheral T cells:

1 x 107 purified CD4+, CD8+, or CD3+ T cells were injected retroorbitally into 6-8 week old Rag deficient mice in 200 µl PBS. Mice were sacrificied and histological analysis of organ sections was performed one month after transfer. Successful transfer of lymphocytes was confirmed by staining of peripheral blood for transferred lymphocytes at the time of killing.

2.D.4 *In vivo* BrdU labeling:

6-8 week old mice given tap water containing 0.8 mg/ ml Bromodeoxyuridine (Sigma) and 1% sucrose. A stock of 500 ml of BrdU containing water was made and kept at 4C, and the drinking water of mice was changed every two days. After ten days, mice were sacrificed and flow cytometric analysis of BrdU incorporation was performed.

Intracellular staining for BrdU was performed using the instructions provided by the manufacturer (BD Pharmingen).

2.D.5 Oral tolerization:

C57Bl/6 x Balb/c mixed genetic background D011.10 dxd or Itch fl/fl CD4 Cre D011.10 dxd mice were given drinking water containing 20 mg/ml Ovalbumin (Sigma) for five days. Mice were then sacrificed and CD4+ T cells were purified separately from spleen and mesenteric lymph nodes. 5×10^4 CD4+ cells were cultured with 5×10^5 irradiated syngeneic APCs and either 0, 15nM, 150 nM, or 1.5mM ovalbumin peptide per triplicate well in a 96 well round bottom plate. Cells were cultured for 72 hours at 37C, and 1μCi/well of [^{3}H] thymidine was added for the final 8 hours of culture. Incorporated [^{3}H] thymidine was measured using a scintillation counter.

2.D.6 Histology:

Haematoxilin and eosin stainings of organ sections were performed by the Genetically Engineered Mouse Phenotype Core at the Rockefeller University. Dr. Krista La Perle provided descriptions of the pathology with representative pictures.

Chapter 3: The role of Itch in peripheral tolerance

3.A Introduction:

3.A.1 Tolerance:

Tolerance refers to the ability of the immune system to recognize and accept both self-antigens and nonpathogenic foreign antigens to which it is exposed. Problems with tolerance can lead to a wide spectrum of pathologies, ranging from allergies to autoimmune diseases. Key mechanisms in maintaining tolerance include T cell anergy, regulatory T cells, and immunomodulatory cytokines such as IL-10 and TGF-β.

3.A.2 E3 ligases in T cell anergy:

Recently, experimental data from several groups has suggested a critical role for E3 ligases in both clonal and *in vivo* anergy. Several lines of evidence support a role for the RING finger E3 ligase Cbl-b in T cell anergy. Cbl-b deficient mice spontaneously develop systemic autoimmunity (Bachmaier, Krawczyk et al. 2000). This spontaneous autoimmune disease was initially linked to the ability of Cbl-b deficient T cells to bypass the requirement for CD28 co-stimulatory signals for their activation (Chiang, Kole et al. 2000). The function of Cbl-b was defined as abrogating signals from the TCR, such that only stimulation with co-stimulatory ligands allows for full signal strength and activation. Since anergy has been linked to stimulation in the absence of co-stimulatory signals, Cbl-b became a natural target to consider in anergy induction. Further support for a role for Cbl-b in the anergy phenotype came from studies demonstrating that Cbl-b mRNA and protein levels were upregulated in T cells upon anergy induction (Heissmeyer and Rao 2004). The most convincing data has come from recent experiments using Cbl-b

deficient mice, which demonstrate that Cbl-b deficient T cells are incapable of being anergized both *in vitro* and *in vivo* (Jeon, Atfield et al. 2004).

Another E3 ligase which is selectively upregulated in anergic cells and thus may regulate T cell anergy is GRAIL (Gene Related to Anergy in Lymphocytes) (Anandasabapathy, Ford et al. 2003; Seroogy, Soares et al. 2004). T hybridoma cells overexpressing GRAIL show a defect in both IL-2 and IL-4 production, similar to anergic cells (Anandasabapathy, Ford et al. 2003). Furthermore downregulation of GRAIL in primary T cells transduced with otubain 1 (a GRAIL-binding protein that increases its rate of turnover) demonstrate enhanced IL-2 production, while T cells transduced with otubain 1 ARF-1 (an isoform that inhibits GRAIL degradation) show reduced IL-2 inducibility (Soares, Seroogy et al. 2004). While these data are suggestive for a role of GRAIL in anergy, definitive proof awaits the analysis of GRAIL deficient mice.

Several indirect lines of evidence also implicate Itch in anergy induction. Induction of clonal anergy *in vitro* using ionomycin leads to an upregulation of Itch mRNA and protein levels (Heissmeyer, Macian et al. 2004). Furthermore, stimulation of anergic but not activated T cells causes Itch to translocate to the detergent insoluble fraction of the plasma membrane (rafts) (Heissmeyer, Macian et al. 2004). The reported higher levels of Jun B in the nucleus of Itch deficient T cells (Fang, Elly et al. 2002) may also effect anergy induction. In addition to its role in Th2 differentiation, JunB is also responsible for AP-1 dependent IL-2 production, and JunB levels are specifically reduced after clonal anergy induction (Mondino, Whaley et al. 1996). The increased levels of JunB in Itch deficient T cells could therefore affect anergy induction as well.

The most direct evidence for the involvement of Itch in the anergic state is that Itch deficient T cells fail to become anergic after treatment with ionomycin (Heissmeyer, Macian et al. 2004). The caveat in this experimental protocol is that ionomycin induction of anergy requires stimulation of T cells under Th1 polarizing conditions. Since Itch deficient T cells display a bias towards Th2 differentiation, it is possible that the observed differences in anergy reflect the Th differentiation bias more than a problem with anergy induction *per se*.

3.A.3 The E3 ligase Cbl-b and suppressor cell function:

The molecular mechanisms by which CD4+CD25+ regulatory T cells exert their suppressive effects on CD4+CD25- effector cells remains unclear. Proteins that negatively regulate signaling in effector cells are excellent candidates to consider as targets that are required for suppressor cells to function. Recently, it has been shown that CD4+CD25- effector T cells deficient in the RING-finger E3 ligase Cbl-b have an impaired ability to be suppressed by CD4+CD25+ regulatory T cells. Thus, although Cbl-b deficient mice have functional suppressor cells, suppression fails due to the inability of effector cells to be suppressed (Wohlfert, Callahan et al. 2004).

3.A.4 E3 ligases and TGF-β signaling:

Upon ligation of TGF-βRII with TGF-β, TGF-β RII heterodimerizes with TGF-β RI and phosphorylates it. Smad 2 and Smad 3 are recruited to this activated receptor complex and are in turn phosphorylated by TGF-β RI. Phosphorylated Smad2 and Smad3 then heterodimerize with Smad4 and translocate to the nucleus. Phosphorylation of Smad proteins is critical for their nuclear import. In addition ubiquitin mediated degradation of a repressor protein bound to Smads (SnON) is required for TGF-β

signaling. Once in the nucleus Smad proteins activate transcriptional complexes through cis-regulatory Smad binding sequences (Bottner, Krieglstein et al. 2000; Wahl, Swisher et al. 2004). TGF-β signaling is silenced by dephosphorylation and ubiquitin mediated degradation of Smad proteins by Smad ubiquitin regulatory factors (Smurfs) (Zhu, Kavsak et al. 1999; Lin, Liang et al. 2000).

T cells from mice deficient in the RING finger ligase Cbl-b show defects in TGF-β mediated suppression of proliferation (Wohlfert, Callahan et al. 2004). The biochemical pathway that leads to this insensitivity is presently unclear. The authors analyzed both TGF-β receptor expression and proximal downstream signaling events such as Smad phosphorylation, but found no difference between Cbl-b deficient and wild-type T cells (Wohlfert, Callahan et al. 2004).

Recently, the murine E3 ligase Itch and its human homologue Atrophin-1 Interacting Protein 4 (AIP-4) have been implicated in regulating TGF-β signaling. In a Smad 3 dependent fashion, AIP-4 was found to bind and ubiquitinate human enhancer of filamentation (HEF-1), a member of the multiple domain docking protein Cas family, leading to its proteosome mediated degradation *in vivo* (Feng, Guedes et al. 2004). Since HEF-1 degradation has been observed in response to TGF-β signling in both fibroblasts and a T cell line (Liu, Elia et al. 2000), this data suggests that AIP-4 may be responsible for TGF-β mediated HEF-1 degradation. Though this data is intriguing, it should be considered with the caveat that no genetic proof was provided for the role of AIP-4 in regulating HEF-1.

In a separate study, Itch was shown to directly regulate TGF-β signaling by modulating Smad2 activation (Liu, Elia et al. 2000). Itch deficient mouse embryonic

fibroblasts were found to have reduced susceptibility to TGF-β induced cell growth and arrest. Interestingly, Itch ubiquitin-mediated modulation of signaling was in a proteolysis-independent manner. Instead, TGF-β insensitivity was found to correlate with decreased Smad 2 phosphorylation and association with the TGF-βR. Further proof that the activity of Smad2 was changed was provided by analysis of SnON levels. Upon Smad2 phosphorylation and nuclear accumulation, SnON is degraded by Smurf2 mediated ubiquitination. SnON levels remained high in Itch deficient fibrolasts, providing further evidence that Samd2 phosphorylation was decreased. Transfection experiments revealed that Itch was capable of ubiquitinating Smad2 and that Smad2, Itch, and TGF-βR form a complex in TGF-β stimulated fibroblasts. Surprisingly, the poly-ubiquitination of Smad2 occurred on K48. Although K48 polyubiquitination is generally associated with proteasomal degradation, Smad2 ubiquitination did not lead to its degradation. Rather, Smad2 ubiquitination affected its ability to bind to the TGF-β receptor and consequently Smad2 phosphorylation. Whether this change in binding is due to a conformational change due to ubiquitination or due to Itch localizing Smad2 to the membrane via its C2 domain remains to be determined.

3.A.5 Aims of this study:

The inflammatory phenotype seen in the a^{18H} naturally occurring Itch deficient mice suggests that there is at some level inappropriate regulation of the immune response. To establish the role of T cells in the inflammatory pathology seen in naturally Itch deficient mice, conditionally Itch deficient mice were generated. The aim of the experiments described in this section of the thesis was to provide evidence for the importance of CD4+ T cells in the inflammatory phenotype and to provide insight into

which tolerance mechanisms may be affected in Itch fl/fl CD4 Cre mice. In particular, the role of CD4+CD25+ suppressor cells and the cytokine TGF-β in modulating Itch deficient T cells was addressed. In addition, the ability of Itch deficient T cells to be anergized by oral tolerance was assessed.

3.B Results:

3.B.1 C57BL/6 Itch fl/fl CD4 Cre mice develop a spontaneous inflammatory disease

As reported previously for the naturally occurring Itch mutant mouse strain a^{18H} (Perry, Hustad et al. 1998), Itch fl/fl CD4 Cre mice developed a visible dermatitis by 20 weeks of age. In order to ascertain the type and extent of the inflammatory pathology, organ sections from two, five, and seven month old mice were stained with hematoxylin and eosin (**Figure 3-1**). Analysis of sections from two-month old mice revealed inflammatory lesions surrounding the epithelial lining of the respiratory tract, including the nasopharynx and alveoloi, the gastrointestinal tract, including the eosophagus and stomach, and the urinary tract, including the ureters. The inflammatory lesions were marked by hyalinosis of the epithelial lining. Neutrophil infiltrates and plasma cells were present in the lesions, along with eosinophilc crystalline granules. The lungs revealed the most severe accumulation of eosinophilic granules as well as infiltrating macrophages, neutrophils, and plasma cells. No lesions were present in other internal organs, including the brain, heart, thyroid gland, parathyroid gland, pancreas, liver, small intestine, large intestine, adrenal gland and kidneys. The kidneys did show signs of hydronephrosis, but this may be secondary to the inflammation in the ureters, as no inflammatory lesions

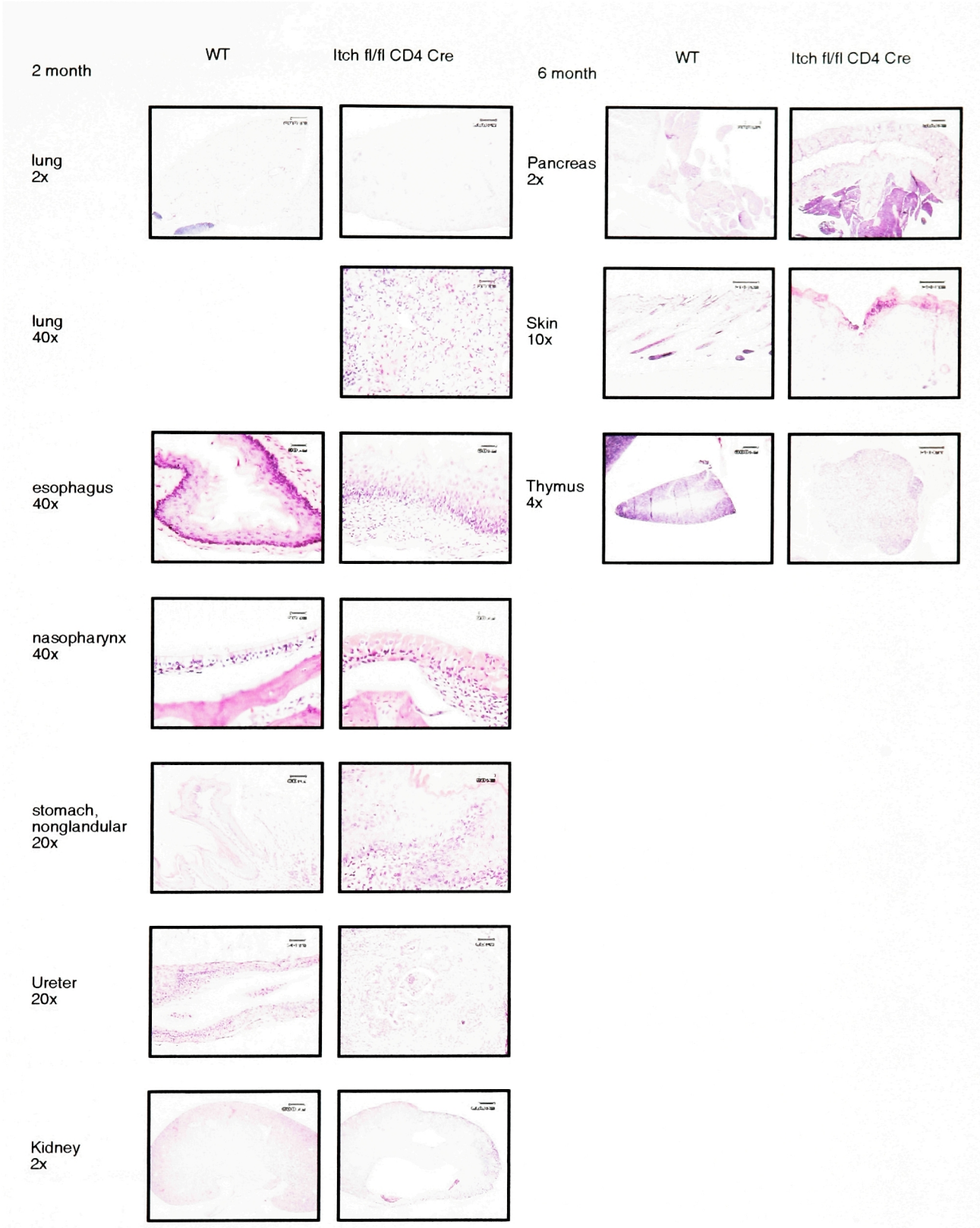


Figure 3-1. Inflammatory lesions along mucosal linings in Itch fl/fl CD4 Cre mice. **Left panel.** Heaematoxilin and eosin stainings of organ sections from 2 month old Itch fl/fl CD4 Cre mice and littermate controls. Analysis of lung sections reveals perivascular infiltrates and acidophilic macrophage pneumonia. Analysis of sections of nasopharynx, esophagus, nonglandular sections of the stomach, and ureters reveals acidophilic neutrophilic infiltrates, plasma cell infiltrates, and hyalanosis. Kidney sections revealed hydronephrosis but no signs of inflammation. **Right panel.** Six month old mice develop further infiltrates in the pancreas, skin, and thymus. Dermatitis becomes grossly visible at this time.

were present in the kidneys themselves.

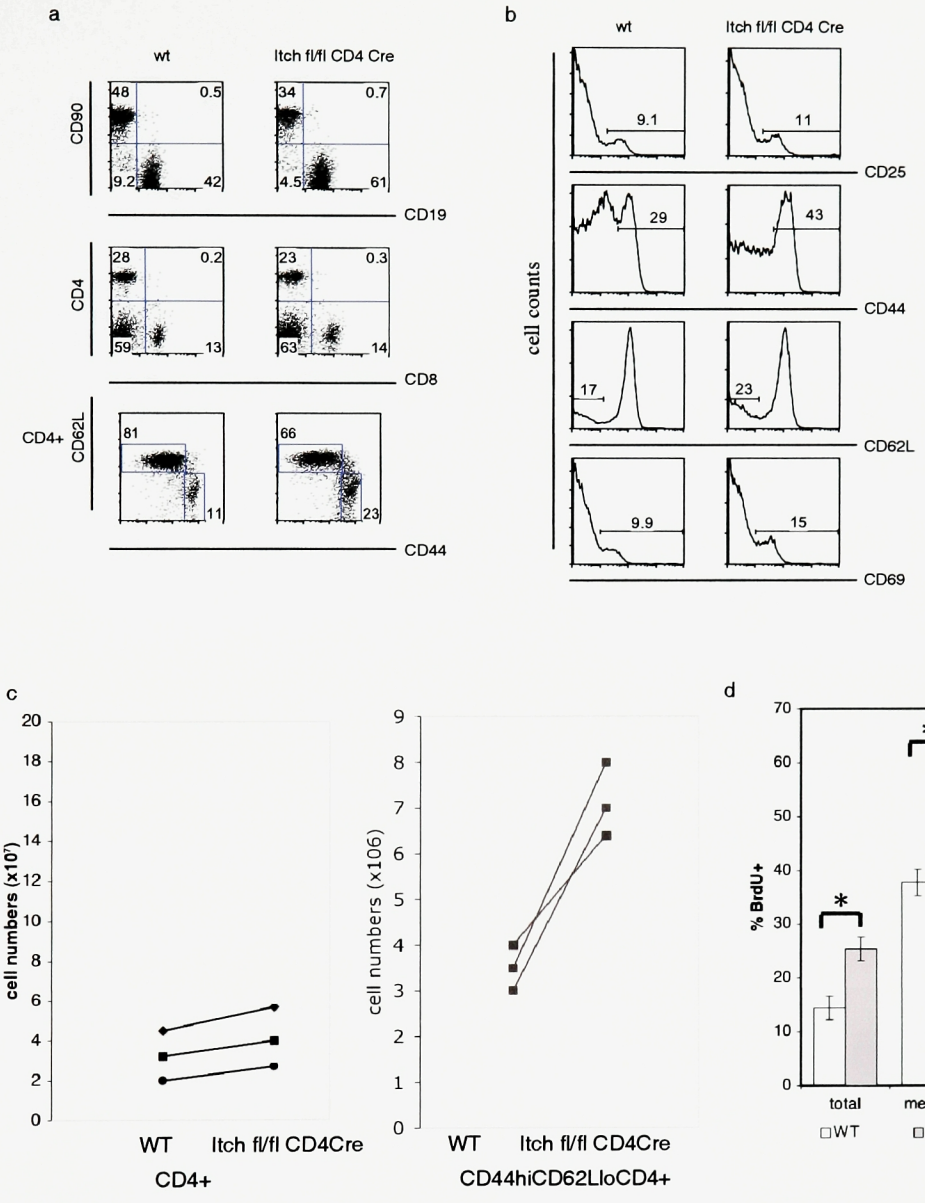
By 5 months of age, neutrophil, macrophage, and plasma cell infiltrates were additionally seen in the pancreas, thymus, and skin (**Figure 3-1**). Dermatitis was grossly visible at this time. In addition, the corticomedullary boundary of the thymus was obliterated.

Since Itch fl/fl CD4 Cre mice were housed in a pathogen free environment, and since CD4 Cre leads to the deletion of Itch in only T cells and CD4+ NKT cells, it can be concluded that the inflammatory phenotype is due to either a cell autonomous, spontaneous activation of T cells or the inappropriate activation of T cells by self or non-pathogenic environmental antigens.

3.B.2 Itch fl/fl CD4 Cre accumulate effector/memory CD4+ cells and B cells in the spleen and lymph node

By eight weeks of age, Itch fl/fl CD4 Cre mice had developed splenomegaly and lymphadenopathy. In particular, the mandibular lymph nodes tended to be prominently enlarged as compared to wild type mice upon gross inspection. Flow cytometric analysis of lymphocyte populations revealed that there was an increase in the percentage as well as absolute number of B cells in both the spleen and lymph node (**Figures 3-2a** and **3-3a**). In contrast there was a decrease in the percentage of CD4+ T cells in the spleen and lymph nodes, although the absolute numbers were similar or greater (**Figure 3-2c**). In the spleen, and to a lesser extent in the lymph nodes, a greater percentage of CD4+ T cells showed an effector/memory phenotype, as ascertained by CD44 and CD62L surface marker levels (**Figure 3-2a**). Analysis of stainings from three independent

Spleen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wt
Itch fl/fl CD4 Cre
CD90
CD19
48
0.5
9.2
42
34
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CD4
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59
13
23
0.3
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14
CD4+
CD62L
CD44
81
11
66
23
b
wt
Itch fl/fl CD4 Cre
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CD25
CD44
CD62L
CD69
9.1
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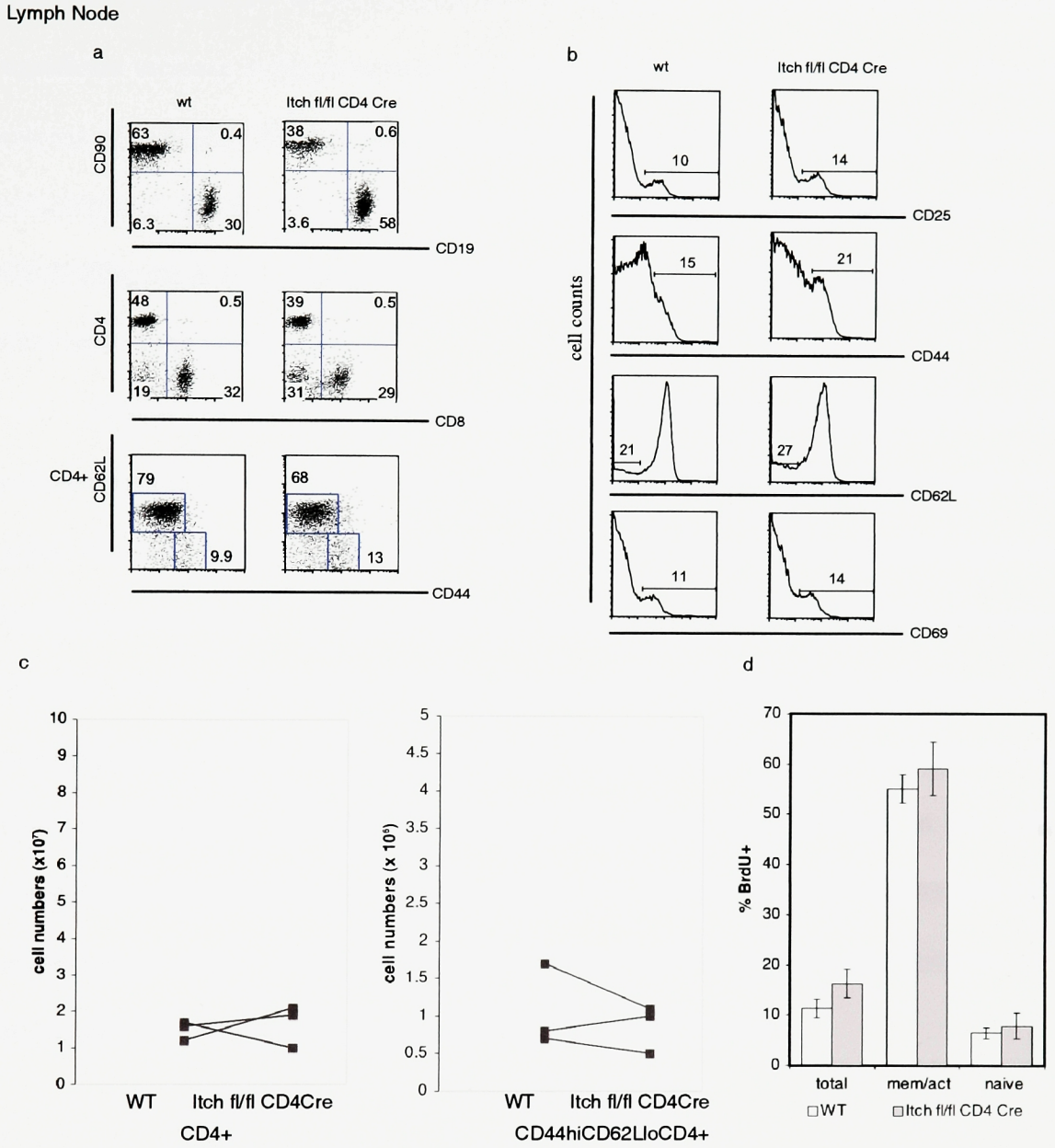


Figure 3-2. Accumulation of B cells and activated T cells in secondary lymphoid organs of 8 week old Itch fl/fl CD4 Cre mice. **First panel.** Spleen **Second panel.** Lymph nodes **a.** Flow cytometric analysis of lymphocyte populations. Numbers indicate the percentage of cells in the gated subpopulation. **b.** Cell surface expression of activation markers of CD4+ cells. **c.** Increased numbers of effector/memory CD4+ cells in Itch fl/fl CD4 Cre mice. Increase in absolute numbers of total CD4+, and effector memory CD4+ (CD44hiCD62Llo) cells. Absolute CD4+ cell numbers were calculated using absolute splenic and lymphnode numbers and percentages of gated subpopulations. Each data point represents the average of two Itch fl/fl CD4 Cre and two littermate controls analyzed on the same day. **d.** Increased percentage of actively dividng effector/memory CD4+ cells in Itch fl/fl CD4 Cre mice. Proliferation rate of peripheral CD4+ T cells in WT (open bars) and Itch fl/fl CD4 Cre (grey bars) mice *in vivo*. BrdU incorporation among total, effector/memory (CD44hiCD62Llo), and naive (CD44loCD62Lhi) CD4+ cells after mice were given drinking water containing 0.8 mg/ml BrdU for 10 days is shown. *The proliferation rate of splenic total CD4+ and effector/memory CD4+ cells as measured by BrdU incorporation is significantly higher in Itch fl/fl CD4 Cre mice ($p<0.001$ by student's t-test). Four mice were used per experimental group. Flow cytometric data is representative of three independent experiments.

groups of two Itch fl/fl CD4 Cre mice and two littermate controls revealed a statistically significant increase in the percentage of CD44hi CD62L low effector/memory cells in the spleens of Itch fl/fl CD4 Cre mice ($p<0.01$ by student's t-test). In addition, an increased percentage of splenic CD4+ cells showed recent activation markers such as CD25 and CD69 (**Figure 3-2c**). No statistical difference was seen in the percentage of activated CD4 cells in the lymph nodes.

Further confirmation that total CD4+ cells in the spleens of Itch fl/fl CD4 Cre mice contained more activated CD4+ cells came from *in vivo* studies in which mice were given drinking-water containing 0.8 mg/ml of the DNA analog bromodeoxyuridine (BrdU) for ten days. On the tenth day, mice were sacrificed and flow cytometric analysis of the percentage of BrdU positive cells in naïve and effector/memory subpopulations of CD4+ cells was performed. Itch fl/fl CD4 Cre mice contained 1.5 fold more proliferating CD4+ cells in the effector/memory compartment in the spleen compared to littermate controls (**Figure 3-2d**). No difference in BrdU uptake was found in the lymph nodes. These data are consistent with the hypothesis that there are more activated CD4+ T cells in the spleens of Itch fl/fl CD4 Cre mice.

3.B.3 The inflammation in Itch fl/fl CD4 Cre mice does not require B cells

Although conditional deletion of Itch using CD4 Cre insures that the disease pathology is not initated by Itch deficiency in B cells, it remained possible that B cells were critical for driving its progression. This hypothesis was consistent with data showing increasing accumulation of B cells in the spleen and lymph nodes of Itch fl/fl CD4 Cre mice with increasing age and pathology (**Figure 3-2**, data not shown). To

address the role of B cells in the Itch pathology, Itch fl/fl CD4 Cre mice were crossed to C57BL/6 J_HT mice. J_HT mice lack functional B cells due to a targeted disruption of the heavy-chain joining region, which leads to developmental arrest of B cells at the pro-B cell stage (Gu, Zou et al. 1993). Itch fl/fl CD4 Cre J_HT mice developed dermatitis and acidophilic macrophage pneumonia with similar severity and kinetics as Itch fl/fl CD4 Cre mice (**Figure 3-3**). Haematoxilin and eosin stainings of organ sections revealed similar levels of neutrophil infiltrates in the mucosal linings of the respiratory and GI tract. There was a large decrease in the number of lymphocytes cuffing blood vessels in effected areas of the lung, consistent with previous data showing that the majority of lymphocytes in inflamed areas were CD19+ B cells (Jonsson 2002). These data support the view that the activation of B cells and the increase in serum IgG1 and IgE titres in Itch fl/fl CD4 Cre mice are secondary events in the pathology and not essential for the inflammatory disease present in these mice.

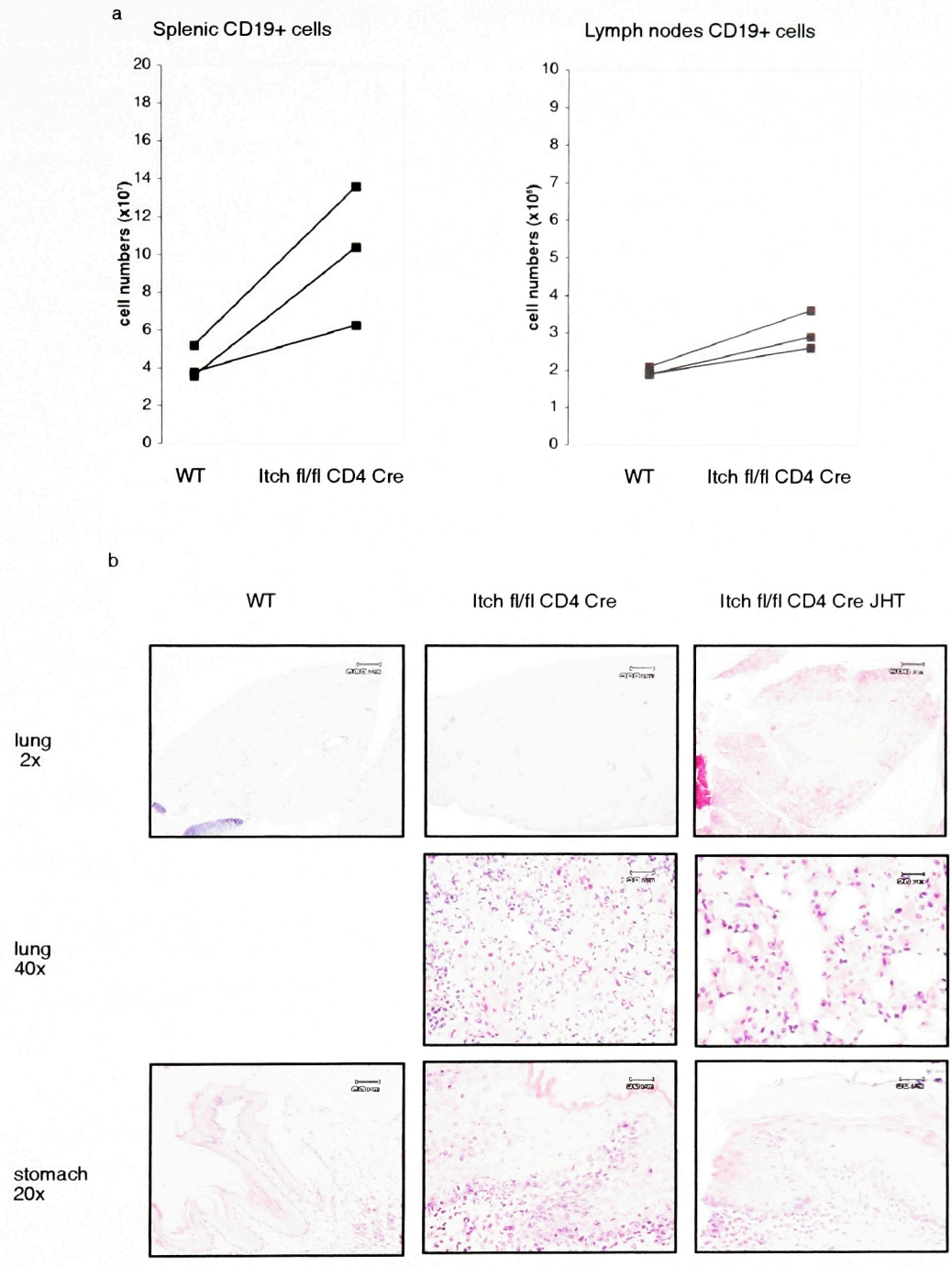


Figure 3-3. B cell accumulation is not essential for the Itch inflammatory pathology. **a.** Increased numbers of B cells in spleen and lymph nodes of Itch fl/fl CD4 Cre mice. Each point represents an independent experiment of cell counts from the average of two wild-type and two Itch fl/fl CD4 Cre mice. Absolute B cell numbers were calculated from absolute splenic and lymph node numbers and the percentage of CD19+ B cells as assessed by flow cytometric analysis. **b.** B cell deficient Itch fl/fl CD4 Cre JHT mice develop an inflammatory pathology similar in extent and kinetics to Itch fl/fl CD4 Cre mice. Haematoxilin and eosin stainings of lung sections and stomach sections are shown.

3.B.4 Transfer of CD4+ T cells transfers the Itch pathology.

Although CD4 Cre is thought to delete specifically in the double positive stage of thymocyte development, CD4 is also expressed at low levels in macrophages and dendritic cells. In addition, CD4 Cre leads to Itch deletion in CD8+ cells as well as CD4+ cells. In order to further substantiate the role of CD4+ cells in the Itch pathology, 1x 10^7 purified T cells or CD4+ T cells were transferred from 8-week old Itch fl/fl CD4 Cre mice and littermate controls into Rag-/- recipients. Transfer of Itch deficient total T cells and CD4+ T cells lead to a recapitulation of the Itch pathology one month after transfer into Rag deficient mice (**Figure 3-4**). Recipients of Itch deficient CD4+ T cells were visibly cachectic and had obvious dermatitis, whereas recipients of wild-type CD4+ T cells showed no signs of pathology. Haematoxilin and eosin stainings of organ sections from Rag deficient mice that received Itch deficient CD4+ T cells revealed alveolar macrophages pneumonia in the lungs, as well as esophagitis, gastritis, and nasopharyngitis. Notably, transfer of Itch deficient CD4+ T cells into unirradiated wild-type hosts lead to no visible pathology and no change in the percentage of activated CD4+ cells or B cells in the spleen and lymph node of recipient mice (data not shown).

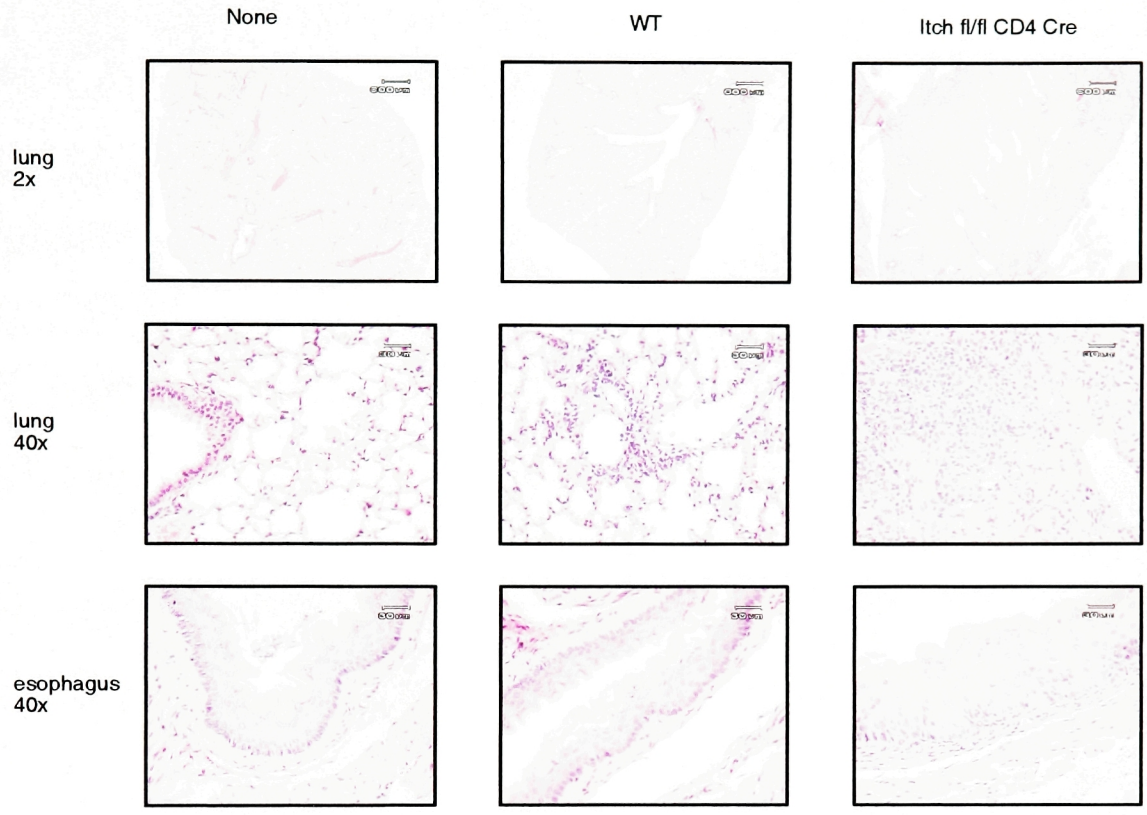


Figure 3-4. Itch deficient CD4+ T cells are directly responsible for the Itch pathology. Haemotoxilin and eosin staining of organ sections from Rag deficient mice that were retroorbitally injected with no cells, 1 x 10^7 wild-type CD4+ T cells, or 1 x 10^7 Itch deficient CD4+ T cells . Mice were sacrificied one month after transfer.

3.B.5 The activation of Itch fl/fl CD4 Cre T cells is not spontaneous

In order to address the role of antigen in the activation of CD4+ T cells in Itch fl/fl CD4 Cre mice, Itch fl/fl CD4 Cre mice were crossed to the AND TCR transgene on a C57BL/6 b haplotype background (Kaye, Hsu et al. 1989). The AND transgene encodes a T cell receptor made from Vα11.1 alpha chain and Vβ3 beta chain which recognizes a peptide derived from pigeon cytochrome C on k haplotype MHC II molecules. T cells expressing this receptor are expected to maintain a naïve phenotype, as CD4+ T cells have no access to pigeon cytochrome C in an otherwise unmodified mouse. Flow cytometric analysis of Itch fl/fl CD4 Cre AND+ mice revealed that there was still an accumulation of effector/memory phenotype T cells in Itch fl/fl CD4 Cre AND+ mice in comparison to AND+ littermates; however, analysis of Vα11.1+, Vβ3+ CD4+ T cells revealed no difference in the percentage of naïve cells between wild-type and Itch fl/fl CD4 Cre mice (**Figure 3-5**). Overall, these data support the conclusion that Itch deficiency in CD4+ T cells does not lead to spontaneous or antigen independent activation, but rather modulates the signal from the TCR upon antigen engagement in a manner that leads to an inflammatory disease.

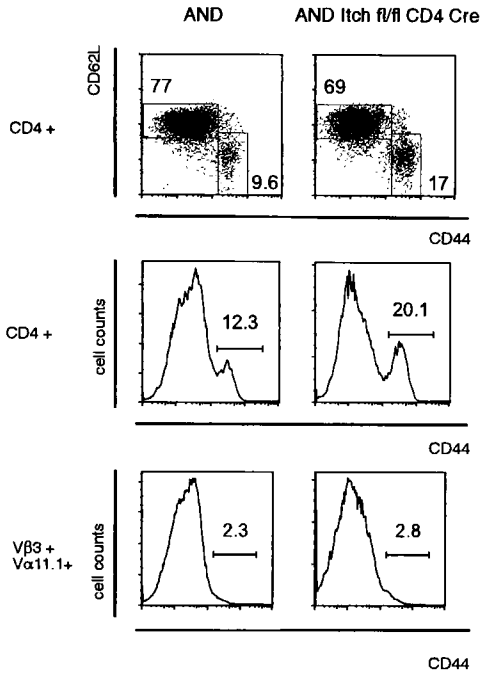


Figure 3-5. Itch deficiency does not lead to spontaneous activation of CD4+ T cells. Flow cytometric analysis of CD4+ T cells from AND transgenic Itch fl/fl CD4 Cre mice and littermate controls. Cell surface expression of activation markers in total CD4+ T cells and in CD4+ T cells expressing the Vβ and Vα chains of the AND transgene is shown. Results are representative of two independent experiments.

3.B.6 Itch fl/fl CD4 Cre mice develop and maintain CD4+CD25+ suppressor cells.

The pathology seen in Itch fl/fl CD4 Cre mice is clearly due to an unregulated inflammatory response. Though this inflammatory response, which is predominantly seen at mucosal sites and the skin, does not resemble the multi-organ autoimmune disease described in mice deficient in CD4+CD25+ suppressor cells, it never-the-less remained a formal possibility that the inflammatory pathology in Itch deficient mice was in some way connected to an absence of suppressor T cells. In order to address this possibility, splenocytes and lymphocytes were co-stained for CD4 and CD25 cell surface markers and the percentage of CD4+CD25+ cells was assessed by flow cytometry. 8-week old Itch fl/fl CD4 Cre mice showed an increase in the percentage of CD4+CD25+ cells compared to littermate controls (**Figure 3-6a**). Since CD25 is also upregulated on activated CD4+ cells, it remained a possibility that the CD4+CD25+ cells seen in Itch fl/fl CD4 Cre mice represented activated rather than suppressor T cells. A more selective marker for suppressor T cells is Foxp3. Semi-quantitative RT-PCR from purified CD4+ cells from Itch deficient mice and littermate controls revealed no difference in the expression levels of Foxp3 (**Figure 3-6b**). Thus by two independent means of assessment, Itch deficient mice contain CD4+CD25+ suppressor T cells. From this data, one can conclude that the both the generation and maintenance of CD4+ CD25+ suppressor T cells is not affected in Itch deficient mice.

3.B.7 CD4+CD25+ suppressor cells from Itch fl/fl CD4 Cre mice are functionally anergic and suppressive:

Although the previous data established that Itch deficient mice phenotypically have suppressor T cells, it was possible that these suppressor cells were not functional. The ability of Itch deficient CD4+CD25+ cells to suppress effector CD4+ cells was tested using an *in vitro* suppressor assay. This assay is routinely used to measure suppressor function, and in the case of Cbl-b deficient cells, provided definitive proof of a failure of suppression (Wohlfert, Callahan et al. 2004). To address the ability of Itch deficient CD4+CD25+ to suppress, CD4+CD25+ were sorted from Itch fl/fl CD4 Cre mice and wild-type littermate controls. The mice used for these experiments were 8-12 weeks old, with significant evidence of pathology both grossly and also by accumulation of activated T cells and B cells in their secondary lymphoid organs. Older mice were used to circumvent the argument that suppressor function ceases to work only later in the life of the mice, when disease is visibly present. CD4+CD25+ cells from Itch fl/fl CD4 Cre or control mice were co-cultured with wild-type CD4+CD25- responder cells in the presence of irradiated wild-type APCs and soluble α-CD3 antibody. To assess the anergic phenotype of the suppressor cells, CD4+CD25+ cells were also cultured alone with irradiated APCs and soluble α-CD3 antibody. Both wild-type and Itch fl/fl CD4 Cre CD4+CD25+ cells failed to proliferate by themselves, providing evidence that Itch deficient CD4+CD25+ cells are functionally anergic (**Figure 3-6c, left panel**). Furthermore, co-culture of either wild-type or Itch fl/fl CD4 Cre CD4+CD25+ suppressor cells with wild-type CD4+CD25- effector cells lead to a titratable decrease in the proliferation of CD4+CD25- effector cells (**Figure 3-6c, left panel**). Thus, one can conclude that Itch deficient CD4+CD25+ cells are indeed functional suppressor cells.

3.B.8 CD4+CD25- effector cells from Itch fl/fl CD4 Cre mice are capable of being suppressed by CD4+CD25+ regulatory T cells:

The ability of CD4+CD25+ cells from Itch fl/fl CD4 Cre mice to suppress did not rule out the converse possibility that effector cells from Itch fl/fl CD4 Cre mice were incapable of being suppressed. To address this possibility, an *in vitro* suppressor assay was performed with wild-type CD4+CD25+ suppressor cells and CD4+CD25- effector cells from either wild-type or Itch fl/fl CD4 Cre mice. Again, older mice with clear signs of pathology (8-12 weeks old) were used for these experiments. CD4+CD25- effector cells from both wild-type and Itch fl/fl CD4 Cre mice were capable of being suppressed to an equal degree (**Figure 3-6c, right panel**). Finally, to rule out the possibility that suppression fails between mutant CD4+CD25+ suppressor and mutant CD4+CD25- effector cells, the *in-vitro* suppressor assay was performed with Itch fl/fl CD4 Cre CD4+CD25+ suppressor cells and Itch fl/fl CD4 Cre CD4+CD25- effector cells. Suppression again was obvious in a titratable fashion (**Figure 3-6c, right panel**). This set of data combined with the previously mentioned data show that regulatory T cell suppression as measured by an *in vitro* suppressor assay is not effected by Itch deletion in T cells.

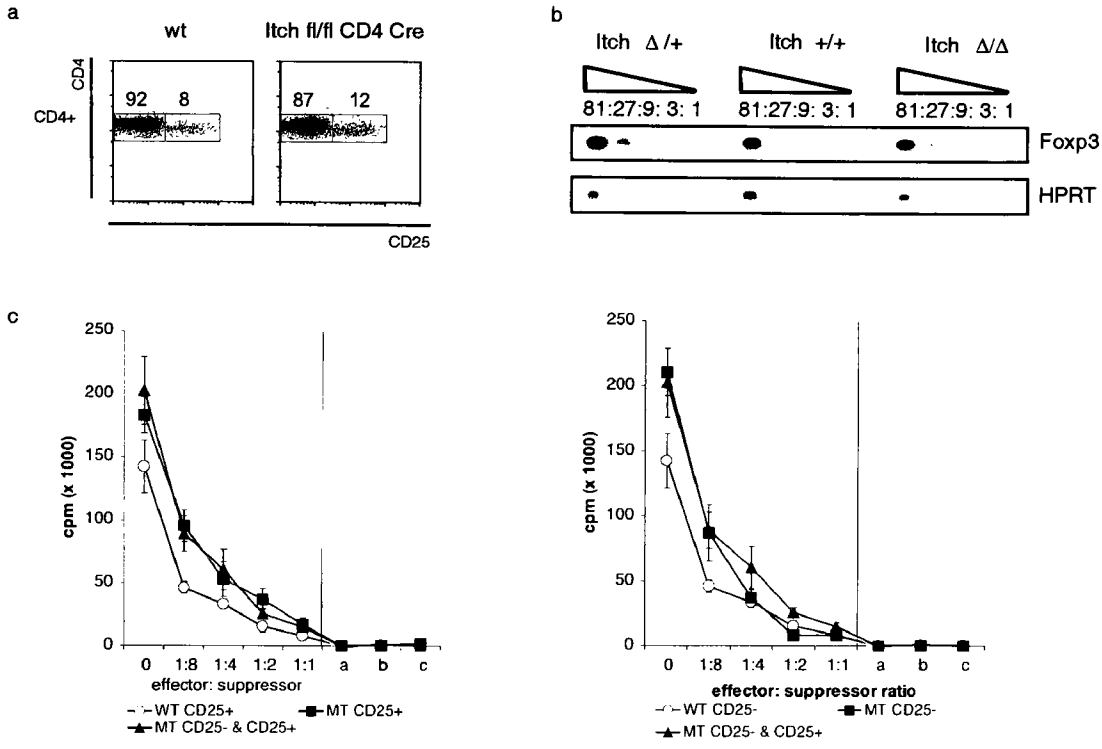


Figure 3-6. CD4+ CD25+ regulatory T cell function is intact in Itch fl/fl CD4 Cre mice. **a.** CD4+CD25+ cells are present in Itch fl/fl CD4 Cre mice. Flow cytometric analysis of the CD4+CD25+ subpopulation in the spleen. Numbers indicate the percentage of cells in the gated CD4+ subpopulation. Similar results were obtained for lymph nodes (data not shown). **b.** A population of Itch deficient CD4+ cells express Foxp3. Semi-quantitative RT-PCR analysis of Foxp3 expression in purified CD4+ T cells from wild-type and Itch deficient mice. Total RNA was reverse transcribed using oligo(dT) primers and threefold serial dilutions of cDNA were used for PCR reactions. The PCR products were visualized by southern blotting with Foxp3 and HPRT specific probes. RT-PCR analysis of HPRT expression was used as a loading control. **c.** Itch deficient CD4+ cells display suppressor cell function *in vitro*. *In vitro* suppressor assay. **Left panel.** Wild-type CD4+CD25- responder cells were cultured with APCs and soluble anti-CD3ε alone and with wild-type (WT) or Itch fl/fl CD4 Cre (MT) CD4+CD25+ suppressor cells added at a ratio of 1:8, 1:4, 1:2, or 1:1. **Right panel.** Itch fl/fl CD4 Cre CD4+CD25- responder cells were cultured with APCs +soluble anti-CD3ε alone and with wild-type (WT) or Itch fl/fl CD4 Cre (MT) CD4+CD25+ suppressor cells added at a ratio of 1:8, 1:4, 1:2, 1:1. Anergy of CD4+CD25+ suppressor cells was confirmed by co-culturing with APCs + soluble anti-CD3ε (a). Culture of APCs alone (b) and APCs and responder cells without soluble anti-CD3 (c) provided negative controls for proliferation. Cells were cultured for 72 hours, and [^{3}H] thymidine was added 8 hours before cell harvesting. Proliferation was determined by the analysis of the [^{3}H] thymidine incorporation.

3.B.9 CD4+ T cells from Itch fl/fl CD4 Cre mice are insensitive to TGF-β mediated suppression of proliferation:

Peripheral tolerance is maintained by numerous mechanisms, only one of which is CD4+CD25+ suppressor T cells. TGF-β is an immunomodulatory cytokine that has an unclear role in suppressor T cell function *in vivo*, but has a clear role in regulating T cell activation and modulating inflammatory responses (Gorelik and Flavell 2000). The recent demonstration that Itch and its human homolog AIP-4 are involved in TGF-β signaling in fibroblasts and cell lines (Bai, Yang et al. 2004; Feng, Guedes et al. 2004) suggested that it may also play a role in modulating TGF-β signaling in T cells. In order to address this possibility, purified CD4+ T cells were isolated from either spleen or lymph nodes of wild-type and Itch fl/fl CD4 Cre mice and stimulated *in vitro* with plate-bound α-CD3 and α-CD28 antibodies in the presence of increasing concentrations of TGF-β. Wild-type CD4+ T cells showed a titratable decrease in proliferation in response to exogenous TGF-β. In stark contrast, proliferation of CD4+ T cells from Itch fl/fl CD4 Cre mice was not inhibited by TGF-β, even at ten fold higher concentrations than those necessary to inhibit wild-type CD4+ T cells (**Figure 3-7a**). These data suggested that CD4+ T cells from Itch fl/fl CD4 Cre mice may be insensitive to TGF-β.

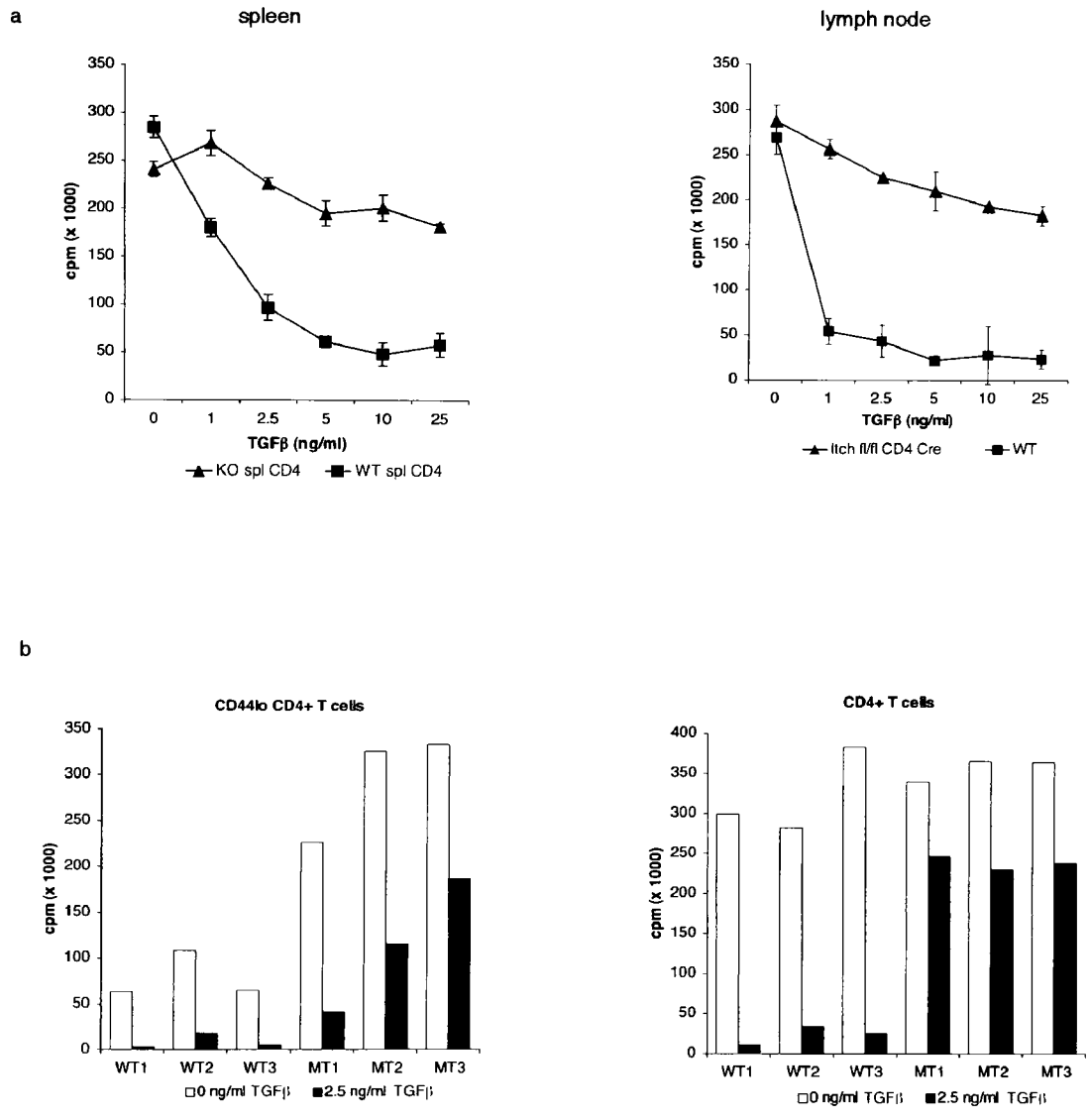


Figure 3-7: Itch regulates TGF-β sensitivity. **a.** Total CD4+ T cells from Itch fl/fl CD4 Cre mice are insensitive to TGF-β mediated suppression. Purified CD4+ T cells from wild-type (squares) or Itch fl/fl CD4 Cre (triangles) were cultured with 10 μg/ml α-CD3 and 5 μg/ml α-CD28 platebound antibodies in the presence or absence of indicated concentrations of recombinant human TGFβ. Proliferation was assessed after 72 hours of culture by the incorporation of [^{3}H] thymidine added eight hours prior to cell harvesting. Results are representative of two independent experiments with three mice per experimental group. **Left panel.** Spleen. **Right panel.** lymph nodes. **b.** CD44lo "naïve" CD4+ T cells are sensitive to TGFβ mediated suppression. Wild-type (WT) or Itch fl/fl CD4 Cre (MT) CD44lo CD4+ T cells were purified to >98% purity from pooled spleen and lymph node lymphocytes. Cells were cultured with 10 μg/ml anti-CD3 and 5 μg/ml anti-CD28 platebound antibodies in the presence (grey bars) or absence (open bars) of 2.5 ng/ml recombinant human TGF-β. Proliferation was assessed by [^{3}H] thymidine incorporation during the last eight hours of a 72 hour culture period. Data represent three wild-type and Itch fl/fl CD4 Cre mice. **Left panel.** CD44lo CD4+ T cells. **Right panel.** Total CD4+ T cells from the same mice.

3.B.10 Naïve CD44lo CD4+ T cells from Itch fl/fl CD4 Cre mice are sensitive to TGF-β mediated suppression of proliferation

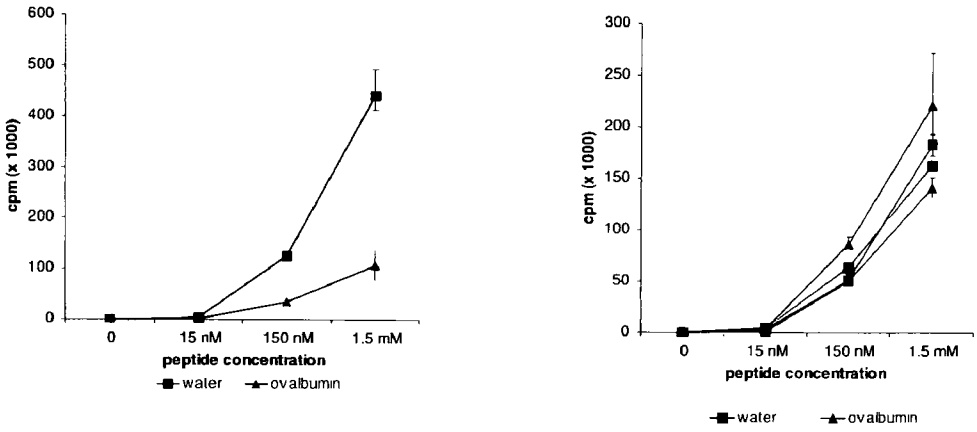
Since TGF-β inhibition is dependent on the levels of IL-2 present (Kehrl, Wakefield et al. 1986), it remained a possibility that the difference seen *in vitro* was compounded by the increase in IL-2 secreting activated/effector cells in Itch fl/fl CD4 Cre mice compared to wild type control mice. In order to address this concern, CD44lo CD4+ cells were purified and stimulated in the presence of TGF-β. CD44lo CD4+ cells from Itch fl/fl CD4 Cre mice proliferated five-fold more than their wild-type counterparts, although proliferation levels of total CD4+ cells were similar. TGF-β mediated suppression was more evident in Itch fl/fl CD4 Cre CD44loCD4+ cells than in total CD4+ cells (**Figure 3-7b**). These data suggest that TGF-β insensitivity seen in Itch deficient CD4+ T cells may correlate with their activated phenotype.

3.B.11 Itch fl/fl CD4 Cre mice do not develop oral tolerance:

Itch mRNA and protein levels are increased in a model of clonal anergy induced by incubation of differentiated Th1 T cells with ionomycin (Heissmeyer, Macian et al. 2004). Although this data suggests that Itch may be upregulated in clonally anergic cells, it does not provide any evidence for the role of Itch in anergy induction *in vivo*. In order to directly address the role of Itch in peripheral tolerance *in vivo*, Itch fl/fl CD4 Cre D011.10 TCR transgene mice were give soluble ovalbumin for 5 days to induce oral tolerance. CD4+ cells from splenocytes were restimulated with ovalbumin peptide presented by irradiated syngeneic splenocytes for three days, and proliferation rates were determined by [^{3}H] thymidine incorporation. Preliminary results indicate that CD4+ T cells from Itch fl/fl CD4 Cre mice fail to tolerize. There was no difference in the extent of proliferation of mutant mice whether they were fed Ovalbumin or not (**Figure 3-8a**).

It should be noted; however, that the proliferation rates of mutant cells were considerably lower than wild-type cells, in striking contrast to what is seen in nontransgenic mice upon antibody stimulation (**Figure 3-8b**).

a



b

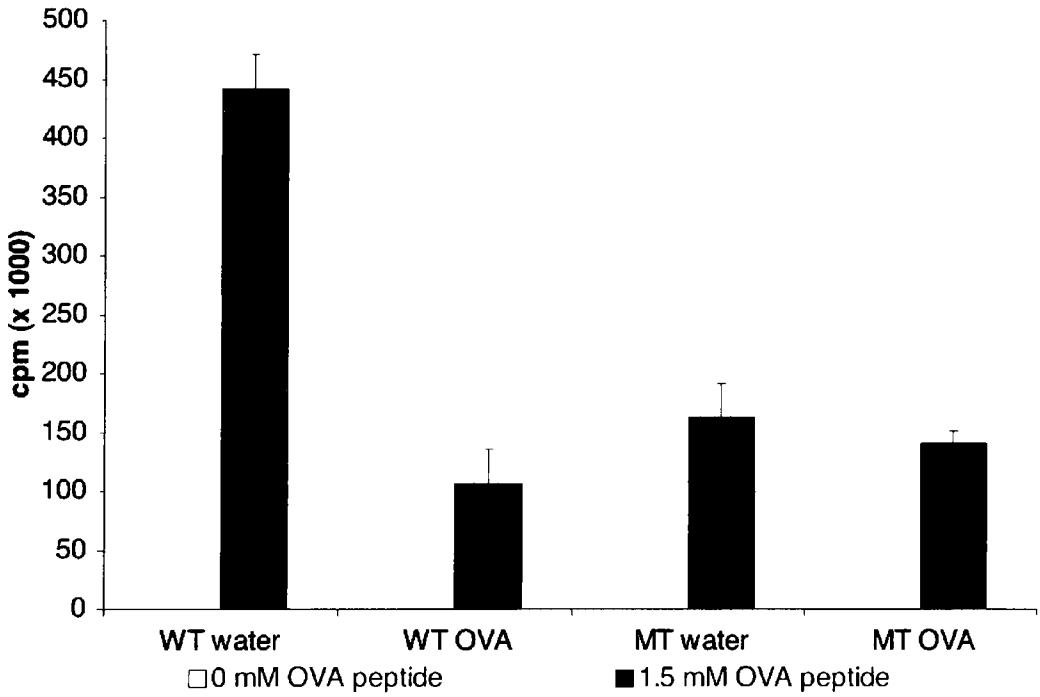


Figure 3-8. Itch deficient cells are not anergized upon oral tolerization. DO11.10 mice (WT) or D011.10 Itch fl/fl CD4 Cre (MT) were given water alone (water) or water containing 20 mg/ml ovalbumin (OVA) for five days. Mice were sacrificed on the sixth day and purified CD4+ splenocytes were cultured with indicated concentrations of OVA peptide presented by irradiated syngeneic splenocytes. Proliferation was measured three days later by addition of [^{3}H] thymidine to cultures eight hours prior to harvesting. **a**. Proliferation in response to titrated concentrations of peptide in mice given water (squares) or ovalbumin (triangles) in their drinking water. Left panel. DO11.10 mice. Right panel. DO11.10 Itch fl/fl CD4 Cre mice **b**. Proliferation rates of DO11.10 mice (WT) and DO11.10 Itch fl/fl CD4 Cre mice (MT) mice in response to 0 mM (empty bars) or 1.5mM OVA peptide (filled bars).

3.C Discussion:

3.C.1 C57BL/6 Itch fl/fl CD4 Cre mice develop a spontaneous inflammatory disease

Histological analysis of 8-week old Itch fl/fl CD4 Cre mice revealed an inflammatory phenotype identical to that described for the naturally occurring Itch mutant strain a^{18H}. The inflammation was characterized by infiltrates consisting of neutrophils, eosinophils, macrophages, and plasma cells along the respiratory tract, gastrointestinal tract and urinary tract. The lungs showed the most striking level of inflammation in the alveoli, where infiltrates of eosinophilic macrophages accumulated to the point of alveolar acidophilic macrophage pneumonia. The inflammation was far from systemic, however, as many internal organs, including the brain, liver, thyroid gland, small and large intestine and kidneys, showed no inflammatory infiltrates in the mice analyzed. Specifically, it seems that the infiltrates accumulated along mucosal linings and eventually along the skin, all areas which are exposed to external antigens. The location of these infiltrates is suggestive of a problem in regulating local inflammatory responses in areas exposed to external antigens. This concept makes the study of Itch deficient mice particularly intriguing from the standpoint of allergies, which are also known to involve dysregulated immune responses to external antigens.

Analysis of lymphocyte populations in secondary organs revealed that while there was visible splenomegaly and lymphadenopathy, there was no difference in the absolute numbers of CD4+ cells. The percentages and absolute numbers of activated/memory phenotype CD4+ T cells were increased in the spleen but not in the lymphnodes. Furthermore, analysis of proliferation rates *in vivo* in Itch fl/fl CD4 Cre mice and littermate controls suggests that the CD44hiCD62Llo cells are indeed activated cells

undergoing proliferation. Why then is there no accumulation of CD4+ cells in the spleen? One reason is that later timepoints need to be assessed in order to see the accumulation. A second potential explanation is that the CD4+ cells emigrate to the periphery upon activation. *In situ* stainings of organ sections for CD4+ cells could address this hypothesis.

3.C.2 The Itch inflammatory phenotype is due to Itch deficient CD4+ T cells:

In addition to the fact that conditionally Itch deficient Itch fl/fl CD4 Cre mice develop an inflammatory phenotype akin to Itch deficient mice, two pieces of data described in this thesis provide compelling evidence that the Itch inflammatory phenotype is due solely to Itch deficiency in CD4+ T cells. Firstly, although Itch deficient mice show an accumulation of plasma cells in inflammatory lesions and increased serum IgG1 and IgE titres, Itch fl/fl CD4 Cre JHT mice developed an inflammatory disease similar in kinetics and severity to that seen in Itch fl/fl CD4 Cre miced. Thus, while B cells are activated in Itch mice, their role in the pathology is secondary. Secondly, transfer of purified Itch deficient CD4+ T cells into Rag deficient mice led to a recapitulation of the inflammatory phenotype within one month of transfer. These data not only provide evidence for a role of CD4+ T cells in the Itch pathology, but also demonstrate that Itch deficient CD8+ T cells are not required for the pathology. Strikingly, the location of inflammatory lesions mirrored that seen in Itch fl/fl CD4 Cre mice, suggesting that the location of inflammation is not arbitrary and must provide insights into the role of Itch in modulating immune responses.

Genetic proof that CD4+ cells are required for the Itch pathology can be obtained from the analysis of Itch fl/fl CD4 Cre MHC II -/- mice. Breedings to generate these

mice are currently underway. In addition, genetic evidence of the role of Itch defcient CD8+ T cells in the Itch pathology can be obtained from the analysis of Itch fl/fl CD4 Cre β_2M -/- mice. Breedings of these mice have been hampered by the linkage between β_2M and Itch, but eventually will yield conclusive results.

The transfer of CD4+ T cells into unirradiated syngeneic hosts failed to cause any visible pathology, in striking contrast to transfer into Rag deficient mice. There are several potential explanations for this, none of which are addressed by the experiments performed thus far. Firstly, the discrepancy between transfer into Rag deficient mice and unirradatiated syngeneic hosts may simply be a matter of the number of CD4+ T cells that survived the transfer. It is much easier for lymphocytes to survive in a lymphopenic host than to compete with existing lymphocytes for niches. Secondly, transfer of CD4+ T cells into lymphopenic hosts leads to their homeostatic expansion. This expansion may trigger activation of CD4+ T cells (King, Ilic et al. 2004). Although transfer of CD4+ T cells from WT mice caused no obvious pathology in recipient mice, it may be that Itch deficiency coupled with further activation of T cells is required for the Itch pathology to occur. This scenario seems unlikely, as the transferred Itch deficient CD4+ T cells came from mice with visible splenomegaly, lymphadenopathy, and inflammation, which speak in favor of the T cells already being sufficiently activated to cause disease. The third and most intriguing possibility is that the discrepancy between transfer into Rag deficient hosts and unirradiated syngenic hosts is due to an underlying problem with regulatory T cell suppressor function in Itch deficient mice. Transfer of mixed wild-type and Itch deficient CD4+ T cells into Rag deficient mice should provide a way to tease apart some of these potential explanations. If the transfer of mixed wild-type and Itch deficient

CD4+ T cell populations leads to disease, then one could argue that the absence of disease in unirradiated syngeneic hosts is more likely due to the percentage of cells that survived transfer rather than absent suppressor functions *in vivo*.

3.C.3 The activation of Itch fl/fl CD4 Cre T cells is not cell autonomous

While transfer of CD4+ T cells from diseased Itch fl/fl CD4 Cre mice transferred the disease, it remained unclear whether the activation of Itch deficient CD4+ T cells occurred cell autonomously or in an antigen-dependent manner. Analysis of Itch fl/fl CD4 Cre AND transgenic mice revealed that AND TCR expressing CD4+ T cells remained naïve as judged by surface marker staining. Although AND Itch fl/fl CD4 Cre mice became sick and accumulated activated CD4+ T cells, these CD4+ cells did not express the AND transgene. These data support the notion that Itch deficiency in CD4+ T cells does not lead to spontaneous activation. Instead these data are consistent with the hypoethesis that Itch deficiency modulates signaling such that TCR signaling leads to inappropriate activation and inflammatory responses in these mice. Itch may regulate TCR signaling itself or may regulate co-stimulatory ligand signaling such that upon antigen encounter, Itch deficient CD4+ T cells respond inappropriately. It is also possible that Itch regulates CD4+ T cell effector function upon T cell activation.

One potential caveat in this interpretation of the data is that surface markers were used to assess the naïve phenotype of CD4+ T cells. Although cell surface markers are commonly used to identify a cell's activation status, it remains a formal possibility that Itch deficient AND transgenic CD4+ T cells, while appearing naïve, were none-the-less activated and contributing to the disease phenotype. These concerns will be addressed with the generation of Itch fl/fl CD4 Cre mice on a Rag1 deficient TCR transgenic

background. Crosses are already underway for both the AND and DO11.10 transgenes. If Itch deficient cells truly require cognate antigen stimulation to become inappropriately activated, then one would expect Itch fl/fl CD4 Cre Rag-/- AND+ and Itch fl/fl CD4 Cre Rag1 -/- DO11.10 mice to remain free of pathology. Conversely, if Itch deficient CD4+ cells secrete cytokines even in the absence of antigenic stimulation, then one would expect the Rag deficient TCR transgenic mice to also succumb to disease.

3.C.4 CD4+CD25+ *in vitro* suppressor function is intact in Itch fl/fl CD4 Cre mice:

The inflammatory pathology seen in Itch fl/fl CD4 Cre mice, which is restricted primarily to mucosal areas and does not affect internal organs, is very different from the multi-organ pathology described for mice deficient in CD4+CD25+ regulatory T cells. None-the-less, it could be formally argued that the dysregulated inflammatory response seen in Itch deficient mice was due to either an absence or a problem in the function of CD4+CD25+ suppressor T cells. Itch fl/fl CD4 Cre mice contain CD4+CD25+ cells, as judged both by surface marker staining and expression of the regulatory T cell specific transcription factor Foxp3. In addition, *in vitro* suppressor assays using wild-type and Itch deficient suppressor and responder cells revealed no qualitative difference in the ability of Itch deficient suppressor cells to suppress and Itch deficient responsder cells to be suppressed. It should be noted that there was a small but consistent difference in the suppressor assay curves between wild-type and Itch deficient cells. Assays in which Itch deficient cells were added had higher proliferation counts and required higher numbers of suppressor cells to bring the counts down. This difference can easily be explained by the

contaminating fraction of activated CD25+ cells in the CD4+CD25+ suppressor cell population of Itch deficient mice.

Although Itch deficient CD4+ T cells show no obvious defect in suppressor function as assessed by *in vitro* suppressor assays, it remains quite possible that there is a problem with suppressor function *in vivo*. How CD4+CD25+ regulatory T cells function *in vivo* remains a matter of controversy, but it is unlikely that direct contact-mediated suppression is the mechanism by which these cells globally suppress inflammatory responses in an organism. Currently, the only *in vivo* assessment of CD4+CD25+ suppressor cell function comes from a SCID colitis model. Transfer of purified CD45RBhi CD4+ cells into SCID mice causes colitis, which can be suppressed by co-transfer of CD45RBlo CD4+CD25+ cells (Mottet, Uhlig et al. 2003). It would be interesting to see if transfer of Itch deficient CD45RBhi and either wild-type or Itch deficient CD45RBlo CD4+CD25+ cells leads to colitis, as this data would indicate a problem with suppression of Itch deficient responder cells *in vivo*. Conversely transfer of wild-type CD45RBhi and either wild-type or Itch deficient CD45RBlo CD4+ CD25+ suppressor cells could address the suppressive potential of Itch deficient suppressor cells. These experiments will require backcrossing the Itch conditional deletion onto a Balb/c background first, but will eventually provide definitive data regarding suppressor T cell function *in vivo* in the absence of Itch.

It is also increasingly clear that the naturally occurring CD4+CD25+ regulatory T cells are not the only T cells with suppressor function *in vivo*. Tr-1 and Th3 suppressor cells, which are both induced upon exposure to antigen under tolerizing conditions, also play key roles in modulating immune responses. The suppressive capabilities of these

cells may be affected by Itch deficiency. The generation of Tr-1 cells and their IL-10 dependent suppressive capabilities *in vitro* have been described for D011.10 mice (Groux, O'Garra et al. 1997). It would be interesting to repeat these experiments with CD4+ T cells from Itch fl/fl CD4 Cre D011.10 mice. Problems with either the generation of Tr-1 cells or their suppressive functions *in vitro* could provide an interesting explanation for the Itch inflammatory phenotype.

3.C.5 TGF-β sensitivity may be affected in Itch deficient CD4+ T cells:

Recently, Itch has been implicated in regulating TGF-β signaling. Analysis of purified CD4+ T cells from Itch fl/fl CD4 Cre mice initially seemed to support a role for Itch in TGF-β signaling in CD4+ T cells; however, analysis of CD44lo CD4+ cells provided data inconsistent with the initial findings. Although there was a great deal of variation between the purified Itch deficient CD44lo CD4+ T cells from different Itch fl/fl CD4 Cre mice, they showed a greater sensitivity to TGF-β than total Itch deficient CD4+ T cells. These data may be interpreted to suggest that the TGF-β insensitivity seen in total CD4+ T cells reflects their activated status more than direct TGF-β insensitivity. TGF-β inibits *in vitro* T cell proliferation in an IL-2 dependent manner (Kehrl, Wakefield et al. 1986), and the enhanced proliferation seen in Itch deficient CD4+ T cells suggests that there may be an increase in IL-2 production, either due to an intrinsic change in IL-2 production in response to Itch deficiency, or due to the activated status of CD4+ T cells purified from Itch fl/fl CD4 Cre mice. Definitive answers can only be provided by purification of naïve CD4+ T cells from Itch fl/fl CD4 Cre Rag deficient TCR transgenic mice, once it is established that these mice do not develop an inflammatory pathology.

One puzzling feature of the comparison of total CD4+ T cells from wild-type and Itch deficient mice with regards to sensitivity to TGF-β is that the difference in TGF-β sensitivity is far more striking than the difference in activated CD4+ cells as assessed by surface marker staining. One explanation, which has already been discussed, is that surface marker analysis underestimates the number of activated CD4+ T cells. Another possibility is that the cells that are insensitive to TGF-β suppression not only proliferate but also cause bystander activation of other CD4+ T cells through their secretion of IL-2. It is known that TGF-β mediates its suppression in an IL-2 dependent manner. But why don't the activated CD4+ T cells from wild-type mice also cause this bystander activation of naïve CD4+ T cells? One possible reason is that only Itch deficient activated CD4+ cells are insensitive to TGF-β mediated suppression. One possible way to address this issue would be to activate CD4+ T cells for 24-48 hours prior to the addition of TGF-β.

3.C.6 The role of Itch in T cell anergy:

Recent experimental evidence supports a role for E3 ligases, including Itch in regulating T cell anergy (Heissmeyer, Macian et al. 2004). Tolerization of Itch fl/fl CD4 Cre DO11.10 transgenic mice supports this hypothesis, as Itch deficient CD4+ T cells failed to tolerize. These data are preliminary, however, and much more definitive experiments are needed before a compelling case for the role of Itch in anergy induction *in vivo* can be made. Itch deficient CD4+ DO11.10 T cells proliferated to the same extent, whether or not the mice were tolerized to OVA using an oral tolerization protocol. Although these data are consistent with a role for Itch in anergy induction, it should be noted that the proliferation rates of Itch deficient CD4+ T cells were low, whether or not the mice from which they were derived were tolerized. These data are in striking contrast

to what is seen upon polyclonal stimulation of Itch deficient T cells *in vitro*. It is presently unclear whether this discrepancy is due to the stimulation of Itch deficient T cells with more physiologically relevant APCs and peptide or is related to the TCR transgene.

Analysis of Itch fl/fl CD4 Cre DO11.10 Rag -/- mice should provide a cleaner sytem for anlayis, as all CD4+ cells should be OVA sensitive and naïve. Comparison of proliferation rates between DO11.10 Rag -/- and Itch fl/fl CD4 Cre DO11.10 Rag -/- mice either upon polyclonal antibody stimulation or APC and peptide stimulation should provide important control data regarding the effects of the type of stimulus on proliferation rates. Furthermore, analysis of oral tolerance in Rag deficient mice should also remove the confounding variable that T cells in Itch fl/fl CD4 Cre mice do not anergize because of the inflammatory environment they are in rather than because of problems with anergy induction *per se* in the absence of Itch.

Experimental evidence also exists for a role of suppressor cells in oral tolerization protocols (Thorstenson and Khoruts 2001). Many of the existing oral tolerization protocols may lead to tolerance through both suppressor cells as well as anergy. Definitive experiments regarding anergy induction will therefore require transfer of CD4+ T cells into transgenic mice expressing soluble OVA, where the only mechanism for tolerance is anergy (Abul Abbas, personal communication). Unfortunately, these transfers require Itch fl/fl CD4 Cre DO11.10 mice on a Balb/c background. As such, they will require 10 generations of backcrossing to DO11.10 Balb/c mice before these assays can be performed.

3.D Summary:

Collectively, the results presented in this chapter of the thesis demonstrate that Itch deficient CD4+ cells are sufficient to phenocopy the pathology of Itch deficient mice. Activation of Itch deficient CD4+ T cells requires antigen. CD4+CD25+ regulatory T cell suppressor function is intact, at least by *in vitro* assays. TGF-β sensitivity may be reduced in Itch deficient CD4+ T cells but this may be secondary to the inflammatory pathology. Finally, peripheral tolerance mechanisms may be affected in Itch fl/fl CD4 Cre mice *in vivo*.

Chapter 4: The role of Th2 cells in the pathology of Itch mice

4.A Introduction:

4.A.1 Th1 versus Th2 immune responses:

CD4+ helper T cells can differentiate into two well define subsets called T helper type 1 (Th1) and T helper type 2 (Th2) T cells during an immune response. Th1 cells preferentially produce inflammatory cytokines such as interferon gamma (IFNγ) and lymphotoxin alpha (LTα) and are generally mediators of cellular immunity. Th2 cells produce cytokines such as interleukins 4,5,9,10, and 13 (IL-4,IL-5,IL-9,IL-10,IL-13) and are critical for humoral responses as well as immune responses against extracellular parasites such as helminthes (Heinzel, Sadick et al. 1989; Finkelman, Pearce et al. 1991). During dysregulated immune responses, the dominance of either helper cell type shapes the outcome of immunologically mediated disease conditions, including autoimmune diseases and allergic responses (Abbas, Murphy et al. 1996).

4.A.2 Evidence for a role of Itch in regulating Th2 differentiation:

The inflammatory pathology and increased serum IgG1 and IgE titres in Itch deficient mice prompted investigators to look at Th2 differentiation in Itch deficient CD4+ T cells. Analysis of CD4+ T cells from Itch deficient mice revealed a bias towards Th2 differentiation *in vitro* (Fang, Elly et al. 2002). Biochemical analysis suggested that this bias was due to increased JunB levels in Itch deficient CD4+ T cells. Recent experimental data has provided further mechanistic details regarding the regulation of JunB by Itch. Gao and colleagues have found that Itch is phosphorylated upon TCR engagement in a Janusk Kinase (JNK) dependent manner (Gao, Labuda et al. 2004). This

phosphorylation leads to increased E3 ligase activity of Itch, leading to increased JunB turnover in recently stimulated T cells. The models the authors propose is that in the absence of either JNK signaling or Itch, JunB accumulates in activated T cells, favoring Th2 differentiation. This model is consistent with the similar Th2 bias and the moderate Jun B accumulation seen in both Itch deficient and JNK1 deficient mice (Dong, Yang et al. 1998; Fang, Elly et al. 2002). What remains unclear from these findings is how Itch autoubiquitination, which is also increased upon JNK mediated phosphorylation of Itch and which leads to Itch degradation, relates to the overall Itch activity in stimulated T cells. Furthermore, it should be noted that JNK1 deficient cells, while displaying moderate accumulation of nuclear JunB, show a far more drastic increase in nuclear NFATc. This drastic increase could also increase the Th2 bias observed in JNK1 deficient mice. Finally, the Th2 bias seen in Itch deficient and JNK deficient mice may be secondary to other changes in the mice due to complete ablation of the respective genes in all cell types.

4.A.3 Aims of this study:

Previous work has shown that Itch deficient CD4+ T cells have an increased propensity to differentiate into Th2 cells *in vitro*. While these data were consistent with the increased IgG1 and IgE serum titres in older mice, it was unclear whether the bias observed was secondary to existing pathology in the mice or the actual cause of the pathology. The experiments described in this chapter provided a causal link between Itch deficiency in CD4+ T cells and the Th2 bias observed in Itch deficient mice. Furthermore, the importance of this Th2 bias in the inflammatory pathology was addressed using genetic techniques. Overall the experiments were designed to

definitively determine the link between Itch deficiency, dysregulated Th2 immune responses, and the pathology seen in Itch deficient mice.

4.B Results:

4.B.1 Increased Th2 differentiation of Itch deficient naïve CD4+ T cells:

In order to determine whether Itch deficient CD4+ T cells have an inherent bias towards Th2 differentiation, CD44lo naïve CD4+ T cells were purified from wild-type and Itch fl/fl CD4 Cre mice at 4 weeks of age. At this age, no dermatitis is visible and FACS analysis reveals minimal changes in lymphocyte populations or activation status of CD4+ cells as compared to wild type littermate controls. Purified naïve CD44lo CD4+ T cells were stimulated *in vitro* with plate bound α-CD3ε (10 μg/ml) and α-CD28 (5 μg/ml) antibodies in the presence of 25 U/ml IL-2 for two days, rested for three days, and restimulated with plate bound α-CD3ε and α-CD28 antibodies in the presence of Brefeldin A for four to six hours. Cytokine production by the cells was then assessed by intracellular staining. Analysis of these experiments revealed that naïve Itch deficient CD4+ T cells had the same propensity towards Th2 differentiation as published for total CD4+ populations of naïve and activated cells (**Figure 4-1a,b**).

4.B.2 Itch deletion does not effect Th1 differentiation:

Th1 and Th2 differentiation are reciprocally regulated pathways, such that differentiation towards Th1 inhibits Th2 differentiation, and vice versa. It thus seemed possible that the increased Th2 differentiation in Itch deficient CD4+ cells was due to an inability to differentiate towards the Th1 lineage. In order to address this issue, naïve wild type and Itch deficient CD4+ cells were stimulated under Th1 polarizing conditions.

No difference was seen in the ability of Itch deficient cells to become Th1 cells (**Figure 4-1a**).

4.B.3 Itch deletion *in vitro* leads to a bias towards Th2 differentiation:

Although purification of naïve cells from Itch fl/fl CD4 Cre mice suggested that Itch deficiency in CD4+ T cells was directly responsible for the Th2 skewing bias observed, it remained possible that the naïve T cell as judged by surface marker stainings had received non-TCR mediated signals *in vivo* that influenced their differentiation *in vitro*. To further substantiate the claim that Itch can directly modulate CD4+ Th1 versus Th2 lineage commitment decisions, CD4+ T cells from Itch fl/+ and Itch fl/fl mice were treated with cell permeable Cre (TAT-Cre) *in vitro*. Cells were allowed to proliferate for 7 days in medium in the presence of anti-IFNγ and anti-IL-4 antibodies to inhibit differentiation. At this time, cells that had deleted Itch at the genomic level had also reduced Itch protein levels as measured by Western (**Figure 4-1c**). Cells were then subjected to a second round of stimulation under Th1, Th2, or unpolarizing conditions. Intracellular cytokine analysis for IFNγ and IL-4 after α-CD3ε and α-CD28 stimulation revealed that TAT-Cre treated Itch fl/fl CD4+ cells showed an increased frequency of Th2 cells compared to TAT-Cre treated Itch fl/+ CD4+ cells (**Figure 4-1c**).

4.B.4 Itch modulation of Th2 differentiation is Stat 6 dependent:

Stat6 is a transcription factor critical for signaling downstream of the IL-4 receptor, and its presence is critical for TH2 differentiation of wild-type CD4+ cells.

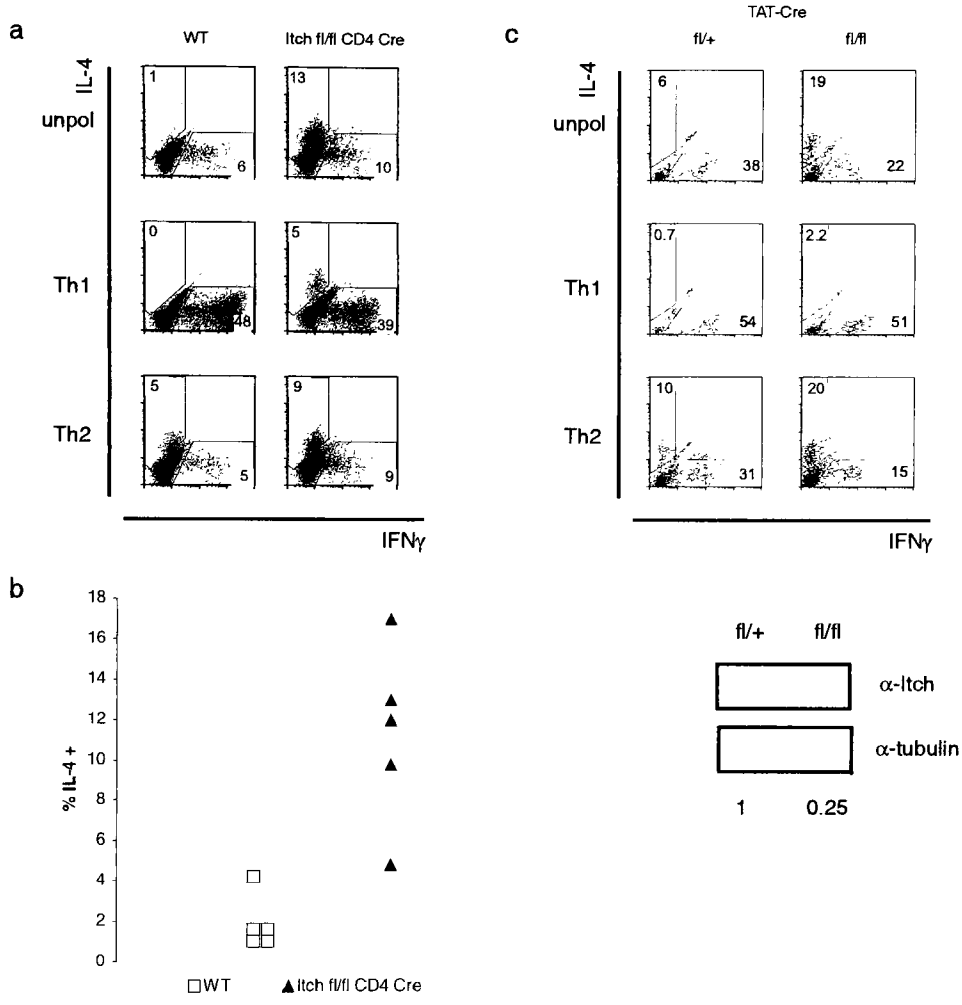


Figure 4-1. Itch regulates Th1/Th2 differentiation. **a.** *In vitro* differentiation of Itch deficient CD44lo naïve CD4+ T cells leads to a Th2 bias. Purified CD44lo CD4+ T cells were stimulated with plate bound anti-CD3ε and anti-CD28 antibodies in medium containing IL-2 alone (nonpolarizing medium), IL-2, IFNγ, and anti-IL-4 antibodies (Th1 polarizing medium) or IL-2, IL-4, and anti-IFNγ antibodies (Th2 polarizing medium). Cells were stimulated for 2 days, rested for four days, washed, and restimulated for four hours in the presence of Brefeldin A. Cytokine production was assessed by intracellular staining for IFNγ and IL-4. Numbers indicate the percentage of cells in the gated subpopulation. **b.** Summary of the percentage of IL-4+ cells from purified wild-type (open sqaures) and Itch fl/fl CD4Cre (closed tringles) CD4+ cells cultured under nonpolarizing conditions as described in a. Each dot represents an individual mouse. The percentage of IL-4+ cells was significantly increased in Itch fl/fl CD4 Cre mice compared to littermate controls ($p=0.001$ by student's t-test). **c.** *In vitro* deletion of Itch leads to Th2 bias in differentiation. Purified CD4+ cells from fl/+ or fl/fl mice were incubated with cell permeable Cre (TAT-Cre) for one hour. Cells were stimulated for 3 days with plate bound anti-CD3ε and anti-CD28 antibodies under T helper differentiation blocking conditions (10μg/ml anti-IFNγ and anti-IL-4 antibodies). After resting cells for four days, Itch protein levels in TAT-Cre treated cells were assessed by Western blot of half the cells **(lower panel)**. Numbers indicate relative Itch protein levels normalized to tubulin and quantified using NIH Imager. Remaining cells were stimulated and cyotkine production was assessed one week later as described in a.

The requirement for Stat6 can be overcome when CD4+ cells are stimulated through the Notch pathway and also upon certain genetic modifications. For example, CTLA-4 deficient CD4+ cells are capable of generating Th2 effector cells even in the absence of Stat 6, as demonstrated by *in vitro* assays of CD4+ T cells from double deficient mice. To determine if Itch modulation of Th2 differentiation was Stat6 dependent, C57Bl/6 Itch fl/fl CD4 Cre mice were crossed to Balb/c Stat 6 deficient mice. Itch fl/fl CD4 Cre Stat 6 -/- mice were born at normal mendelian ratios. Strikingly, Itch fl/fl CD4 Cre Stat 6 -/- cells were incapable of differentiating into Th2 cells *in vitro*, even under Th2 polarizing conditions (**Figure 4-2**).

4.B.5 Itch pathology *in vivo* is Stat6 dependent

The pathology of Itch deficient mice has been ascribed to an overwhelming Th2 immune response in these mice. As Stat6 is critical for Th2 cell differentiation *in vivo* and *in vitro*, and as Stat6 deficiency inhibited Th2 differentiation of Itch deficient CD4+ T cells *in vitro*, Itch fl/fl CD4 Cre Stat 6-/- mice were compared to Itch fl/fl CD4 Cre Stat6 +/+ mice for the extent of their pathology. C57Bl/6 x Balb/c mixed genetic background Itch fl/fl CD4 Cre mice developed a similar inflammatory pathology as their C57Bl/6 counterparts. Itch fl/fl CD4 Cre Stat6 -/- mice, however, showed no grossly visibly signs of pathology. Analysis of organs sections from Itch fl/fl CD4 Cre Stat 6 -/- mice further revealed that the Itch pathology was absent in Itch fl/fl CD4 Cre Stat 6 -/- mice (**Figure 4-3**). All mucosal linings were free of inflammatory infiltrates, though only lung and esophageal sections are shown. The occasional focal infiltrates seen in Itch fl/fl CD4Cre Stat 6 -/- mice were consistent with the age (6 month old) of the mice and did not indicate pathology. Surprisingly, the inflammatory infiltrates were also

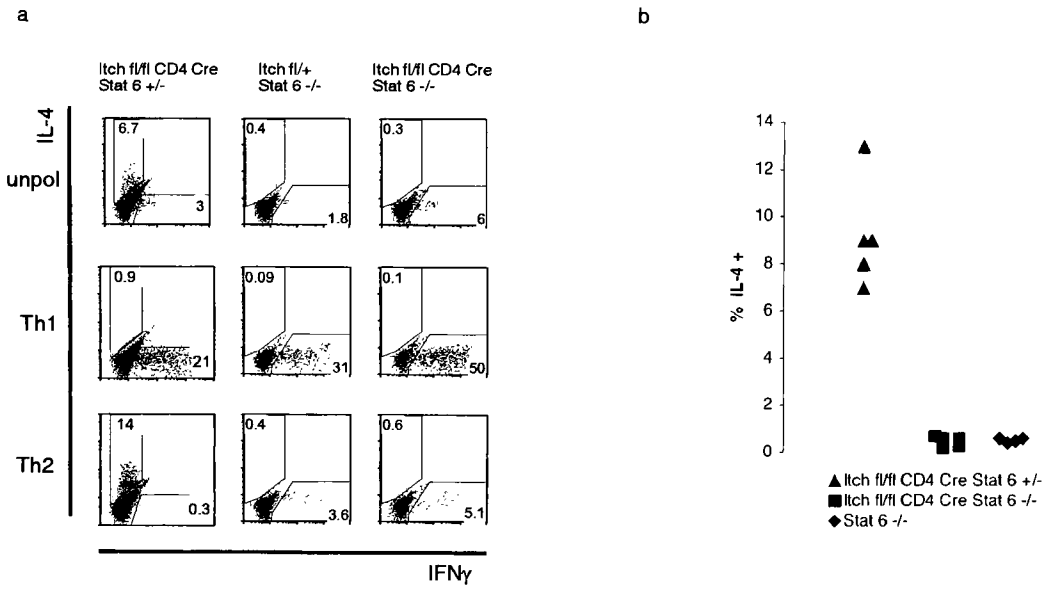


Figure 4-2. Stat6 is required for Itch dependent Th2 differentiation *in vitro*. **a.** CD4+ T cells were purified from Itch fl/fl CD4 Cre Stat 6 +/-, Itch fl/+ Stat 6 -/-, and Itch fl/fl CD4 Cre Stat 6 -/- mice. Cells were stimulated with plate bound anti-CD3ε and anti-CD28 antibodies in medium containing IL-2, IL-4, and anti-IFNγ antibodies (Th2 polarizing medium) for 2 days, rested for four days, washed, and restimulated for four hours in the presence of 10μg/ml Brefeldin A. Cytokine production was assessed by intracellular staining for IFNγ and IL-4. Numbers indicate the percentage of cells in the gated subpopulation. **b.** Graph summarizing 3 independent experiments carried out as described in a. for Th2 polarizing conditions. Each dot represents CD4+ T cells purified from an Itch fl/fl CD4 Cre Stat 6 +/- (triangle), Itch fl/fl CD4 Cre Stat 6 -/- (square) or Stat 6 -/- (diamond) mouse.

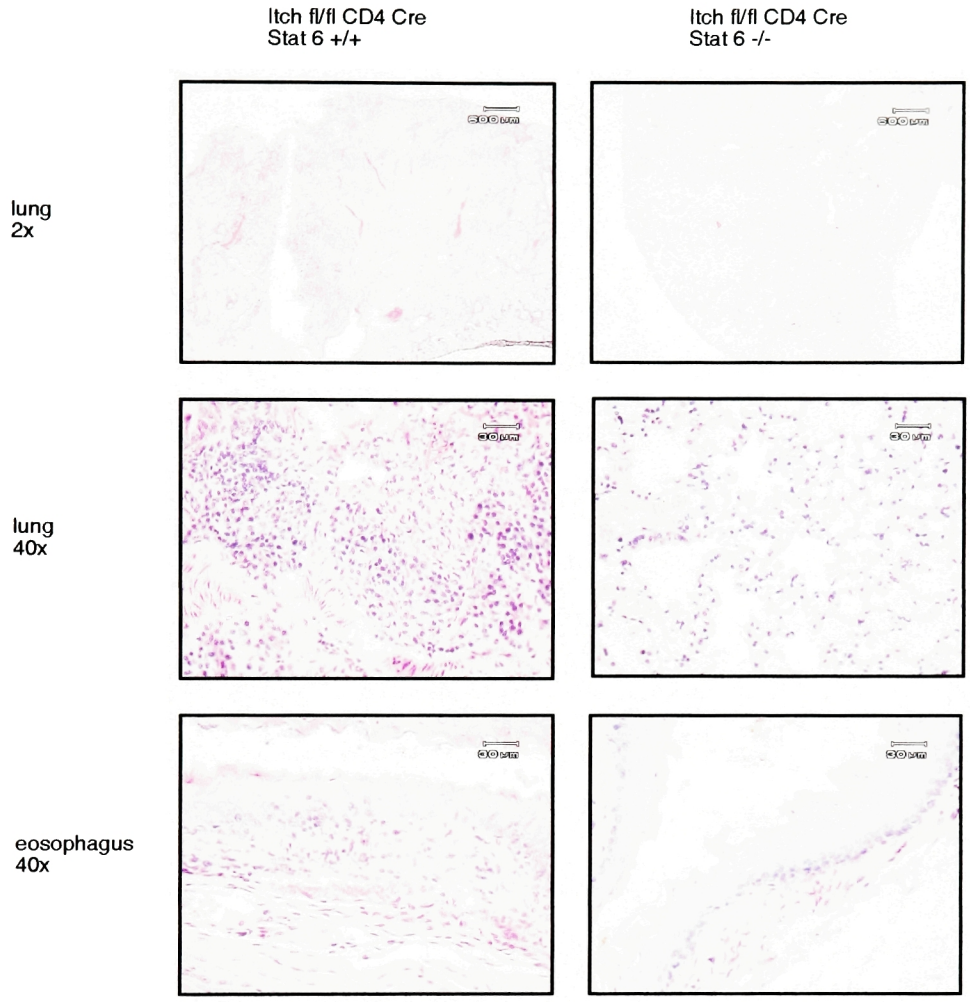


Figure 4-3. Stat6 is required for the pathology seen in Itch fl/fl CD4 Cre mice. Haematoxilin and eosin stainings of organ sections from mixed C57Bl/6 x Balb/c genetic background 3-month old Itch fl/fl CD4 Cre Stat 6 +/+ mice and 6-month old Itch fl/fl CD4 Cre Stat 6 -/- mice. Lung sections (2x and 40x) reveal acidophilic macrophage pneumonia in Itch fl/fl CD 4 Cre stat 6 +/+ but not Itch fl/fl CD4 Cre Stat 6 -/- mice. Esophagus sections reveal hyalinosis and eosinophilic infiltrates in Itch fl/fl CD4 Cre Stat 6 +/+ mice but not Itch fl/fl CD4 Cre Stat 6 -/- mice. Three Itch fl/fl CD4 Cre Stat 6 +/+ and six Itch fl/fl CD4 Cre Stat 6 -/- mice were analyzed. Sections depicted are representative for each experimental group.

moderately decreased in Itch fl/fl CD4 Cre Stat 6 +/- mice as well

4.B.6 Increased antigen sensivity in Itch deficient CD4+ T cells:

The decision to differentiate into a Th1 or Th2 helper cell is in part determined by the strength of the signal from the TCR. To address the role of Itch in modulating the strength of the TCR signal, naive CD4+ T cells were purified from wild-type and Itch fl/fl CD4 Cre mice and cultured with titrated doses of plate bound α-CD3 or α-CD3 and α-CD28 antibodies. Sensitivity to antigen was measured by the proliferation of cells in response to different amounts of antigen (**Figure 4-4, Table 4-1**). CD44loCD4+ Itch deficient cells showed increased sensitivity to antigenic stimulation as assessed by proliferation.

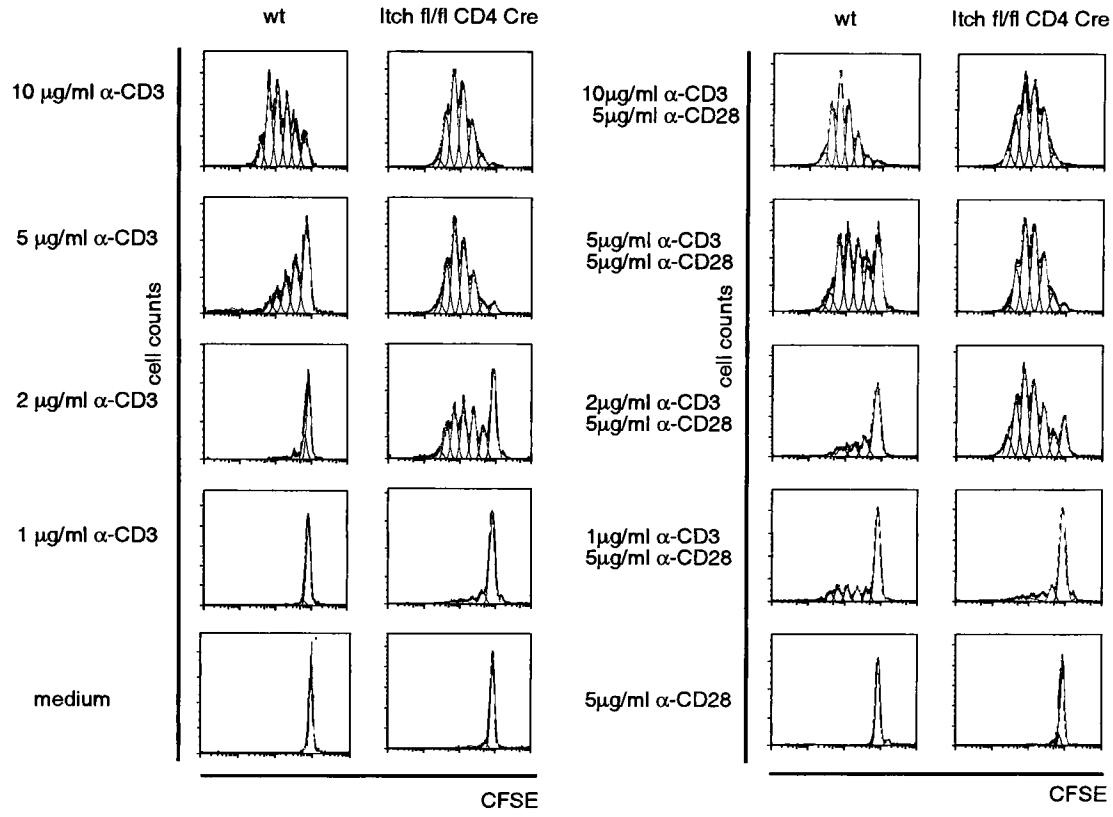


Figure 4-4. Itch deficiency leads to an increased proliferative responses in response to antigenic stimulation *in vitro*. Purified CD44loCD4+ cells were labeled with CFSE and cultured with indicated concentrations of plate bound anti-CD3ε **(left panel)** or plate bound anti-CD3ε and anti-CD28 antibodies **(right panel)**. The extent of proliferation was measured after three days by flow cytometric analysis of CFSE dilution.

Table 4-1: Increased proliferative responses in purified CD44lo CD4+ cells from Itch fl/fl CD4 Cre mice shows variation between mice. The percentage of cells that divided and the proliferation index (the number of divisions a cell that had begun dividing made) in the experiment described above were calculated using flow cytometric software (Flow Jo). Data from six mice is shown.

	Percent division		Proliferation Index	
μg/ml α-CD3ε	wt	Itch fl/fl CD4 Cre	wt	Itch fl/fl CD4 Cre
10	61.5	91.4	1.98	2.54
	57.1	73.1	1.87	2.9
	47	87.9	1.58	2.26
5	28.9	75.6	1.39	2.57
	24.9	30.5	1.41	2.13
	18.6	66.4	1.33	2.3
2	14.3	30	1	2.01
	19.5	6.65	1	1
	18.7	38.1	1	2.28

4.C Discussion:

4.C.1 Itch regulates Th2 differentiation in CD4+ T cells:

Previous work has shown that CD4+ T cells from Itch deficient mice show a bias towards Th2 differentiation *in vivo*. Because of the inherent limitations of working with Itch deficient mice, it was unclear whether this bias was the cause of the Itch inflammatory pathology or a consequence of it. The data provided in this chapter provide the first definitive evidence that Itch deficiency directly leads to a bias towards Th2 differentiation. Firstly, purified CD44lo CD4+ cells from four-week old Itch fl/fl CD4 Cre mice, which displayed no visible signs of inflammation, showed a bias towards Th2 differentiation. Secondly, *in vitro* deletion of Itch using cell permeable TAT-Cre also led to a bias towards Th2 differentiation. Combined, these data strongly argue that Itch is directly involved in modulating signals that regulate Th1 versus Th2 differentiation decisions. Furthermore, the data generated using TAT-Cre argue that the bias towards Th2 differentiation is not secondary to differences that may occur in the selection of Itch deficient thymocytes.

4.C.2 Itch modulation of Th2 differentiation is along the IL-4-Stat6 pathway:

There are numerous pathways that regulate Th2 differentiation. Broadly speaking, these include signals downstream of cytokine receptors, signals from the TCR, signals from co-stimulatory ligands, and signals involving the Notch pathway. The role of Stat6 in Th2 differentiation is well established for *in vitro* differentiation of wild-type cells; however, it is also clear that Notch signaling, constitutive Stat5 signaling, and the absence of CTLA-4 regulation of TCR signaling all lead to Stat6 independent Th2 differentiation *in vitro*. Itch deficiency does not modulate signaling along these

pathways, as Th2 differentiation in the absence of Itch is Stat6 dependent. This observation is particularly relevant for the Notch pathway, where data exists to support a role for Itch in negatively regulating Notch signaling (Qiu JBC 2000) and recently a role for the Notch pathway in regulating Th differentiation has been established (Maekawa, Tsukumo et al. 2003; Amsen, Blander et al. 2004). There is no data that speaks definitively on the connection between JunB and Stat6 in Th2 differentiation. Thus it is difficult to ascertain whether the increased JunB levels reported in Itch deficient mice are indeed the mechanism by which the bias towards Th2 differentiation occurs.

4.C.3 Stat6 dependence of inflammatory pathology seen in Itch deficient mice:

Although a bias towards Th2 differentiation was consistent with the Itch pathology, it remained unclear whether this bias was sufficient to cause the inflammatory phenotype seen in these mice. Indeed, recent data using an *in vitro* model system has suggested that Itch deficient T cells may have a more general problem with anergy induction (Heissmeyer, Macian et al. 2004). Analysis of Itch fl/fl CD4 Cre Stat6-/- mice provides data in support of a dysregulated Th2 immune response and speaks less favorably towards a general problem in tolerance. Itch fl/fl CD4 Cre Stat 6-/- mice have shown no visible signs of inflammation at the latest time points that have been assessed thus far (8 months). Histological analysis of six-month old mice revealed no acidophilic macrophage pneumonia in the lungs and no significant accumulation of lymphocytes in any organs. The lack of changes are not due to the mixed genetic background of these mice, as Itch fl/fl CD4 Cre Stat 6 +/+ from the same mixed genetic background show similar inflammation as C57Bl/6 Itch fl/fl CD4 Cre mice. Surprisingly, Itch fl/fl CD4 Cre Stat 6 +/- mice also showed reduced inflammation compared to

Itch fl/fl CD4 Cre Stat 6 +/+ controls. These data suggest that there may be a dosage effect for Stat 6 in Th2 differentiation

It is important to note that no novel inflammatory lesions or novel immunopathologies were seen in Itch fl/fl CD4 Cre Stat 6 -/- mice. If Itch deficiency caused a general problem with inappropriate activation of T cells, then crossing Itch fl/flCD4 Cre mice to Stat 6 -/- mice might have been expected to cause a severe autoimmune pathology, as activated T cells would have been forced to differentiate along a Th1 pathway. Examples exist of Th1 mediated pathologies that are exacerbated in Stat 6 deficient mice (Chitnis, Najafian et al. 2001; Wang, Ostlie et al. 2004). The fact that Itch fl/fl CD4 Cre Stat 6-/- mice seem healthy argues against a problem with general tolerance mechanisms.

So, is the inflammatory response in Itch deficient mice entirely due to the Th2 bias? While the data speak clearly in favor of a Th2 mediated pathology, it is presently unclear why these cells become activated Th2 effector cells. Although JNK1 deficient, MEKK7 deficient, and DR6 deficient CD4+ T cells all display a bias towards Th2 differentiation similar to or greater than the bias mediated by Itch deficiency, no accompanying pathology has been reported in these mice. Thus tolerance mechanisms specific to regulation of Th2 cells must be addressed.

4.C.4 Itch deficient CD4+ cells display increased antigen sensitivity:

At lower doses of titrated antigen, there was a clear increase in the sensitivity of Itch deficient purified CD44loCD4+ cells as compared to wild-type cells. These results can be interpreted in two different ways. If one takes the cell surface activation marker phenotype as a true reflection of the naïve status of these cells, then one would have to

conclude that Itch deficient cells are more sensitive to antigen. As Th2 differentiation occurs at the extremes of antigenic stimulation, this data could suggest that more CD4+ cells from Itch fl/fl CD4 Cre mice are stimulated sufficiently to favor Th2 differentiation. These data are also consistent with the data described above with Itch fl/fl CD4 Cre Stat 6 -/- mice, as signals from the TCR and IL-4 receptor need to be integrated for Th2 differentiation to occur.

The caveat that must be considered with this model of increased antigen sensitivity in Itch deficient CD4+ T cells is that cell surface marker expression may not necessarily reflect the activation status of a cell. The wide variation in the antigen sensitivity of CD44loCD4+ cells purified from Itch fl/fl CD4 Cre mice suggests that the populations that are being purified are not homogenous. This data could be interpreted to suggest that the variations in antigen sensitivity noted are reflective of the purity of the naïve Itch deficient CD4+ cells used for the assay. Conclusive data regarding changes in antigen threshold will come from Rag deficient TCR transgenic Itch fl/fl CD4 Cre mice, assuming that these mice do not develop spontaneous inflammation. CD4+ cells purified from Rag deficient TCR transgenic mice can be functionally defined as antigen inexperienced. Furthermore, the cells will be homogenous with regards to the TCR that is expressed on their surface. Therefore, proliferation analysis of CD4+ T cells from these mice will provide more decisive answers regarding the role of Itch in antigen sensitivity.

4.D Summary:

The results presented in this chapter of the thesis provide definitive evidence that Itch directly modulates Th1 versus Th2 differentiation decisions in naïve CD4+ T cells.

In addition, evidence is provided that this modulation occurs upstream of the Stat6 pathway in Th2 differentiation. Finally, genetic proof is provided for the importance of Th2 differentiation in the pathology of Itch mice. Combined, these data argue strongly that the pathology of Itch deficient mice is driven by a dysregulated Th2 immune response.

Chapter 5: The role of Itch in CD8+ memory cell generation

5.A Introduction:

5.A.1 CD8+ memory cells:

A critical difference between the adaptive and the innate immune systems is the generation of immunological memory during an immune response. Because of the importance of CD8+ cells in the generation of immune responses against intracellular pathogens, the generation of CD8+ memory cells is of key interest in the field of vaccination. Although the cellular events and cytokines involved in CD8+ memory cell formation have been elucidated, the molecular pathways that regulate CD8+ memory cell generation and maintenance remain poorly understood.

5.A.2. Factors regulating CD8+ memory cell generation

Many factors influence the generation of CD8+ memory cells. The amount of antigen encountered initially is important. CD8+ cells undergo a programmed proliferative response after antigen encounter, and the extent of proliferation is determined in part by the antigen dose (Ludewig, Ehl et al. 1998; Wherry, Puorro et al. 1999; Kedl, Rees et al. 2000; Kaech and Ahmed 2001). The number of memory CD8+ cells is reflective of the initial expansion (Hou, Hyland et al. 1994; Busch, Pilip et al. 1998). Part of the signal for generating memory cells comes from CD4+ cells, as in their absence CD8+ cells generate effector cells but not memory cells (Janssen, Lemmens et al. 2003; Shedlock and Shen 2003; Sun and Bevan 2003). Survival of memory precursors during the contraction phase following the proliferative phase is also important. After surviving the contraction phase, memory precursor cells continue to differentiate into memory cells. They become able to proliferate slowly. This proliferation is important

for homeostatic maintenance of memory CD8+ cells for the lifetime of the organism. Proliferation occurs in response to the cytokines IL-7 and IL-15 (Goldrath, Sivakumar et al. 2002; Kieper, Tan et al. 2002).

5.A.3 Aims of this study:

Conditional deletion of Itch using CD4-Cre leads to deletion of Itch at the double positive stage of thymocyte development. Therefore both CD4+ and CD8+ cells are Itch deficient in Itch fl/fl CD4 Cre mice. During the course of analysis of Itch fl/fl CD4 Cre mice, it was serendipitously noted that there was a dramatic increase in CD44hiCD8+ cells in Itch fl/fl CD4 Cre mice. Since the role of Itch in CD8+ cells has not been previously addressed, the aim of experiments described in this section was to provide evidence for a direct role for Itch in regulating CD8+ T cells.

5.B. Results:

5.B.1 Accumulation of memory-like CD8 cells in secondary lymphoid organs of 8-week old Itch fl/fl CD4 Cre mice.

Cell surface marker stainings of CD8+ cells from the spleen and lymph node of Itch fl/fl CD4 Cre mice showed a dramatic increase in the percentage of CD44hi CD8+ cells. Although CD44 upregulation can be a marker for activated cells, these cells were neither larger by forward scatter nor positive for other activation markers such as CD25, CD69, or CD62L (**Figure 5-1a,b**). These preliminary surface stainings suggested that there was an increase in memory-like CD8+ cells in Itch fl/fl CD4 Cre mice. Along with CD44 upregulation, CD122 (the β subunit of the IL-15 receptor) and Ly6C upregulation also occurs selectively on memory CD8+ cells. Surface staining of CD8+ cells with CD122 and Ly6C showed similar increases in their surface expression (**Figure 5-1a,b**).

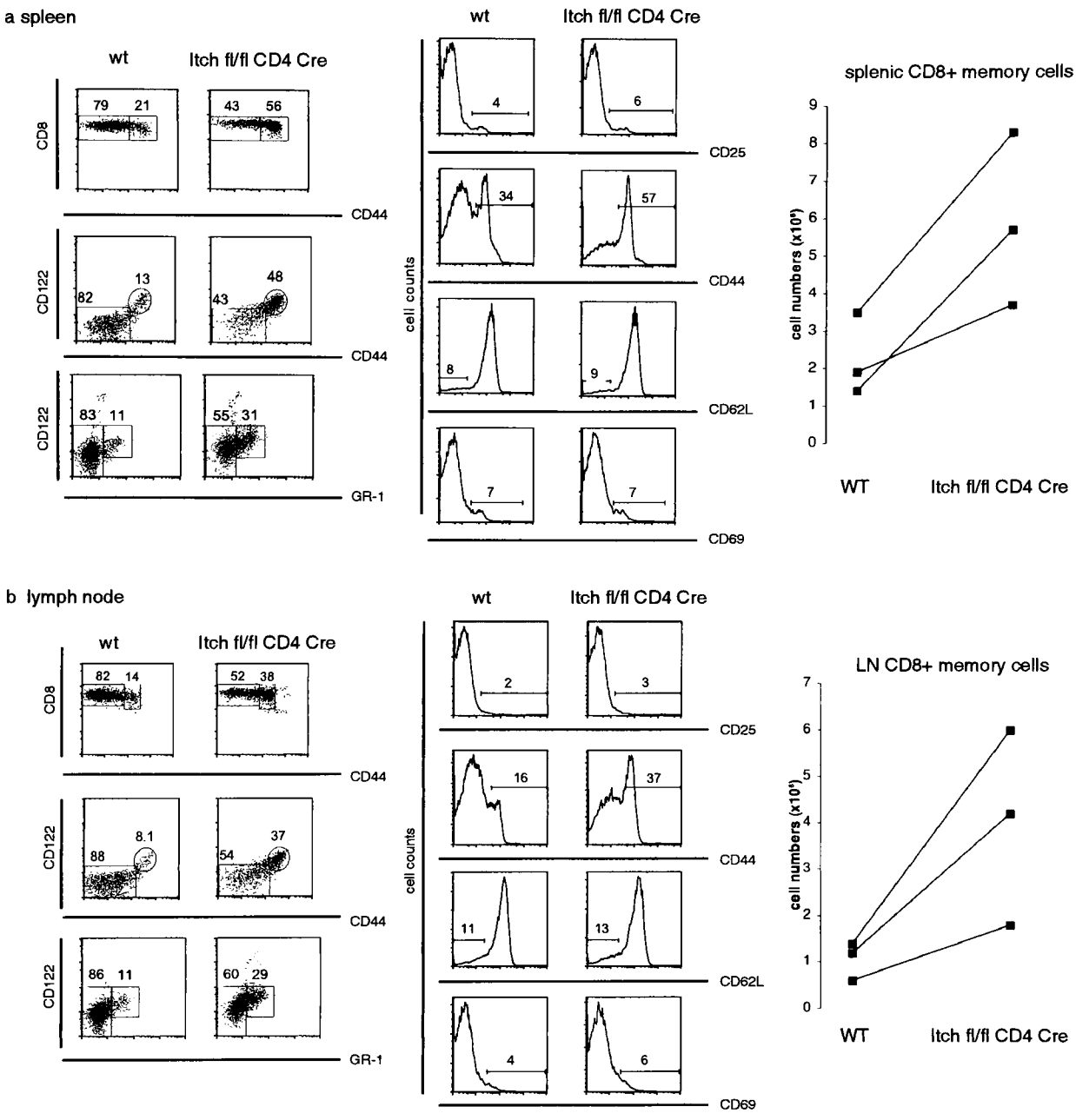


Figure 5-1. Expansion of CD8+ memory cell population in 8 week old Itch fl/fl CD4 Cre mice. **a.** spleen **b.** lymph nodes. **Left panel.** Flow cytometric analysis of lymphocyte populations. All stainings are gated on live CD8+ cells. Numbers indicate the percentage of cells falling within the gated subpopulation. **Middle panel.** Cell surface expression of activation markers in CD8+ cells. **Right panel.** Increase in absolute numbers of memory CD8+ cells in Itch fl/fl CD4 Cre mice. Absolute CD8+ cell numbers were calculated using absolute splenic and lymph node numbers and percentages of CD8+ lymphocytes in the spleen and lymph node. Each data point represents an independent experiment consisting of average cell counts of two Itch fl/fl CD4 Cre mice and two littermate controls.

Calculation and comparison of absolute numbers of memory-like CD8+ cells in spleenand lymph nodes of Itch fl/fl CD4 Cre mice and littermate controls reveals an average 1.6 fold increase in CD8+ cell numbers in both the spleen and lymph node of Itch fl/fl CD4 Cre mice, and a 4 fold increase in the absolute number of memory like-cells in both spleen and lymph nodes. Collectively, these data demonstrate a significant ($p < 0.001$ by student's t-test) accumulation of memory-like CD8+ cells in Itch fl/fl CD4 Cre mice as judged by surface marker staining.

5.B.2 Increased frequency of CD44hi CD8 SP cells in the thymi of Itch fl/fl CD4 Cre mice.

Normally, memory cells are generated in response to antigen in the periphery. In the absence of Itch, it was possible that the activation of CD8+ cells could occur prior to the cell encountering its cognate antigen in the periphery. Preliminary analysis of thymocyte development based on thymocyte population analysis and cell surface expression of CD3, CD5, CD69, and CD24 revealed no significant differences between wild-type and Itch fl/fl CD4 Cre mice (**Figure 5-2a,b**). To determine if Itch deficiency is sufficient to cause an increase in CD44 expression in the absence of cognate antigenic stimulation, recently positively selected CD4lo CD8lo double positive thymocytes, CD4 SP thymocytes and CD8+ SP thymocytes were stained for CD44. These stainings reveal that Itch fl/fl CD4 Cre have a small but significant increase in the percentage of CD44hi cells amongst recently positively selected

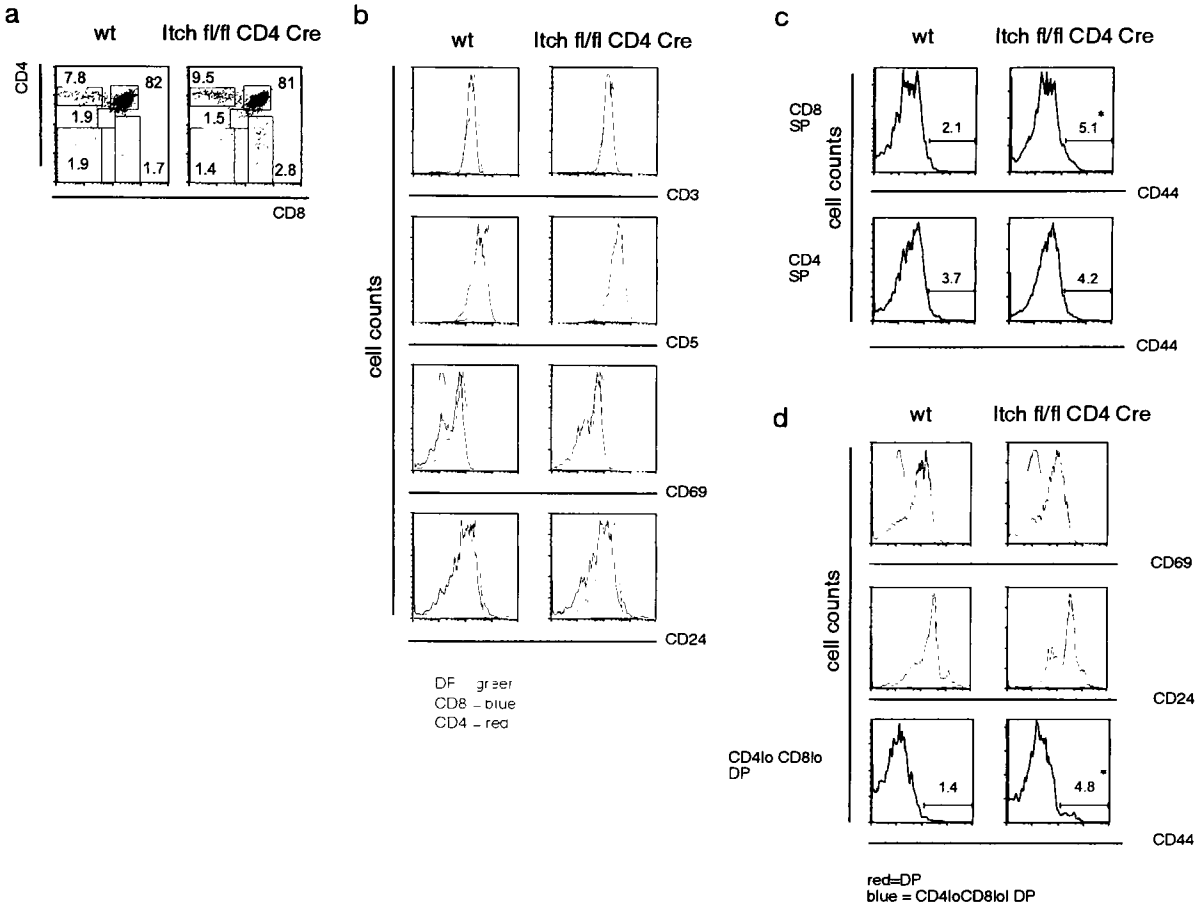


Figure 5-2. Normal T cell development but early CD8+ cell activation in Itch fl/fl CD4 Cre mice. Flow cytometric analysis of thymocyte populations. **a.** Percentages of Double Negative (DN), Double Positive (DP), CD4loCD8lo positively-selected Double Positive (CD4loCD8lo DP), CD4 Single Positive (CD4 SP), and CD8 Single Positive (CD8 SP) populations are equivalent in wild-type and Itch fl/fl CD4 Cre mice. **b.** Upregulation of cell surface expression of CD3, CD5, and CD69 and downregulation of CD24 during transition from DP to SP stages of development is equivalent between wild-type and Itch fl/fl CD4 Cre mice. **c.** Significant increase in frequency of CD44hi CD8 SP thymocytes in Itch fl/fl CD4 Cre mice. CD44 expression in CD8 SP (top panel) and CD4 SP (bottom panel). * denotes $p<0.01$ by student's t-test. The percentages of CD44hi CD4 SP cells is not significantly increased in Itch fl/fl CD4 Cre mice ($p=0.3$ by student's t-test). d. CD44 expression in recently positively selected CD4loCD8lo DP thymocytes is increased in Itch fl/fl CD4 Cre mice. Positive selection of CD4loCD8lo DP thymocytes was judged by CD69 upregulation and CD24 downregulation. Itchfl/fl CD4 Cre mice have a significant increase in the percentage of CD44hi CD4loCD8lo DP thymocytes (* $p<0.01$ by student's t-test). Stainings are representative of two independent experiments of three 6-8 week old mice.

CD4lo CD8lo double positive thymocytes and CD8+ SP thymocytes, but not among CD4 SP thymocytes (**Figure 5-2c,d**). These data are consistent with the hypothesis Itch deficiency modulates signaling such that CD8+ cells are activated in the thymus, prior to encountering their cognate antigen in the periphery.

5.B.3 The increased frequency of memory like CD8+ cells is independent of the inflammatory pathology in Itch fl/fl CD4 Cre mice

The cytokine milleu can play a role in the proliferation rate of memory like CD8+ cells. Naïve cells placed into a lymphopenic environment acquire a memory-like phenotype and proliferate. Furthermore, naïve cells from adult mice placed into a neonate also develop a memory phenotype and undergo proliferation as if they were in a lymphopenic environment (Ichii, Sakamoto et al. 2002), suggesting that the cytokine environment in neonates leads to expansion of memory like cells through a process potentially similar to a lymphopenic expansion. In particular, IL-15 has been shown to play a critical role in CD8+ memory cell proliferation (Zhang, Sun et al. 1998), and one of the main producers of IL-15 are macrophages (Kennedy and Park 1996). The accumulation of macrophages in Itch fl/fl CD4 Cre mice (Jonsson 2002) made it important to consider the possibility that the accumulation of memory phenotype CD8+ cells is secondary to the inflammatory disease in these mice.

Three pieces of evidence speak against this argument. Firstly, CD8+ memory cell accumulation is seen in two-week old Itch fl/fl CD4 Cre mice, prior to any visble activation of CD4+ T cells as judged by surface marker stainings (**Figure 5-3a**). Transfer studies have made it clear that CD4+ T cells initiate the inflammation in Itch fl/fl CD4 Cre mice. Secondly, Itch fl/fl CD4 Cre mice do not show an increase in NK

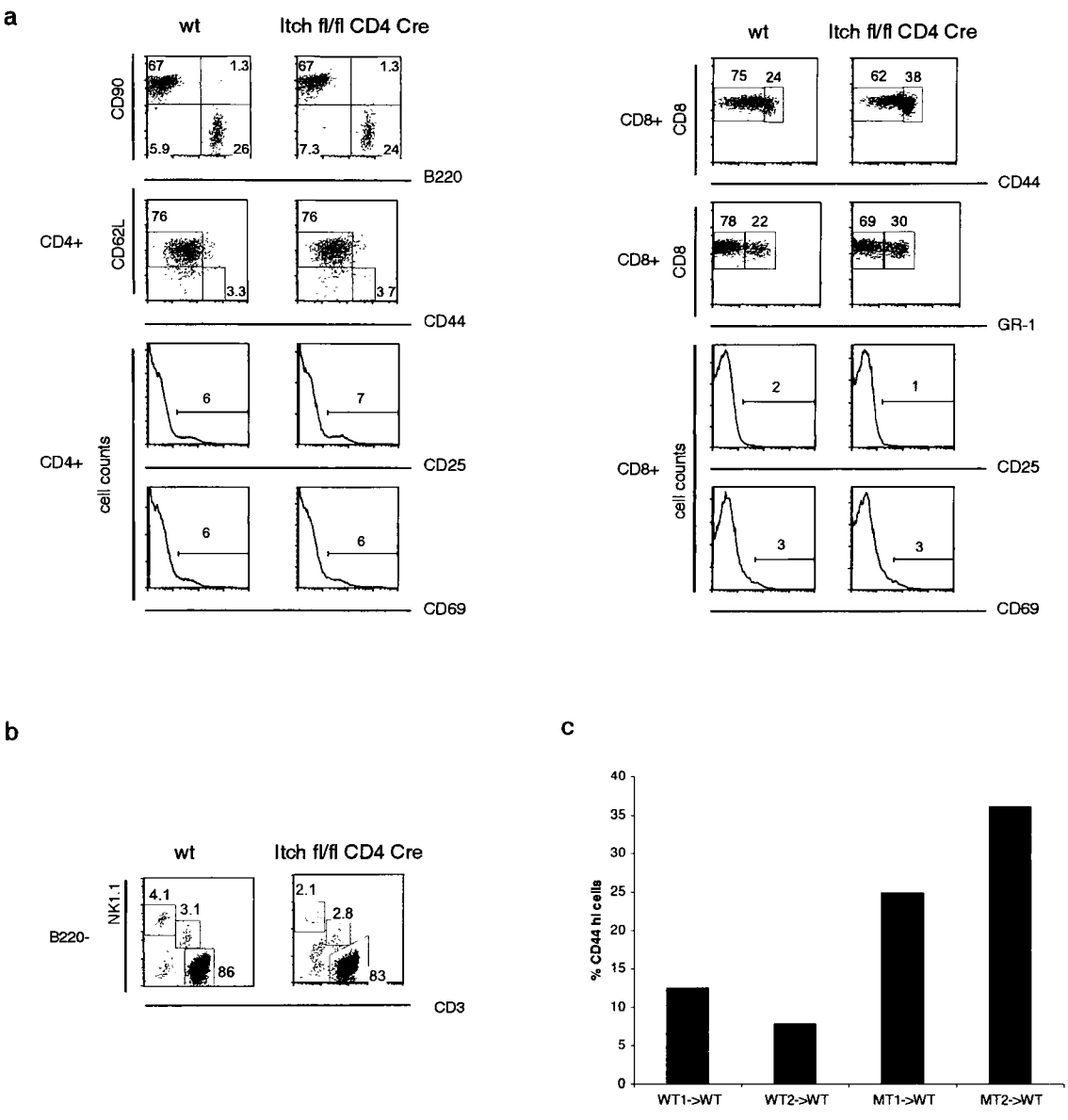


Figure 5-3. CD8+ memory-like cell accumulation in Itch deficient mice is independent of the Itch inflammatory pathology. **a.** Increased frequency of memory-like CD8+ cells is the first population change in Itch fl/fl CD4 Cre mice. Flow cytometric anlaysis of two week old Itch fl/fl CD4 Cre mice. B cell accumulation and CD4+ T cell activation is not evident **(left panel)** whereas memory-like CD8+ cell accumulation is evident **(right panel)** **b.** NK and NKT cell accumulation is unaffected in Itch fl/fl CD4 Cre mice **c.** Maintenance of Itch deficient CD44hi CD8+ cells in the periphery of wild-type mice. CFSE labeled Itch deficient (MT) or wild-type (WT) CD8+ SP cells were transferred into wild-type mice. Recipient mice were sacrificed four weeks after transfer and splenocytes were stained for surface expression of CD8 and CD44. The percentage of CD44hi CFSE labeled CD8+ cells is shown.

and NKT cells (**Figure 5-3b**), whose homeostatic proliferation is also controlled by IL-15 (Ranson, Vosshenrich et al. 2003); nor do they develop fatal leukemias like IL-15 transgenic mice (Fehniger, Suzuki et al. 2001). Finally, transferred CD8+ SP thymocytes from Itch fl/fl CD4 Cre mice accumulate as CD44hi transferred cells in wild-type recipients (**Figure 5-3c**).

5.B.4 Itch deficient memory-like cells CD8+ cells function like memory cells in response to IL-15 stimulation *in vitro.*

Although cell surface marker stainings suggested an accumulation of memory-like CD8+ cells in Itch fl/fl CD4 Cre mice, it remained a possibility that these cells were only "masquerading" as memory cells but had no functional properties of memory cells. In the absence of MHC I restricted TCR transgenic mice lacking Itch, it was impossible to track *in vivo* CD8+ memory cell generation in response to an antigen or measure memory cell function in secondary responses to an antigen. Therefore, proliferation in response to IL-15 was used as a functional readout of the memory like cells seen in Itch fl/fl CD4 Cre mice. CD44lo and CD44hi CD8+ cells were purified and stimulated with titrated concentrations of IL-15. As is the case for wild-type CD8+ cells, Itch deficient CD44lo CD8+ cells proliferated minimally in response to IL-15, whereas CD44hi cells proliferated vigorously (**Figure 5-4a**). Comparison of CD44lo and CD44hi proliferation rates from Itch fl/fl CD4 Cre mice to wild-type mice revealed a slight increase in the sensitivity of Itch deficient CD8+ cells to IL-15 *in vitro* (**Figure 5-4b**); however, this data was confounded by the fact that the purification procedure left more contaminating CD44lo cells in the CD44hi population of wild-type cells. Thus no conclusive arguments

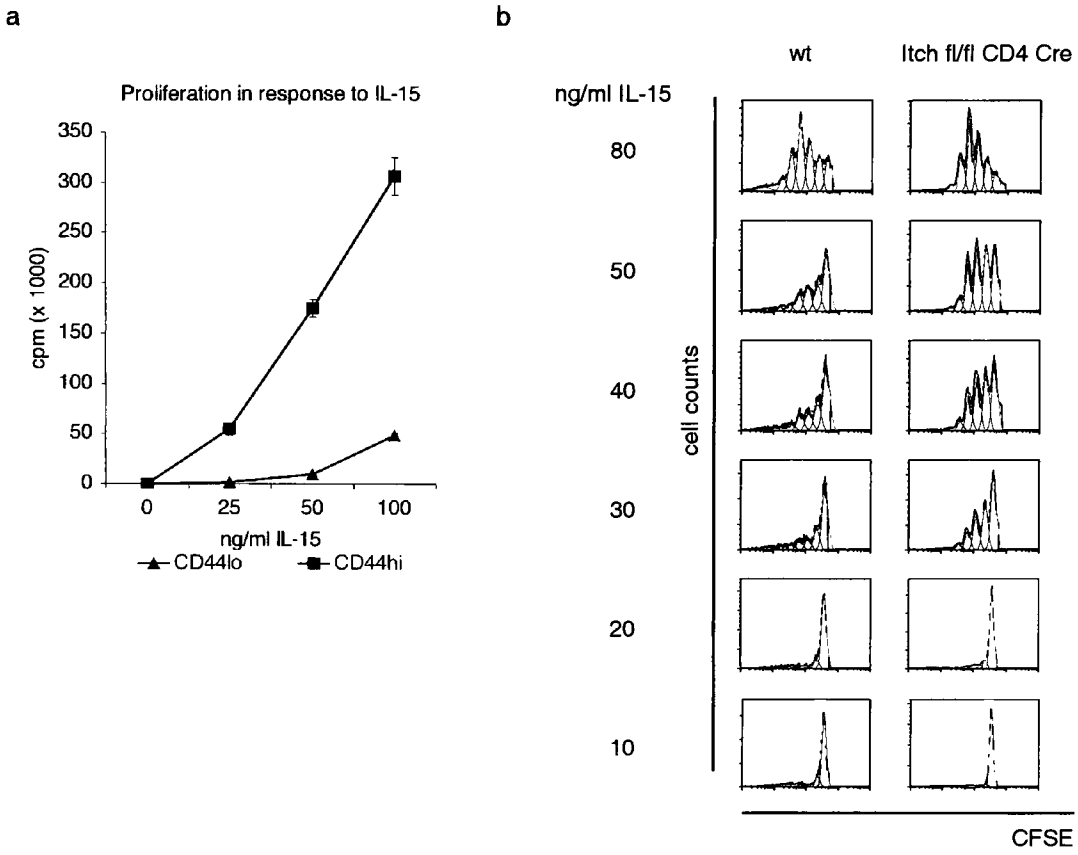


Figure 5-4. The memory-like CD8+ cells accumulating in Itch fl/fl CD4 Cre mice have functional properties of memory CD8 cells. **a.** Proliferation of CD44hi but not CD44lo CD8+ cells in response to IL-15. Purified CD44lo (traingle) and CD44 hi (square) CD8+ cells from Itch fl/fl CD4 Cre mice were incubated with indicated concentrations of recombinant IL-15 for three days. Proliferation was measured by [^{3}H] thymidine incorporation during the last eight hours of culture. Standard error of the mean of triplicates is provided. **b.** Equivalent proliferation of wild-type and Itch fl/fl CD4 Cre CD44hi CD8+ cells in response to titrated concentrations of IL-15. Purified and CFSE labeled cells were cultured for three days prior to flow cytometric analysis of proliferation. Results are representative of two independent experiments.

can be made about the sensitivity of Itch deficient CD8+ cells to IL-15 *in vitro*.

5.B.5 Itch deficient memory-like CD8+ cells have the same basal homeostatic proliferation rate as wild-type cells

To determine if the increased proliferation in response to IL-15 seen *in vitro* had any correlate *in vivo*, Itch fl/fl CD4 Cre mice and littermate controls were fed 0.8 mg/ml of the DNA precursor analog bromodeoxyuridine (BrdU) in their water for 10 days. Mice were then sacrificed and the percentage of BrdU positive cells was ascertained by intracellular staining. Concomittant staining of surface markers allowed for the analysis of proliferation rates of naïve and memory cells separately. Although Itch fl/fl CD4 Cre mice had an increase in the proliferation rate of CD8+ cells in total, no difference was seen between the memory and naïve compartments when compared to wild-type controls (**Figure 5-5**). The overall difference reflects simply the increased percentage of memory cells, which have a higher proliferative rate than naïve cells. These data show that there is no increase in the homeostatic proliferation rate of memory-like CD8+ cells in Itch fl/fl CD4 Cre mice.

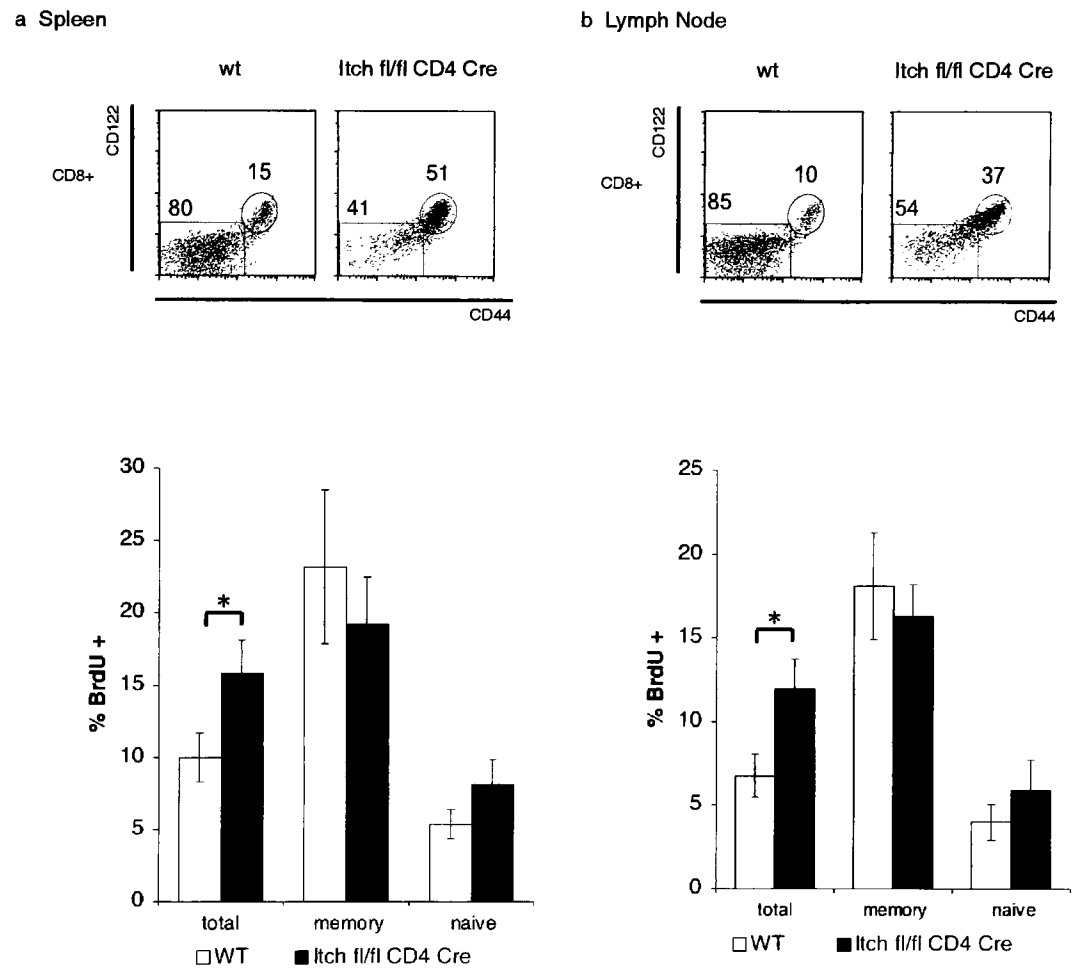


Figure 5-5. The rate of homeostatic proliferation of memory CD8+ cells is unaffected in Itch fl/fl CD4 Cre mice. **a.** Spleen **b.** Lymph nodes. Mice were given 0.8 mg/ml of the DNA analog bromodeoxyuridine (BrdU) in their drinking water for ten days. The percentage of cells that proliferated in this time period was assessed by flow cytometric analysis of BrdU incorporation. **Top panel.** Flow cytometric analysis of naive (CD44loCD122lo) and memory (CD44hiCD122hi) CD8+ subpopulations in wild-type and Itch fl/fl CD4 Cre mice given BrdU. Numbers indicate the percentage of cells falling in the gated subpopulations. **Bottom panel.** BrdU incorporation in total CD8+, naïve CD8+, and memory CD8+ of wild-type (open bars) and Itch fl/fl CD4 Cre (closed bars) mice is shown. Four mice were used per experimental group. Error bars denote the standard deviation of the mean of groups. *The percentage of proliferating CD8+ cells is significantly increased in both the spleen and lymph nodes of Itch fl/fl CD4 Cre mice ($p<0.01$ by student's student's t-test). No difference was observed in the proliferation rate of memory CD8 cells.

5.B.7 Itch does not regulate antigen sensitivity in CD8+ cells

The generation of memory cells is dependent on the initial proliferation of naïve cells. If more naïve cells were triggered in Itch fl/fl CD4 Cre mice, then presumably the number of memory CD8+ cells would also be increased. To address the role of antigen sensitivity in the extent of proliferation and hence memory cell formation in Itch fl/fl CD4 Cre mice, CD44lo naïve CD8+ cells were purified from Itch fl/fl CD4 Cre mice and littermate controls and stimulated with titrated doses of plate bound α-CD3 antibody or plate bound α -CD3 and α -CD28 antibodies. Antigen sensitivity was assessed by proliferation (**Figure 5-6**). No difference was seen between naïve WT and Itch deficient CD8+ cells. These data are consistent with the hypothesis that Itch does not modulate antigen sensitivity in CD8+ cells.

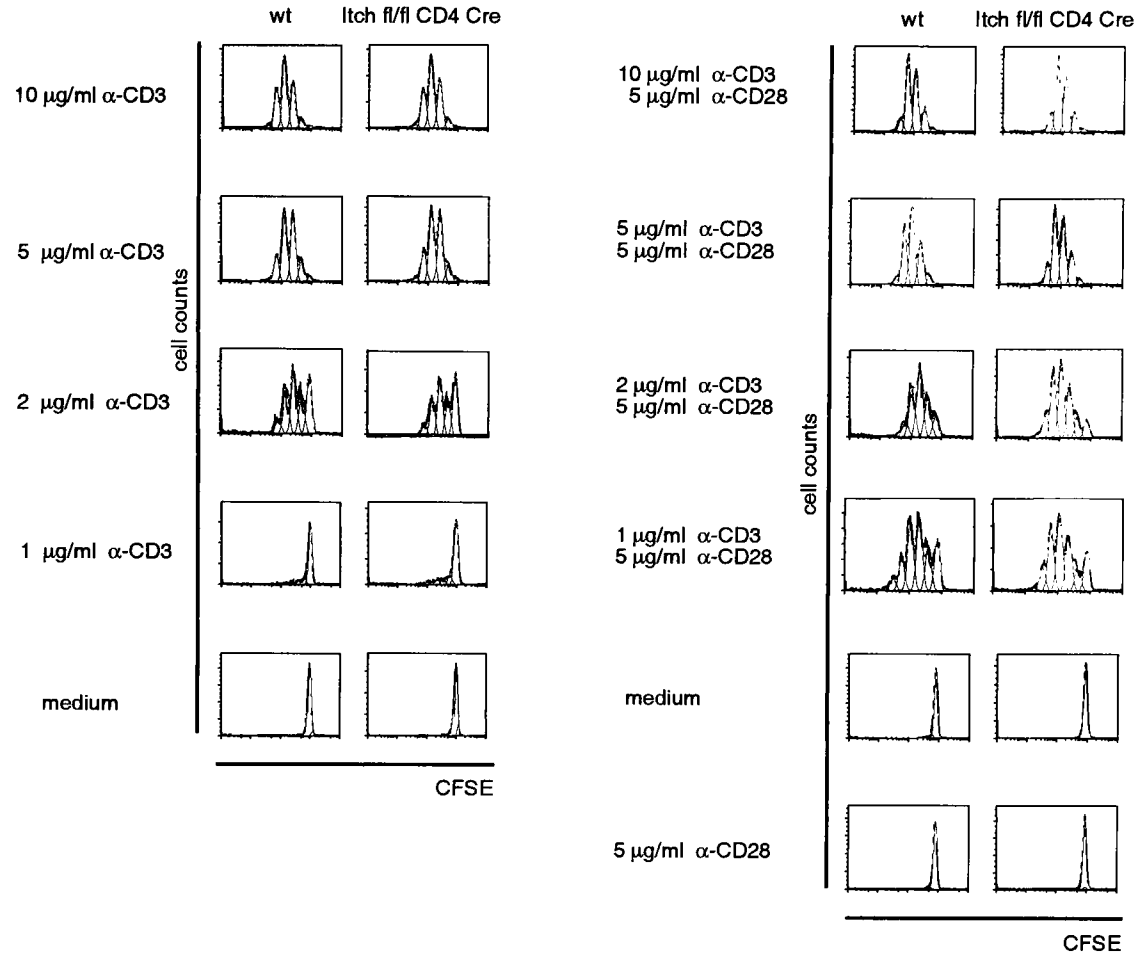


Figure 5-6. Proliferative capacity in response to antigenic stimulation is unaffected in Itch deficient CD8+ cells. Purified CD44lo naïve CD8+ were labeled with CFSE and incubated with indicated concentrations of plate-bound anti-CD3ε (left panel) or plate-bound anti-CD3ε and anti-CD28 (right panel) antibodies. Flow cytometric analysis of CFSE dilution upon proliferation was performed after three days of culture. The results are representative of two independent experiments.

5.C Discussion:

5.C.1 Accumulation of memory-like CD8+ cells in the secondary lymphoid organs of Itch fl/fl CD4 Cre mice:

Accumulation of $CD25^{-}CD62LhiCD69^{-}CD44hiCD122hiLy6C+$ memory-like CD8+ cells occurred in both the spleen and lymph nodes of Itch fl/fl CD4 Cre mice. Itch is expressed at similar levels between CD4+ and CD8+ cells and Itch conditional deletion using CD4 Cre leads to its deletion in both T cell subpopulations. Thus, it was possible that Itch played an as yet unidentified role in regulating CD8+ memory cell generation. The expression of CD62L and the accumulation of these memory-like cells in the lymph nodes as well as in the spleen suggest that these are central memory cells. Although these memory-like cells phenotypically resemble CD8+ memory cells and even have functional attributes of CD8+ memory cells, their definitive description as memory CD8+ cells will require crossing Itch fl/fl CD4 Cre mice to an MHC I restricted TCR transgenic background and infection of these mice so that memory responses can be traced. An ideal TCR transgene to cross these mice to is P14, which encodes a MHC I restricted TCR specific for an epitope of the LCMV virus (Pircher, Burki et al. 1989). Numerous CD8+ memory studies have been performed using this system, making it an ideal model system for defining the function of Itch.

One can invisage two potential outcomes from this cross. In one scenario, TCR transgenic mice will have no CD8+ memory cells, which will suggest that Itch regulates memory cell formation through an antigen-dependent manner. Infection of mice will provide insight into which aspect of CD8+ memory cell generation is affected in Itch deficient mice.

A second scenario is that Itch fl/fl CD4 Cre mice will continue to accumulate CD8+ memory like cells even in the absence of cognate antigen stimulation. This result will imply that Itch regulates CD8+ memory cell generation in a TCR independent manner, perhaps through cytokine signaling. The role of Itch in regulating memory responses can still be studied by adoptively transferring CD44lo naïve TCR transgenic CD8+ cells into recipient hosts before viral infection. Of more interest will be determining which cytokine is responsible for the TCR independent memory cell accumulation in the absence of Itch. The two cytokines that are critically important for memory CD8+ cell generation are IL-7 and IL-15. Both may play a role in homeostatic proliferation, but IL-7 is also critical for the survival of memory-precursor cells. Since Itch has been implicated in regulating the protein levels of the IL-7 receptor in B cells (Liu 2004), it is an important target to test in Itch deficient CD8+ cells.

Recently, a role for CD4 cells in CD8+ memory cell generation has been described. The requirement for CD4 cells in the accumulation of CD8+ memory like cells in Itch fl/fl CD4 Cre mice could be addressed by the analysis of Itch fl/fl CD4Cre MHC II-/- mice. Breedings to generate these mice are currently underway.

5.C.2 Itch deficiency leads to upregulation of CD44 on CD8 SP cells in the thymus:

Stainings of thymi from Itch fl/fl CD4 Cre mice reveal a small but significant increase in the number of CD44hi CD8+ single positive (SP) cells in the thymus. These data suggest that CD8+ cells are being activated inappropriately soon after their generation. Further multi-color stainings are required to deterimine if these CD44hi cells have also upregulated other memory cell markers such as CD122 and Ly6C or other activation markers such as CD25. Already, this data is consistent with Itch modulating

CD8+ cell development prior to the cells encountering antigen in the periphery. Experiments to determine which cytokine is responsible for this activation will be difficult to design. One possible approach would be to look at signaling events downstream of the IL-15 or IL-7 receptor. Changes in signaling thresholds for these cytokines would lead to memory cell accumulation, and perhaps biochemical analysis of signaling pathways downstream of the cytokine receptors could shed light on the role of Itch in CD8+ memory cell generation In particular, Stat5 signaling should be considered, as it is involved in both CD4+ Th2 differerntiation and CD8+ memory cell generation.

5.C.3 Accumulation of Itch deficient memory-like CD8+ cells is independent of the inflammatory pathology in Itch fl/fl CD4 Cre mice:

Although B cells accumulate in Itch fl/fl CD4 Cre mice, it is clear that this accumulation is secondary to the inflammatory pathology initiated in these mice by CD4+ T cells. The accumulation of memory-like CD8+ T cells could also have been secondary to the Itch pathology. Two pieces of data argue against this hypothesis. Firstly, the accumulation of memory-like CD8+ cells is seen in two week old mice, when neither splenomegaly not CD4+ T cell activation as assessed by surface marker staining is evident. Secondly, transfer of Itch deficient CD8+ single positive cells into wild-type mice leads to an accumulation of CD44hi CD8+ cells even in the periphery of wild-type mice. Thus, it seems that the accumulation of memory-like Itch deficient CD8+ cells is independent of the Itch pathology. Ofcourse, the cytokine environment in the thymus of Itch fl/fl CD4 Cre mice may be altered such that it leads to CD8+ cell activation. Definitive proof that Itch has a direct role in regulating CD8+ memory cells independent of its function in CD4+ T cells requires crossing of Itch deficient mice to a CD8+ T cell specific Cre. Unfortunately, such a Cre transgene does not yet exist.

5.C.4 Itch deficient memory-like CD8+ cells behave like memory CD8+ cells

Although Itch fl/fl CD4 Cre mice accumulated CD8+ cells that looked like memory cells by surface phenotype, proof that they behaved like memory CD8+ cells was missing. In the absence of an *in vivo* model system to study CD8+ memory cell generation, *in vitro* proliferation in response to IL-15 was chosen as a functional read-out. It has been shown that memory CD8+ cells are unique in their ability to proliferate in response to IL-15 (Zhang, Sun et al. 1998). Purification of Itch deficient CD44lo and CD44hi CD8+ cells and culture in the presence of IL-15 demonstrated that Itch deficient CD44hi CD8+ cells proliferated in response to IL-15, whereas CD44lo cells did not. Furthermore, comparison of proliferation rates between wild-type and Itch deficient CD44hi cells revealed that Itch deficient cells were as sensitive if not more sensitive *in vitro*. Thus the memory-like CD8+ cells accumulating in Itch fl/fl CD4 Cre mice have memory CD8+ cell functional characteristics as well as phenotypic similarities.

5.C.5 CD8+ memory cell homeostatic proliferation is unaffected in Itch fl/fl CD4 Cre mice:

One potential mechanism by which CD8+ memory cells might accumulate in Itch fl/fl CD4 Cre mice is through an increased rate of homoestatic proliferation. This hypothesis was consistent with the increased proliferation rate of Itch deficient CD44hi CD8+ cells in response to IL-15 *in vitro*. A potential caveat in the interpretation of the *in vitro* data was the consistent contamination of a higher percentage of CD44lo cells in the CD44 hi population of wild-type cells. To determine if there was an increased rate of homeostatic proliferation of memory CD8+ cells in Itch fl/fl CD4 Cre mice *in vivo*, mice were given drinking water containing the DNA analog Bromodeoxyuridine (BrdU) for ten days, and incorporation of this analog in proliferating cells was assessed. As has been

previously published, approximately 15% of memory CD8+ cells undergo proliferation in this time period. There was no difference between Itch fl/fl CD4 Cre and wild-type memory CD8+ cells in the percentage of BrdU+ cells, suggesting that the proliferation rate is equivalent. Since all existing evidence suggests that the homeostatic proliferation rate is controlled predominantly by IL-15, these data argue that there is neither an increase in the levels of IL-15 in Itch fl/fl CD4 Cre mice nor an increase in the sensitivity to IL-15 of Itch deficient CD8+ cells.

5.C.6 Antigen sensitivity is unaffected by Itch deficiency in CD8+ cells:

Since the number of memory cells generated is dependent on the initial proliferation burst, it seemed plausible that the accumulation of memory-like CD8+ cells in Itch fl/fl CD4 Cre mice was due to an increase in the antigen sensitivity of Itch deficient CD8+ cells. In order to address this hypothesis naïve CD44lo CD8+ cells were purified from wild-type and Itch fl/fl CD4 Cre mice and proliferation in response to titrated concentrations of plate-bound antibodies was assessed. No difference was seen in antigen sensitivity, suggesting that the accumulation of memory-like CD8+ cells is not due to an increase in antigen sensitivity. One potential caveat of this experiment is that purification of CD44lo cells from Itch fl/fl CD4 Cre mice may select for only those cells whose antigen sensitivity was not affected such that they did not proliferate and eventually give rise to memory CD8+ cells. One way to circument this problem would be to delete Itch *in vitro* using cell permeable TAT-Cre and then assess proliferation.

5.D Summary:

The data provided in this chapter of the thesis provide preliminary evidence in favor of a role for the E3 ligase Itch in regulating CD8+ memory cell generation. Collectively these data show that Itch fl/fl CD4 Cre mice have an accumulation of memory-like CD8+ cells, both by phenotype and by function. Furthermore, this accumulation is shown to be independent of the Itch inflammatory pathology. Antigen sensitivity of naïve CD8+ cells and homeostatic proliferation of memory CD8+ cells were found to be equivalent to wild-type in Itch fl/fl CD4 Cre mice. However, accumulation was found to begin in the thymi of Itch fl/fl CD4 Cre mice, shortly after positive selection. These data can be interpreted to suggest two different mechanisms for the accumulation of memory like CD8+ cells. The first is that this accumulation is cytokine driven, perhaps through increased IL-7 sensitivity. The second is that antigen sensitivity may be modified such that upon positive selection CD8+ T cells are becoming activated and acquiring memory like phenotypes.

Chapter 6: Discussion

6.A Itch fl/fl CD4 Cre mice develop a spontaneous inflammatory disorder:

The study of spontaneously occurring inflammatory disorders in mice is considered of particular clinical significance because they resemble idiopathic inflammatory diseases seen in humans (at least in their idiopathic nature). In the case of the E3 ligase Itch, this approach is made all the more appealing based on the highly polymorphic nature of the Itch gene (Jonsson 2002). It would be of great interest to see if Itch polymorphisms within the human population correlate with atopic diseases.

The spontaneous inflammatory disease seen in Itch fl/fl CD4 Cre mice could be explained by a number of models. The least exciting of these is that Itch regulates T cell activation in the most general way possible, such that in the absence of Itch, CD4+ T cells are spontaneously activated. This hypothesis is less appealing to immunologists because it brings the regulation of CD4+ T cells to questions of cell biology rather than systems regulation. Fortunately, the role of Itch in immune response regulation is not so blunt. Analysis of TCR transgenic mice has revealed that TCR transgenic CD4+ T cells are not spontaneously activated, at least as judged by cell surface activation marker analysis. This suggests that Itch regulates immune responses in response to antigens. In addition, the location of the inflammation seen in Itch deficient mice, which occurs along mucosal linings, suggests that Itch may be involved in regulating immune responses to external antigens. Further analysis of Itch deficient mice could provide insight into the physiological mechanisms involved in regulating local inflammatory responses in the absence of pathogens.

The inflammatory phenotype in Itch deficient mice suggested that Itch could also be involved in general processes such as CD4+CD25+ regulatory T cell function. The experiments provided in this thesis also speak against such a role for Itch. At least by *in vitro* assays, no functional defect in CD4+CD25+ regulatory T cells was found. This is consistent with the differences in phenotypes observed between mice deficient in CD4+CD25+ regulatory T cells, which develop multi-organ autoimmune diseases, and mice deficient in Itch, which display inflammatory infiltrates along their mucosal linings.

Itch deficient CD4+ T cells display problems with tolerance induced in response to an antigen. Induction of tolerant CD4+ T cells in response oral tolerization with ovalubmin failed in Itch fl/fl CD4 Cre DO11.10 mice. In addition, Itch deficient CD4+ T cells show decreased sensitivity to TGF-β , a cytokine which has been linked to induced tolerance. Surprisingly, the decrease in sensitivity to TGF-β seemed to be more due to changes in the sensitivity of activated Itch deficient CD4+ cells rather than naïve Itch deficient CD4+ T cells. Combined these data may argue that Itch regulates the extent of immune responses once an antigen is encountered.

6.B Dysregulated Th2 immune response in Itch fl/fl CD4 Cre mice:

Are these problems with peripheral tolerance mechanisms general, or are they more specific? Intriguingly, it seems that Itch fl/fl CD4 Cre mice have only a dysregulated Th2 immune response. Analysis of CD4+ T cells in which Itch was deleted *in vitro* confirmed that Itch deficiency does indeed affect Th2 differentiation. It should be noted, though, that these data and data from naïve Itch deficient CD4+T cells reveal a significant but small increase in the total number of Th2 effector cells, at least *in vitro*. Similar, if not greater, changes in Th2 bias have been reported for DR6 or JNK1 deficient

mice, but no inflammatory pathology has been published for these mice. Overall, these data argue that Itch must regulate more than Th2 differentiation in order to lead to the spontaneous inflammatory pathology seen in Itch deficient mice.

Analysis of Itch fl/fl CD4 Cre Stat 6-/- mice revealed that in the absence of Stat6, Itch deficient CD4+ T cells cannot differentiate into Th2 cells *in vitro*. Furthermore, analysis of Itch fl/fl CD4 Cre Stat 6 -/- mice revealed that the inflammatory phentoype of Itch fl/fl CD4 Cre mice disappeared in the absence of Stat6. Firstly, these data argue strongly that the inflammatory pathology was Th2 driven. Secondly, they argue that Itch deficient T cells are not generally activated. If the Th2 bias were a marginal side issue in Itch fl/fl CD4 Cre mice, and if the underlying reason for the inflammatory disorder were a universal problem in peripheral tolerance, then one might have expected manifestations of auto-immune disease in Itch fl/fl CD4 Cre mice. Since Th1 and Th2 differentiation are reciprocally regulated, an inability to generate Th2 effector cells would have led to Th1 effector cells in these mice. It is widely accepted that dysregulated Th1 immune responses mediated auto-immune diseases while dysregulated Th2 responses lead to inflammatory disorders of an atopic nature. Therefore, one might have expected that Itch fl/fl CD4 Cre Stat 6 -/- mice would develop a sever auto-immune disease. This was not the case. Combined, these data argue that if there is a problem with tolerance mechanisms in Itch fl/fl CD4 Cre mice, it is restricted to tolerance of Th2 effector cells.

Definitive proof regarding the role of Itch in regulation of Th2 effector responses requires the generation of TCR transgenic Rag deficient Itch fl/fl CD4 Cre mice. These mice, which presumably will contain naïve Itch deficient CD4+ T cells, can be analyzed for Th1 and Th2 immune responses generated *in vivo*. It is the author's prediction that

immunization with antigen favoring Th1 differentiation may lead to normal immune responses, assuming that Itch deficient mice don't generate Th2 effector cells under Th1 favoring conditions. In contrast, immunization with antigen that favors Th2 differentiation will lead to an unregulated inflammatory response.

The study of peripheral tolerance mechanisms that are induced upon antigen exposure and are specific to either Th1 or Th2 effector cells is a relatively new concept in immunology (Akbari, Stock et al. 2003). The ability to regulate specific effector responses without affecting others would be of considerable clinical interest, as many diseases are due to dysregulation of either Th1 or Th2 immune responses. Skewing immune responses towards the opposite effector phenotype has been attempted in the past, but brings with it the risk of causing different pathologies. The ability to specifically regulate Th1 or Th2 effector responses would provide a more controlled regulation of the immune system.

6.C The role of Itch in CD8+ memory cell generation:

Serendipitously, it was noticed that Itch fl/fl CD4 Cre mice have an increase in the number of CD44hiCD122hiLy6C+ CD8+ memory-like cells in their secondary lymphoid organs. Although Itch is expressed in CD8+ cells, its role in CD8+ cell function has previously gone unstudied. The experiments described in this thesis attempt to lay the groundwork for further studies about the role of Itch in CD8+ memory cell generation. These data suggest that Itch deficient CD8+ memory cells accumulate not because of the pathology in Itch fl/fl CD4 Cre mice but because of an inherent difference in Itch deficient CD8+ cells. The rate of homeostatic proliferation of Itch deficient CD8+

memory-like cells in the periphery was found to be unaffected, suggesting that Itch does not regulate CD8+ memory cell homeostasis once they are formed. Instead, Itch may regulate the generation of memory-like CD8+ cells. Consistent with this hypothesis is the finding that an increase in CD44hiCD8+ was observed as soon as positive selection occurs in the thymus. The exact mechanims by which Itch regulates the CD8+ memory like formation requires analysis of MHC I TCR transgenic mice, in which antigen dependence of this process will be tested, and MHC II deficient mice, in which the requirement for CD4+ help will be tested. It is hoped that by discovering the mechanisms by which Itch modulates CD8+ memory cell generation, further insight into the general mechanisms whereby CD8+ memory cells are formed will be provided.

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