Conditional inactivation of the ubiquitin protein ligase Itch of the mouse

Inaugural-Dissertation
zur
Erlangung des Doktorgrades
der Mathematisch-Naturwissenschaftlichen Fakultät
der Universität zu Köln

vorgelegt von
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September 2002

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Tag der müntlichen Prüfung: 25 November 2002

To my parents Annagreta och Börge,
whose love and support made this work possible

A. Introduction

Incorrect activation or lack of regulation of signaling cascades in the immune system can induce severe immune disease, such as autoimmunity and inflammatory diseases (for review see (Strober et al., 2002). It has for example, been observed that mice lacking PD-1, an immunoinhibitory receptor, develop autoimmune disorders characterized by production of high titers of autoantibodies (Freeman et al., 2000). In addition, transgenic mice expressing the T cell costimmulatory molecule CD40L, develop a chronic inflammatory disease (Clegg et al., 1997).

Signaling pathways are complex networks of proteins, where biological functions can be regulated by different mechanisms, examples are activation and deactivation of proteins by phoshorylation/dephosphorylation, restriction of protein localization to promote or inhibit protein interactions as well as complete abolishment of activated proteins by degradation. A good example of this is signaling regulation of the transcription factor nuclear factor B (NF B), which involves all these three mechanisms. NF B is inactive when bound to the inhibitors of NF B (I B) in the cytosol. Phosphorylation of I B leads to its ubiquitination and degradation in the proteasome, thereby releasing NF B which can be translocated to the nucleus where it can act in transcription (Chen et al., 1995; DiDonato et al., 1996; Li et al., 1995).

Duration of signaling pathways are normally tightly controlled by a negative feedback loop. In its simplest form a signal induces its own negative regulator leading to a decrease of signaling after the signal has reached a threshold. An example of this is the control of cytokine signaling through the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway, where the intracellular protein tyrosine kinases, JAKs, are recruited to the cytokine receptors and transduce the signal through STATs. This signaling induces the expression of suppressors of cytokine signaling-1 (SOCS-1), which inturn interacts with the receptor-bound activated JAKs, resulting in inhibition of tyrosine kinase activity (Endo et al., 1997; Naka et al., 1997; Nicholson et al., 1999). In addition, SOCS-1 has been shown to interact with the ubiquitination machinery and is thought to target associated signaling molecules to proteasomal degradation (Kamura et al., 1998; Zhang et al., 1999).

A.1. Regulation of protein level by degradation

Degradation of cellular proteins is an important process to control the levels of specific proteins as well as to eliminate damaged or misfolded proteins. There are two main pathways of protein degradation in the cell, lysosomal degradation of mainly membranal proteins and degradation by proteasomes of polyubiquitinated intracellular proteins. Polyubiquitination of substrates is usually needed for recognition by the proteasome, while mono- or di-ubiquitinated proteins can be recognized by and directed to the endocytotic pathway reviewed in (Dunn and Hicke, 2001; Hicke, 1999; Hicke, 1997; Lemmon and Traub, 2000).

Poly-ubiquitinated substrates, which are recognized and degraded in the proteasome, include cell cycle regulators as M-phase cyclins and G1 cyclins (King et al., 1994; Won and Reed, 1996), transcription factors such as, STAT1 (Kim and Maniatis, 1996) and c-Jun (Treier et al., 1994), signal transducers such as Protein kinase C (Lee et al., 1996), and protein tyrosin kinase Src (Hakak and Martin, 1999; Harris et al., 1999) as well as abnormal proteins, which have to be removed from the cells. This pathway is also involved in the regulation of signaling cascades by degradation of inhibitory proteins as observed for the activation of the NF□B transcription factor, upon I□B-□ ubiquitination and degradation (Chen et al., 1995; DiDonato et al., 1996; Li et al., 1995).

Identified ubiquitinated substrates degraded in the lysosome are receptors or receptor subunits such as, platelet-derived Growth factor □-receptor (PDGF □-receptor), (Mori et al., 1993). Thus, ubiquitin-mediated degradation of proteins plays an important role in regulation of signal transduction and in contrast to other mechanisms, for example phosphorylation/dephosphorylation, leads to an irreversible end of the signaling cascade.

A.2. The ubiquitin-proteasome degradation pathway

Ubiquitin-dependent degradation of cellular proteins operates as a two step mechanism. The first step includes attachment of an ubiquitin molecule, a highly conserved 76-amino acid polypeptide, to a substrate. Ubiquitin can then serve as its own substrate for ubiquitin conjugation (ubiquitination), which results in the formation of polyubiquitin chains. The second step of the process includes recognition of the polyubiquitinated protein and its degradation by the 26S proteasome, or internalisation and degradation by the lysosomal pathway, as has been shown for some monoubiquitination membranal proteins, for review see (Dunn and Hicke, 2001; Hicke, 1999; Lemmon and Traub, 2000). Ubiquitination of cellular proteins is a highly regulated process (Fig.1). In this process, ubiquitin, is bound to an ubiquitin activating enzyme also called E1, in an ATP- dependent process resulting in a high energy thiol ester bond between a cysteine in the E1 and the ubiquitin molecule. In the next step of the cascade, the ubiquitin molecule is transferred to an ubiquitin conjugating enzyme also called ubiquitin carrier protein or E2, where it is covalently linked to a conserved cysteine in the E2. Finally, the ubiquitin is transferred to the substrate molecule with the help of a third class of enzymes, the ubiqitin protein ligases or E3s (Hershko and Ciechanover, 1998; Hershko and Ciechanover, 1992; Jentsch, 1992). Ubiquitin can then serve as a substrate itself in the formation of polyubiquitin chains, possibly with the aid of an E4 (Koegl et al., 1999). The ubiquitin system appear to be a hierarchic system with only one functional E1 known so far, several E2s (between 20-30 estimated in mammals) and a large number of E3s (more then hundered revealed by database searches).

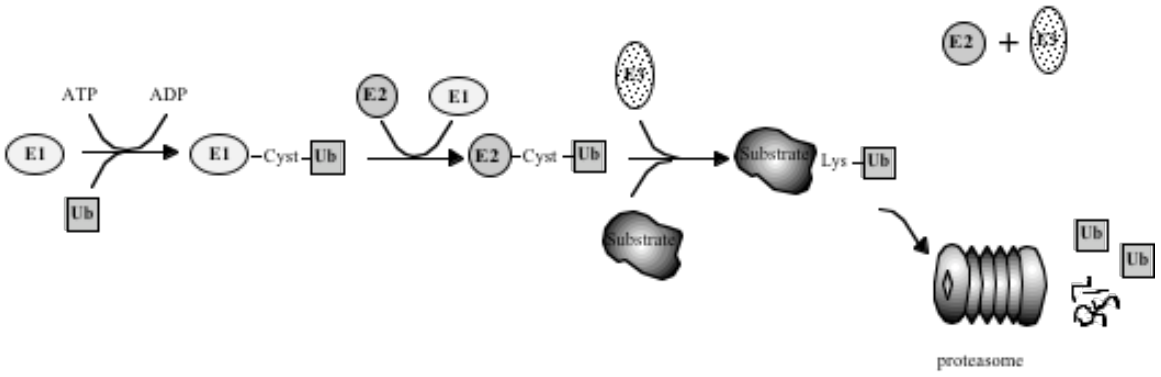


Figure 1. The ubiquitination pathway.

Shown is the specific enzymatic reaction of ubiquitin (Ub) conjugation to a substrate molecule, which involves three types of enzymes, the ubiquitin-activating enzyme (E1), the ubiquitin-conjugating enzyme (E2) and the ubiquitin-protein ligase (E3). The conjugation of Ub to a cysteine in the E1 is an ATP dependent processs, the Ub is then transferred to a conserved cysteine in the E2 and, finally, in interaction with an E3, the Ub is transferred to lysine residues in the substrate molecule. After polyubiquitination, the substrate is degraded in the proteasome leaving Ub and peptides as end products.

A.3. Ubiquitin protein ligases (E3)

E3s have loosely been defined as proteins participating together with E1 and E2s in the ubiquitination of proteins, which cannot be recognized and ubiquitinated by E2 alone (Ciechanover, 1994; Hershko and Ciechanover, 1992). A common feature of all known E3s is that each E3 appears to specifically interact with distinct E2s or distinct subsets of E2s (Feldman et al., 1997; Huibregtse et al., 1995; Kumar et al., 1997; Lisztwan et al., 1998; Moynihan et al., 1999; Nuber et al., 1996; Scheffner et al., 1994; Schwarz et al., 1998). The substrate recognition of the ubiquitination system is achieved by direct interaction between the substrate and the E3 or with E3 as part of a complex with an E2. The interaction between the

substrate and the E3 is likely to contribute to the high substrate specificity in the system. Based on the domain essential for E3 activity, two types of E3s have been defined, the ring finger E3s and the homology to E6-AP C-terminus (hect) domain E3s (Huibregtse et al., 1995; Joazeiro and Weissman, 2000).

The family of ring finger domain E3s can be devided into four different groups, examples of E3s from these groups are the Rbx1, UBR1, Apc11p and c-Cbl. The Rbx1 (or Hrt1/Roc1) protein, is an essential component of the large combinatorial Skp1-cullin-F-box (SCF) E3 complexes, acting in the ubiquitination of phosphorylated proteins (Kamura et al., 1999; Skowyra et al., 1999), reviewed in (Elledge and Harper, 1998)**.** The ring finger protein UBR 1 also named E3, is the E3 of the N-end rule family and is responsible for ubiquitination of N-end rule substrates (Bachmair et al., 1986; Bachmair and Varshavsky, 1989; Bartel et al., 1990; Kwon et al., 1998). The Apc11p is an essential component of the Anaphase Promoting Complex (APC), which is responsible for ubiquitination of cell cycle regulators (Gmachl et al., 2000; Leverson et al., 2000). Finally, the ring finger proteins c-Cbl and Mdm2 are involved in ubiquitination of activated receptor protein tyrosine kinases (RPTKs) and p53 respectively (Fang et al., 2000; Honda and Yasuda, 2000; Waterman et al., 1999). Mammalian genomes encode several hundreds of ring finger proteins, which can potentially act as ubiquitin protein ligases. The number of potential E3s is further enhanced by combinatorial association of some ring finger proteins with proteins providing docking sites for different substrates (Joazeiro and Weissman, 2000).

In contrast to the ring finger domain E3s, the hect domain E3s can directly bind ubiquitin. Hect domain E3s are large enzymes ranging from 90 kDa to more than 500 kDa, which are found in all eukaryotic organisms examined so far. These include yeast, *Arabidopsis*, C. *elegance*, *Drosophila* and mammmals (Huibregtse et al., 1995). Database searches suggest that the human genome encodes for around 50 different hect domain proteins. These enzymes all contain the characteristic hect domain of approximately 350 amino acids situated in the C terminus of the protein, for review see (Pickart et al., 2001). This domain harbors the enzymatic ligase activity and for the mammalian E3, E6-AP, it was shown that the hect domain by itself can form a thiol ester intermediate, indicating that the hect domain is

sufficient for recognition and activation by the E1 and E2 (Schwarz et al., 1998). The conserved cysteine in the hect domain has been shown to be essential for ubiquitin ligase activity. A point mutation of this cysteine completely abolishes the ligase activity of the mammalian hect domain E3 E6-AP (Schwarz et al., 1998) and the yeast E3 RSP5 (Wang et al., 1999). Some of the hect domain E3 ligases also contain ww-domains, protein interaction domains, which interact with small proline rich sequences. These are called PY motifs, of which the most common is PPxY, where x being any amino acid, reviewed in (Einbond and Sudol, 1996; Staub and Rotin, 1996). The ww-domain seems to be the site of interaction between the E3 and its substrate, as shown for some E3-substrate interactions e.g the hect domain E3 Nedd4 and its substrate epithelial sodium channel (Harvey et al., 1999; Staub et al., 1996) and RSP5 and its substrate Rpb1 (the large subunit of RNA polymerase II) (Wang et al., 1999). Some of the hect domain E3s contains a N-terminal C2 domain, a domain found in some proteins functioning in signal transduction or membrane traffic. The C2 domain binds Ca^{2+} and phospholipids and is therefore believed to recruit proteins to membranes reviewed in (Nalefski and Falke, 1996). In fact, it has been demonstrated that the Nedd4 C2 domain is responsible for the Ca^{2+} dependent translocation of the protein to the plasma membrane (Plant et al., 2000; Plant et al., 1997). The ubiquitin conjugating enzymes (E2), which have been shown to act together with the mammalian hect domain E3s are UBCh5 and the E2 UBCh7 (Schwarz et al., 1998).

A.4. The itch gene and protein

The *itch* gene was first discovered in 1998 by Copeland and coworkes, when revealing the genetic defect underlying the auto-immune phenotype in a^{18H} mice (Perry et al., 1998). On C57BL/6 genetic background the a^{18H} mice developed interstitial inflammation of the lung, hematopoeitic cell proliferation in the spleen, hyperplasia of the thymus and lymph nodes as well as ulcers and inflammatory processes on the ears and skin, leading to death of the animals after 4-6 month. On a different genetic background, JU/Ct, the mutation gave rise to a partly different phenotype. These mice developed an interstitial inflammation of the lung and inflammation of the large intestine (Hustad et al., 1995). The phenotype difference seen in

a^{18H} mice on the two different genetic backgrounds could be due to the influence of other genes modifying the effect of the mutation causing immune dysfunction. The genetic defect identified in these mice was an inversion between the agouti locus and the *itchy* locus (Perry et al., 1998). The proximal breakpoint of the inversion is situated between the untranslated agouti exons one and two and the distal break is located in the intronic sequence between exon 1 and 2 of the *itch* gene (Perry et al., 1998). The inversion is predicted to produce an *itch* null allele by separating the promoter from the coding region of the *itch* gene. This leads to a complete abolishment of the Itch expression and to a ten fold reduction of the agouti expression (Hustad et al., 1995; Perry et al., 1998).

Since mutation in the agouti locus had previously not shown induce an auto-immune phenotype, it was believed that the disruption of the *itch* gene was responsible for the auto-immune like phenotype developed by a^{18H} mice (Perry et al., 1998). It is also possible that additional genes located in the inverted segment are affected in these mice and thereby influence the phenotype. The *itch* gene is situated on the mouse chromosome 2 and contains thirteen exons. The cDNA is 5122-bp long, with an open reading frame of 2562-bp, which encodes a 854 amino acid large protein with the predicted molecular weight of 113 kDa and which is ubiquitiously expressed (Perry et al., 1998). A prediction of the amino acid sequence revealed a protein with high homology to the family of hect domain ubiquitin-protein ligases (Perry et al., 1998). The domain structure of Itch resembles a N-terminal C2 domain, followed by four ww protein interaction domains and a C-terminal hect domain encoded by the last 336 amino acids. The conserved cysteine on amino acid position 822 of the hect domain, binds ubiquitin covalently and the capability of Itch to act as an E3 has now also been demonstrated in vitro (Qiu et al., 2000).

A.5. Potential substrates of Itch

Potential substrates of Itch have been perposed, but it is still questionable if these are the real substrates of Itch. Several substrates have so far been suggested and there are probably still more to be discovered. A recently proposed substrate of Itch is JunB. It was shown that JunB degradation in a^{18H} T cells was delayed compared to wild type cells. In addition,

ubiquitination of JunB in Jurkat cells transfected with plasmids expressing JunB and ubiquitin was promoted by cotransfection of an Itch expressing vector but not by a vector expressing anenzymatically inactive Itch (Fang et al., 2002). The transcription factor JunB has been shown to be involved in the regulation of the interleukin-4 (IL-4) expression during T helper cell differentiation (Li et al., 1999). JunB upregulates expression of Th2 cytokines in transgenic mice and synergizes with c-Maf to activate the IL-4 promoter (Li et al., 1999).

Notch-1 is another protein, which may represent a substrate of Itch. The Drosophila protein suppressor of Deltex, is a negative regulator of the Notch signaling pathway (Fostier et al., 1998) and was found to be a hect domain ubiquitin-protein ligase with high homology to the murine E3 ligase, Itch and its human homologue AIP4 and the human proteins WWP2 and WWP1 (Cornell et al., 1999). Moreover, it was shown that recombinant Itch can ubiquitinate Notch-1 in vitro (Qiu et al., 2000). Notch-1 has been shown to play a role in the lineage commitment between B and T cells (Pui et al., 1999; Radtke et al., 1999) and the lineage commitment between □□T cells versus □□T cells (Washburn et al., 1997). Its involvement in the lineage commitment between CD4 single positive versus CD8 single positive thymocytes, however, has been disputed (Robey et al., 1996; Wolfer et al., 2001).

It has been shown that the Epstein-Barr Virus protein Latent Membrane protein 2A (LMP2A) enhances the ubiquitination of the non receptor protein tyrosine kinase Lyn in vivo in a manner dependent on the activity of Nedd-4 family E3 ligases such as AIP4 the human homologue of Itch. In addition, a C830A mutant of the AIP4 protein, harbouring a point mutation in the conserved cysteine essential for ubiquitin binding, can inhibit Lyn and Syk ubiquitination in vitro (Ikeda et al., 2000; Winberg et al., 2000). In this study, LMP2A itself was also identified to act as a substrate of AIP4/Itch, leading to its ubiquitination. The region of the interaction between the two proteins was identified to be the PPPPY motifs in the LMP2A molecule and the ww domains of the AIP4/Itch (Ikeda et al., 2000; Winberg et al., 2000). Both Lyn and Syk are involved in the signaling pathway downstream of the B cell receptor, for review see (Hubbard and Till, 2000). However it is debatable whether Lyn and Syk are natural substrates of Itch since ubiquitination of these two proteins is only observed in the presence of LMP2a. Similarly, viral proteins with the ability to connect E3 ligases to none

natural substrates have also been identified previously for the hect domain ubiquitin protein ligase E6AP. In this case, the papillomavirus protein E6 can connect the E6AP to the p53 protein, which leads to ubiquitination followed by degradation of the p53 protein (Scheffner et al., 1993). No ubiquitination of the protein p53 could be shown in the absence of E6, indicating that the p53 protein is not a natural substrate of E6AP.

A.6. The immune response

Immunity is the state of protection against infectious diseases and has an innate and an acquired component. It is thought that innate defense mechanisms provide the first line of host defense against invading pathogens until an acquired immune response develops. The main players of the innate defense are the monocytes, neutrophils and macrophages, which endocytose foreign macromolecules as well as kill and digest whole microorganisms such as yeast or bacteria. In contrast to adaptive immune responses, innate immune responses are antigen receptor-independent and induced via pattern-recognition receptors like the large Toll like receptor (TLR) family. It is now known that in mammals there are about 10 TLRs and each of them has a specific ligand like gram-negative or gram-positive bacterial cell wall components, or dsRNA from viruses, reviewed in (Kaisho and Akira, 2000).

The acquired immune defense on the other hand, is based on the ability of B and T lymphocytes to specifically recognize and selectively eliminate foreign molecules and microorganisms. The acquired immune defense can further be divided into humoral and cell-mediated immune responses. In the humoral response, the microbial antigens are endocytosed by antigen presenting cells (APC), processed and presented preferentially by class II MHC molecules to CD4+ T helper (Th) cells. Th cells collaborate with B lymphocytes for the production of specific antibodies which can eliminate the pathogen or neutralize their soluble toxic products. The cell-mediated immune response involves various subpopulations of T cells that recognize antigens presented on self-cells. CD4+ Th cells respond to antigen by producing cytokines and CD8+ cytotoxic T (Tc) cells respond to antigen by developing into cytotoxic T lymphocytes (CTLs), which mediate killing of altered self–cells. Defense against infectious intracellular organisms, as viruses and some type of bacteria and protozoans, are usually dominated by a cell-mediated immunity characterized by cytolytic activity. On the

other hand, humoral responses are involved in the destruction of most extracellular pathogens, where large amount of pathogen-specific immunoglobulins are produced in an attempt to neutralize the foreign organism, for review see (Kuby, 1997; Constant, 1997; Glimcher, 2000). Most immune responses involve both humoral and cell-mediated immunity acting in concert, but under some conditions, the two types of effector reaction may be exclusive.

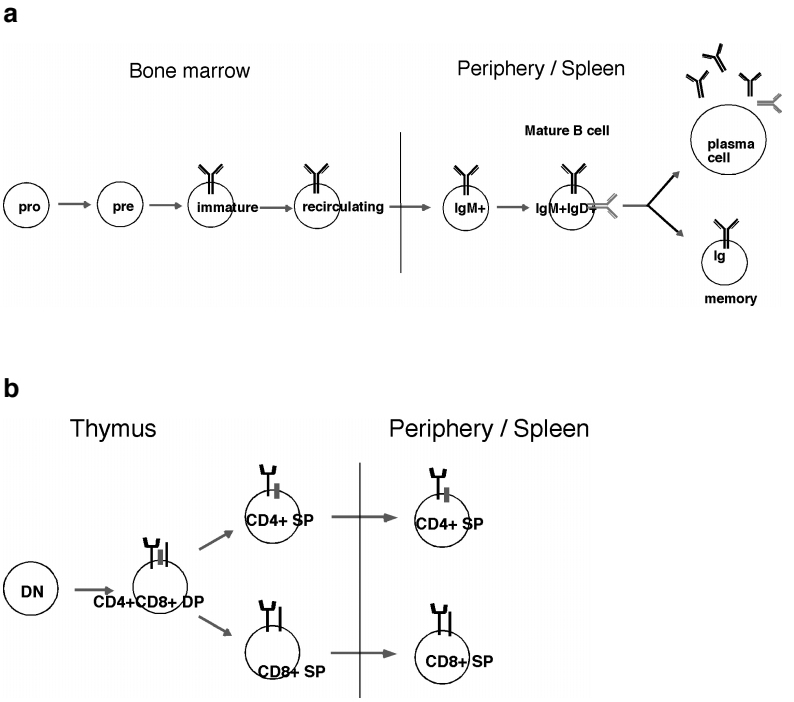


Figure 2. B and T cell development

a Sceme of the different stages of B cell development in the bone marrow, from pro-B to pre-B, to immature- and to recycling-B cells. In the periphery mature-B cell develop to either plasma cell or memory-B cell after antigen stimulation. **b** Sceme of T cell development in the thymus, from double negative (DN) to doubel positive (DP) to CD4 or CD8 single positive (SP) thymocytes, which leave to the periphery and become CD4 SP or CD8 SP T cells.

A.7. Activation of Th cells in the immune response

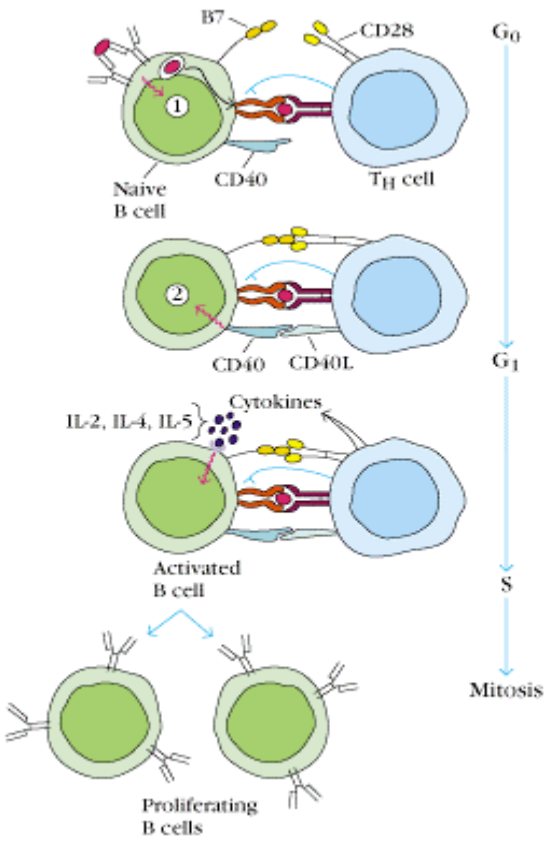
Figure 3. T cell activation

a) The T cell receptor (TCR) is stimulated by a specific peptide-MHC complex on the APC (here B cell).

b) This is followed by a second signaling event involving co-stimulatory signal provided by the interaction of the APC B7 and Th cell CD28 molecules. This interaction induces up-regulation of CD40L on the Th cell. CD40L then binds CD40 on the APC, enhancing B7.1/B7.2 expression and reinforcing the CD28/CD40 positive feedback loop.

c) B cell begins to express receptors for various cytokines. Binding of cytokines released from the Th cell in a directed fashion send signals that supports the progression of the B cell to DNA synthesis.

Figure taken from Kuby Immunology, R.A. Goldsby et al., 2000, 4th edition, Freeman & company.



Two signals are required for optimal activation of T cells. In the initial stage of T cell activation, the T cell receptor (TCR) is stimulated by a specific peptide-MHC complex on the APC, this is followed by a second signaling event involving co-stimulatory molecules to achieve full T cell activation. The co-stimulatory signals include interaction of the T cell CD28 molecule with B7.1 and/or B7.2, which are expressed at low levels on the APC. This interaction induces up-regulation of CD40L on the T cell. CD40L then binds CD40 on the APC, enhancing B7.1/B7.2 expression and reinforcing the CD28/CD40 positive feedback loop (Fig.3). Activation of CD28 also induces up-regulation of the co-stimulatory molecule ICOS on the T cell, which interacts with its ligand B7h on the APC. CTLA4, which binds B7

with a higher affinity than CD28, is induced after T cell activation and is involved in down-regulating T cell responses, for review see (Miceli et al., 2001; Oosterwegel et al., 1999; Thompson and Allison, 1997).

A.8. Th1 versus Th2 response

Upon receiving an antigenic stimulus, the CD4+ T helper lymphocytes differentiate into one of two distinct effector subsets, T helper 1 (Th1) and T helper 2 (Th2), which are defined both by their function and by their unique cytokine profile. The hallmark cytokine of the Th1 subset is IFN, but Th1 cells also secrete interleukin-2 (IL-2), Tumor necroses factor (TNF) and Lymphotoxin (LT). These cytokines induce cell-mediated immune responses including recruitment and activation of macrophages and other non-specific inflammatory cells. The signature cytokine of the Th2 subset is IL-4, but Th2 cells also secrete the cytokines IL-5, IL-9, IL-10 and IL-13, cytokines which can help to induce B cell proliferation and differentiation and are critical in the allergic response (Arthur and Mason, 1986; McKenzie et al., 1998; Mosmann and Coffman, 1989; Paliard et al., 1988; Paul and Seder, 1994). The cell specific response of Th1 effector cells and the humoral response of Th2 effector cells are thereby specialized to protect the organism from different types of pathogens. Both genetic and environmental factors are responsible for the Th1 or Th2 differentiation, although the mechanisms by which the genetic background controls Th-cell differentiation remain elusive (Hsieh et al., 1995). Environmental factors proposed to influence Th1 and Th2 lineage commitment are the nature and strength of the interaction between the TCR/CD3 complex with peptide bound MHC class II molecule as well as the nature of the costimulation (Paul and Seder, 1994). However, neither of these signals is as potent a determinant as the cytokine environment itself. The two critical cytokines in the Th1 and Th2 polarization are IL-12 and IL-4 respectively (Hsieh et al., 1993; Macatonia et al., 1993; Maggi et al., 1992; Manetti et al., 1993; Parronchi et al., 1992; Scott, 1991; Seder et al., 1993; Wu et al., 1993). The requirement for these cytokines has been demonstrated by the phenotype of mice lacking these cytokines, cytokine receptors or downstream effector molecules of these receptors. IL-12 is secreted by APC and activates the Stat4 signaling pathway and mutant mice for one of these molecules can not develop Th1 cells (Kaplan et al., 1996; Magram et al., 1996). On the other

hand mice lacking IL-4, IL-4 receptor or its downstream signaling molecule Stat6, fail to support Th2 differentiation in response to most stimuli (Kühn, 1991; Kopf, 1993; Kaplan, 1996; Shimoda, 1996; Takeda, 1996).

A.9. Autoimmunity and systemic inflammation

The term autoimmunity describes the response of the immune system against self-components. Healthy individuals normally also posses self-reactive lymphocytes, which normally does not result in autoimmune reactions. The activity of these lymphocytes are normally regulated by anergy or suppression, whereas a breakdown of this regulation can lead to activation of self reactive clones of T or B cells and generation of humoral or cell-mediated responses against self antigens. Both host genes and the environment seem to play a role in the development of autoimmune diseases and can both influence the susceptibility to autoimmunity by influencing the over all reactivity. Organ damage in autoimmune diseases can be mediated by T cells as in multiple sclerosis (MS) and type I diabetes, where both CD4+ and CD8+ T cells play crucial roles in disease development (Haskins and McDuffie, 1990; Hutchings et al., 1992; Steinman, 1996; Wong et al., 1996). In other autoimmune diseases such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA), the damage is mediated by autoantibodies and requires CD4+ T helper cells (Kotzin, 1996; Kotzin et al., 2000).

The balance between Th1 versus Th2 cells seems to play an important role in several autoimmune diseases. Multiple sclerosis (MS) is presumed to be an autoimmune disease of the central nervous system. In a mouse model for MS, called experimental autoimmune encephalomyelitis (EAE), most T cells exhibit a Th1 phenotype based on the analysis of IFN-γ/IL-2 production versus IL-4/IL-10 production (Kuchroo et al., 1992; Renno et al., 1995; Renno et al., 1994; van der Veen et al., 1993; Zamvil and Steinman, 1990). In animals with EAE treatment with IL-4 induce leads to polarization of T cells to Th2 and results in a diminution of disease symptoms (Racke et al., 1994; Racke et al., 1995). A polarization towards Th1 also seems to play an important role in the development of other autoimmune diseases, such as Hashimoto's thyroiditis and Crohn's disease. Hashimoto's thyroiditis , being an autoimmune disease characterized by histological changes in the thyroid, including massive

infiltration of lymphoid cells and parenchymal destruction and Crohn's disease, characterized by chronic inflammation of the alimentary canal from mouth to rectum. (Sugihara et al., 1995; Sugihara et al., 1993)

Systemic lupus erythematosus (SLE) is an autoimmune disorder characterized by overproduction of a wide range of autoantibodies and pathological abnormalities involving the kidney, skin, brain, lungs and other organs (Mills, 1994). In SLE patients, there is an inbalance between Th1 and Th2 cytokines in favor of Th2 cytokines, which may be critical for disease induction (Dueymes et al., 1993; Hagiwara et al., 1996; Klinman and Steinberg, 1995). Other results obtained with lupus prone mice, MRL-lpr/lpr, suggests that both Th1 and Th2 cytokines may play a pathogenic role, since the disease is attenuated by administration of anti-IFNγ or anti-IL10 antibody but accelerated by administration of IL-10 (Ishida et al., 1994; Ozmen et al., 1995).

Thus the majority of autoimmune diseases studied, especially organ specific autoimmune diseases, appear to be mediated by Th1 cells. However there are also autoimmune diseases where a Th1/Th2 polarization is not apparent or in which Th2 responses predominate.
Deficiency or overexpression of genes regulating the reactivity of the immune system have been shown to be involved in the development of autoimmune diseases. Examples of gene defects or overexpression affecting the overall immunoreactivity and thereby inducing autoimmune disease are, Fas and Fas ligand knock-out as well as Bcl-2 transgene (Griffith et al., 1995; Strasser et al., 1991; Strasser et al., 1991), which act by inhibiting apoptosis, genes involved in B cell activation such as FcγRIIb, the phosphatase SHP-1, CD22, protein tyrosine kinase Lyn (Cornall et al., 1998), genes involved in T cell activation and regulation, as TGFβ, TGFβR, PD-1 knock-out and CD40L transgene (Boivin et al., 1997; Christ et al., 1994; Clegg et al., 1997; Gorham et al., 2001; Larsson et al., 2001; Nishimura et al., 1998). In addition, genes affecting T cell recognition of peptides, dictating which antigen will be the target of autoimmune disease and thereby which organs will be attacked as well as genes involved in cytokine signaling. In addition, transgenes of IL-4, IFNγ, TNFα, IL-10 and IL-2 deletion

(Bessis et al., 1998; Butler et al., 1997; Horak et al., 1995; Moritani et al., 1994; Sarvetnick et al., 1988; Tepper et al., 1990), thereby inducing inflammatory responses, has also been shown to be involved in autoimmune disease development.

A.10. Conditional gene inactivation

One way to study a gene function in vivo, is the usage of specific mutagenesis of the gene in mice. In this way conclusions about the gene function can be drawn from the phenotype seen in the mutated mice. The prerequisite of such gene targeting experiments is the establishment of embryonic stem (ES) cell and mutation of the ES cells in culture by homologous recombination (Thomas and Capecchi, 1987). This method is based on the ability of the bacteriophage P1 Cre enzyme to recognize and excise a DNA segment between two *loxP* sequences (a specific 34bp sequence), when directed in the same orientation (Gu et al., 1993; Sternberg and Hamilton, 1981). This method can be used to induce the gene mutation in either an inducible or a cell specific manner, by breeding of mice harboring a *loxP* flanked gene with a mouse expressing Cre under the control of an inducible or a cell type specific promoter (Rajewsky et al., 1996).

A.11. Aims of this study

The *itch* gene encodes a hect domain E3, which ubiquitin ligase activity has been demonstrated in vitro. It has been shown that in the a^{18H} mouse strain the *itch* gene is disrupted due to a large inversion of DNA segment between the *itch* locus and the *agouti* locus. The disease in a^{18H} mice take different form on different genetic backgrounds, were the disease on a C57BL/6 background show prominent effects on the immune system and systemic inflammation (Perry et al., 1998).

It is likely that the loss of Itch in a^{18H} mice is the reason for the development of inflammatory disease. However, it is possible that other genes located in the inverted gene segment are also affected and participate in the disease development. In addition, the cell type involved in the initiation of disease and the mechanistic features behind the disease development remain unknown.

The inflammatory/auto-immune like phenotype observed in a^{18H} mice strongly suggests that lymphocytes have a primary role in the initiation of disease (Hustad et al., 1995; Perry et al., 1998). Lack of down-regulation of one or more signaling proteins involved in the activation of lymphocytes, in the absence of Itch may account for the observed phenotype.

The aim of this study was to show whether disruption of the itch gene can lead to the development of an auto-immune like or inflammatory disease in mice and if so identify the cell type responsible for the initiation of the disease. We also hope to shred some light on the mechanism underlying this dysfunction.

In order to address these questions, we generated a mouse strain, which allowed us to conditionally inactivate the *itch* gene. Using the Cre/*LoxP* recombination technology, the *itch* gene could be disrupted specifically in different lymphocyte populations.

B. Material and Methods

B.1. Bacterial transformation

Heat-shock competent E. coli DH5α were used for transformation in all experiments. Bacteria were thawed and incubated with plasmid DNA for 30 minutes on ice, subjected to heat-shock at 42°C for 45 seconds and incubation on ice for 2 minutes. Subsequently, 1 ml SOC medium (2% bacto tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$10 mM $MgSO_4$, 20 mM glucose) was added. Cells were incubated for 30 minutes at 37°C before plating on LB agar plates with proper antibiotics and incubated at 37°C for 12-16 h.

B.2. Isolation of plasmid/BAC DNA

Plasmid DNA was prepared according to the alcaline lysis method descriebed by (Sambrook, 1989). Briefly, Bacteria pellet was resuspended in solution I (50 mM glucose, 25 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0), vortexed and lysed in Solution II (0.2N NaOH, 1% SDS). The lysis reaction was neutralized in ice cold Solution III (5 M potassium acetate, 11.5% glacial acetic acid) by inversion. The DNA was precipitated by propanol and washed in 70% EtOH before resuspention in TE pH 7.5 (10 mM Tris, 1 mM EDTA) Plasmid DNA preparation for sequencing was isolated using GFX™ Micro Plasmid Prep Kit (Amersham Pharmacia Biotech Inc.). Large amount of plasmid DNA was isolated by midi or maxi prep kit (Qiagen). All according to the manufactures protocol. The BAC clone received from GenomeSystemsInc, harbored C57BL/6 mouse genomic DNA inserted into the pBeloBAC 11 vector. The BAC clone DNA was isolated as follows, BAC transformed bacteria were grown 12-14 h in LB 12.5 μg/ml chloramphenicol. Pellet was resuspended in GTE buffer (50 mM glucose, 10 mM EDTA, 0.1 M NaCl)+ 100 μg/ml lysozyme, incubated for 5 minutes at RT and lysed in 0.2 M NaOH, 1% SDS and incubated on ice for 5 minutes. The lysis reaction was neutralized in 3 M KAc buffer (3 M KAc, 11.5% acetic acid) by inversion and 5 minutes incubation on ice. After centrifugation the RNA was removed from the supernatant by addition of 50 μg/ml RNAseA and incubation for1 hour at 37°C. The solution was then

extracted with phenol/chloroform (1:1) to remove proteins. DNA was precipitated with propanol, washed in 70% EtOH and resuspended in TE pH 7.5.

B.3. Cloning of genomic DNA

Cloning was preformed by using DNA ligation kit (Takara) according to the manufacture's instruction or T4 DNA ligase (Gibco BRL) according to the methods of (Sambrook, 1989). Filling of incompatible 3' termini was done with Klenow fragment of E.coli DNA polymeraseI (Boehringer Mannheim), for removal of protruding 3' termini bacteriphage T4 DNA polymerase was used. The vector was dephosphorylated with calf intestinal phosphatase (CIP), followed by extraction with Phenol/Chloroform, precipitated with 2 volumes of EtOH and diluted in H_2O before ligation.

B.4. Restriction enzyme analysis

Restriction enzyme (Boeringer, Gibco, Takara, New England BioLabs) were used according to the manufacture's instruction.

B.6. Synthetic oligonucleotides

Oligonucleotides used for PCR reactions and DNA sequencing were purchased from Sigma ARK or Eurogentec. Oligonucleotides used for PCR on the Light Cycler (Roche) were designed and purchased from mol TIBbiol.

B.7. Purification of DNA fragments

DNA fragments were cut out from agarose gels and purified using JETsorb solutions (Genomed) or QIAquick gel extraction kit (Qiagen) according to the manufacture's instruction.

B.8. DNA sequencing

Agarose gel purified Plasmid DNA (0.5-1 □g) or PCR fragments (0.05-0.2 □g) was used per sequencing reaction using "Tay Dye Deoxy Terminator Cycle Sequencing Kit" (Applied

Biosystems) according to the manufacturer's instructions. The method is based on the dideoxy-chain termination reaction (Sanger et al., 1977). The products were precipitated with 120 mM Sodium acetate in 95% EtOH and washed in 70% EtOH. Sequencing reactions were resolved and analysed on an automated sequencer (A373, Applied Biosystems), according to the manufacture's protocol.

B.9. Isolation of genomic DNA

Genomic DNA was isolated from mouse tail tips. 0.5-1 cm tail tips were digested in 0.5 ml Tail lysis buffer (10 mM Tris(hydroxymetyl)aminoethan (Tris)/HCL pH 8.5, 5 mM ethylene-diamine-tetraacetic-acid (EDTA), 0.2% sodium dodecyl sulfate (SDS), 200 mM NaCl, 100 μg/ml proteinase K) O/N at 56°C and 1000 rpm. Genomic DNA was isolated from ES cells grown to confluence in 96 well plates and washed once with PBS, by O/N digestion in ES cell lysis buffer (10 mM NaCl, 10 mM Tris-HCl pH 7.5, 10 mM EDTA, 0.5% Sarcosyl, 0.4 mg/ml freshly added proteinase K) at 56°C at humid condition according to protocol by (Pasparakis and Kollias, 1995).

B.10. Southern blot analysis

Approximatly 10 μg of genomic DNA was digested 14-16 hours using 150 units of appropriate restriction enzyme. The digested genomic DNA was resolved on a 0.8% agarose gel. Capillary transfer of DNA to nitrocellulose membrane (Hybond-N, Amersham or genescreen Plus, Dupont) was preformed by standard procedures following (Southern, 1975). The DNA was fixed to membranes by UV-crosslinking using a photo-crosslinker (BioLink BLX-254, LTF Labortechnic). Membrane was blocked ON at 65°C in hybridization buffer (50 mM Tris-CL pH 7.5, 1 M NaCl, 1% SDS, 10% dextran sulfate, 300 μg/ml sonicated salomon sperm DNA). The labeled probe was added to the hybridization buffer and incubated at 65°C ON. Finally, the membrane was washed with 0.5-2x SSC (150 mM NaCl, 15 mM tri-sodium citrate) and 0.1-1% SDS, followed by exposure for autoradiography.

B.11. Amplification and purification of probes used for Southern blot analysis

The probe d was amplified from the subcloned genomic *itch* sequence by PCR using primers d2 forward and d2 reversed. Probe e was amplified using the same template and primers e reverse and e forward. The probes were sequenced before use.

B.12. Radioactive labeling of probes

The radioactive labeling of probes were preformed by random primed (Feinberg and Vogelstein, 1983). 50 ng of DNA template and random hexamers in the final volume of 10 μl were denatured at 100°C for 3 minutes and chilled on ice for 5 minutes. Addition of 2.5 μl BCA10x buffer, 3 μl 0.2 mM dNTP (dCTP, dGTP, dTTP), 25 μCi α^{32}P-dATP (Amersham) in the final volume of 24 μl and 1 μl BCAbest DNA polymerase (Takara). The labeling reaction was preformed at 55°C for 50 minutes.

B.13. Polymerase Chain Reaction (PCR)

The polymerase chain reaction was used for the routine genotype screening of mutant mice, amplification of probes for Southern blot analysis and amplification of cDNA for cloning. All PCR reactions were carried out in 30 μl reactions and either Klentherm (Genecraft) or Taq polymerase (home made) was used.

Table 1. shows the PCR conditions of different PCR reactions.

Target	**primers**	**Annealing temp.(°C)**	**Size of product**	**purpose**
itch genomic DNA	d2 forward d2 reverse	50.1°C	d2: 572bp	Southern probe
itch genomic DNA	e forward e reverse	50.4°C	e: 498bp	Southern probe
Itch genomic locus	+itch 1 -itch 2 -itch 3	58°C	wt: 237bp fl: 270bp □: 497bp	Genotyping
CD19-cre	CD19c CD19d Cre7	59.5°C	wt: 450bp Cre: 715bp	Genotyping
Cre	Cre2 Cre7	60°C	Cre: 689bp	Genotyping
Deleter-Cre	DEL F DEL R	60°C	Cre: 600bp	Genotyping
itch cDNA	Itch F Itch R	62°C	327bp	RT-PCR
hprt cDNA	HPRTse HPRTas	62°C	249bp	RT-PCR
itch cDNA	U-itch cDNAB L-itch cDNAB	58.5°C	2584bp	expression

Table 2. Primer sequences

PCR primer	**sequence**
d2 forward	5’ AGC ACT GTG GTT CCT TC 3’
d2 reverse	5’ AGG TCC CCA TTA GAG AA 3’
e forward	5’ GCT TGA TTT GAT TTG GTT 3’
e reverse	5’ TAT CTC ACA GCA GCA AGG 3’
+a probe	5’ GAG CCT ATT TGA TTG TCT GC 3’
-a probe	5’ CTA CCA TGA GAC CCT ACC TT 3’
+itch 1	5’ACA AGA GGT AGG AGA CAA GCA TT 3’
-itch 2	5’ TGC AGC TTA TTT ATC ATT CCT TA 3’
-itch 3	5’ TGG TTA CTT TAT TTG GCTTTgACTC3’
CD19-Cre d	5’ CCA GAC TAG ATA CAG ACC AG 3’
CD19-Cre c	5’ AAC CAG TCA ACA CCC TTC C 3’
Cre7	5’ TCA GCT ACA CCA GAG ACGGG 3’
Cre2	5’ CAA TTT ACT GAC CGT ACA 3’
U-itch cDNAB	5’ CGC GGA TCC GGT AGT CTG ACC ATG 3’
L-itch cDNAB	5’ CGG GAT CCC GCA ATT ACT CTT GTC C 3’

B.14. Isolation of RNA

Total RNA was isolated from organs or sorted lymphocyte populations using Trizol reagent (Gibco BRL) according to the manufacture’s protocol.

B.15. cDNA synthesis

cDNA was synthesized from total RNA using "First Strand cDNA synthesis kit" (Gibco BRL). RNA from 5 x 10^5 sorted cells or from 6 μg RNA isolated from organ was incubated with random hexamers (Boeringer) in a total volume of 11 μl at 70°C for 10 minutes. Samples were chilled on ice before addition of the reaction master mix (2 μl 0.1 M DTT, 1 μl 10 mM dNTP, 4 μl 5x first strand buffer, 0.5 μl RNAsin ribonuclease inhibitor (Promega)) to the samples. After 2 minutes of pre heating 1 μl (200 units) Superscript reverse transcriptase was added. Samples were incubated at 42°C for 90 minutes followed by inactivation at 95° for 5 minutes.

B.16. Reverse Transcript-PCR using the Light Cycler

As control template for *hprt* cDNA the EST clone 1007097 (2512-p10) from HGMP Resource Center was used and the *itch* control template was the *itch* cDNA clone, a kind gift from Prof. Neal G. Copeland.

Table 3. Primers and probes used for RT-PCR

cDNA	**primers**	**Probe LC**	**Probe FL**	**Annealing temp. (°C)**
itch	Itch F Itch R	Itch LC	Itch FL	62°C
HPRT	HPRT se HPRT as	HPRT 640	HPRT FL	62°C

Table 4. Sequence and reference of RT-PCR primers and probes

Primer/probe	**sequence**	**reference**
Itch F	5' AGG AAT ACA TCA GGA TGG TAG CT 3'	Tib Molbiol
Itch R	5' TCA GCA AAT CCT CCC ACT G 3'	Tib Molbiol
Itch LC	5' LC Red640-ACA TGC CAT CTA CCG CCA CTA CAC p	Tib Molbiol
Itch FL	5' AGG AGA TTG ATT TGA ATG ACT GGC AGA X3'	Tib Molbiol
HPRT as	5' CAC AGG ACT AGA ACA CCT GC 3'	Tib Molbiol
HPRT se	5' GCT GGT GAA AAG GAC CTC T 3'	Tib Molbiol
HPRT 640	5' LC Red640-TCT GCA AAT ACG AGG AGT CCT GTT G p	Tib Molbiol
HPRT 3FL	5' AAA GCC TAA GAT GAG CGC AAG TTG A X	Tib Molbiol

B.17. Amplification of itch cDNA for cloning of the Itch expression vector

The *itch* coding sequence was amplified from the *itch* cDNA containing vector by PCR using primers U-itch cDNAB and L-itch cDNAB. A BamHI restriction enzyme site was introduced in the 5' and 3' of the primers respectively, which enabled for digest of the PCR product with BamHI and in frame cloning into the BamHI site of the pGEX-4T-1 expression vector (Pharmacia biotech). The L-itch cDNAB primer also codes for an in frame stop codon.

B.18. Expression and purification of recombinant GST- Itch fusion protein

The open reading frame of the *itch* cDNA was cloned in frame in the pGEX-4T-1 expression vector (Pharmacia biotech). DX5α bacteria were transformed with the GST-Itch expression vector and cultures were grown in LB medium at RT. At OD_{600} 0.4 the culture was induced with 0.1 mM IPTG and grown for additional 2 hours. The bacteria pellet was washed once with PBS and resuspended in PBS/0.1 mg/ml lysozyme/1% Trition X-100 and incubated at RT for 5 min and then sonificated. After centrifugation the supernatant was incubated with Glutathione Sepharose 4B (Pharmacia Biotech) ON at 4°C. The next day the Glutathione Sepharose beads were washed 3x with PBS and the bound GST-Itch fusion protein was eluted with elution buffer (10 mM reduced glutathione, 50 mM Tris pH 8.0). Protein was stored at –20°C in protein freezing buffer (50 mM KCl, 10 mM Tris-HCl pH 7.5, 0.1 mM EDTA, 1 mM DTT, 50% Glycerol).

B.19. Western Blot Analysis

Lysates from organs were prepared by dounzing the organs together with 3 ml lysis buffer (50 mM Tris-HCL, 150 mM NaCl, 4 mM EDTA, 10% glycerol, 1% NP-40, 0.2 mg/ml aprotenin, 0.1 mg/ml leupeptin, 1 mM PMSF)/g tissue in a dounzer. From lymphoid organs, lysates were prepared from single cells. One ml lysis buffer/10^8 cells was used. Total protein concentration of lysates was determined by Bicinchoninic Acid (BCA) assay (Pierce) against a bovine serum albumine (BSA) standard according to the manufacture's protocol. Lysates were resolved by SDS-poly-acrylamid-gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose (Schleicher & Schuell) or polyvinylidene-difluorid (PVDF) membrane Immobilon-P, Millipore). To confirm equal loading and to identify the size marker (Bio-Rad),

the membranes were stained with Ponceau-Red solution (Sigma). To block unspecific binding, membranes were blocked with 5%BSA fraction V (Boehringer Mannheim) in 1 x TBS-T (100 mM Tris-HCl pH 7.6, 140 mM NaCl, 0.02% Tween 20) ON at 4°C. Membranes were incubated with the primary anti-body for 1-2 hours at RT washed in TBS-T and incubated with Horseradish peroxidase (HRP) conjugated secondary antibody for 1 hour at RT, washed in TBS-T and detected by chemiluminescence system (super-ECL, pharmacia biotech) according to the manufactures protocol.

Table 5. Antibodies used for Western blotting

Antibody	**Peptid specificity or clone name**	**Antigen size**
Anti-Itch-N-terminus	SAKLKENKKNWFGPC	113 kDa
Anti-Itch-N-terminus	CLFAIEETEGFGQE	113 kDa
Anti-Tubulin	DM1A	55 kDa

B.20. Immunohistochemistry

Organs of mice were frozen at –80°C in Jung tissue freezing media (Leisa Instruments GmbH), sections of Spleen, meseteric and inguinal lymph nodes, thymus, kidney, liver, lung, skin/ears, stomach and intestine were stained with F4/80, anti-Gr-1(Ly-G6), Mac-1, anti-CD19 and anti-CD3. Briefly, sections were air dried, fixated in cold aceton at –20°C for 10 min and air dried. Slides were incubated with PBS for 5 min at RT. Prior to blocking incubation with quenching buffer (0.3% H_2O_2, 1% BSA in PBS) for 20 min to remove endogenous peroxidase actvity. Sections were blocked using Blocking kit (Vector Laboratories) according to the manufacture's protocol, sections were incubated with primary antibody and Alkaline Phosphatase (AP) conjugated secondary antibody in 5% mouse serum. Sections were developed using Alkaline Phosphatase Substrate kit III (Vector Laboratories) according to the manufacture's protocol. For double staining the primary antibodies biotinylated hamster anti-mouse CD3 (Pharmingen) and rat anti-mouse CD19 was used and as secondary reagents Peroxidase-Strept-Avidin and Alkaline Phosphatase Goat anti-Rat was used. Sections were developed with POX (acetate buffer pH 5.2, hydrogen peroxidase , AEC reagent) and Alkaline

Phosphatase Substrate kit III according to the manufactures instructions. Sections were washed and immediately imaged.

B.21. ELISA

B.21.1. Isotype ELISA

Enzyme-linked immunosorbent assay (ELISA) was performed essentially as described (Sedgwick and Holt, 1983)

B.21.2. Anti-DNA ELISA

Antibodies against single stranded (ss) and double stranded (ds) DNA was measured in the sera by ELISA, which was performed as described (Fukuyama et al., 1998). The anti-DNA titers were quantitated as dilution reaching 50% binding of MRL/lpr serum (EC50). Serum from MRL/lpr mice has previously been identified to have very high levels of anti-DNA antibodies (Andrews et al., 1978).

B.21.3. IFN-γ ELISA for serum

IFN-γ concentration in serum was measured by mouse IFN-γ ELISA kit (Endogen), according to the manufacture's protocol. Sera were diluted 1:2 immediately before use.

B.21.4. IFN-γ and IL-4 ELISA for cell culture supernatant

IFN-γ and IL-4 in cell culture supernatant was measured by ELISA. For coating of the plates, anti-mouse-IFN-γ antibody (R46A2) and anti mouse IL-4 antibody (11B11). For standards recombinant IFN-γ and recombinant IL-4 (R&D) was used. For detection, biotin rat anti-mouse IFN-γ antibody, Streptavidin-Alkaline Phosphate (Roche) and substrate ALP (Roche) were used. Both samples and standards were diluted in 1:5 steps in 6 measure points. The highest concentration used was 1000 µg/ml IFN-γ and 10 000 U/ml IL-4. The sampels were measured undiluted and the in 1:5 dilution steps. The absorbance was measured at 404-650 nm.

B.22. Generation of mutant mice

B.22.1. Embyonic Stem Cell Culture

Embryonic stem (ES) cell cultures was preformed as described (Torres and Kühn, 1997). For this experiment the mouse C57BL/6 ES cell line Bruce4 was used. The ES cells have to maintain pluripotent during culturing and genetic modification for their later contribution to germeline in the mouse, therefor they were cultured on a monolayer of neo^r embryonic feeder (EF) cells and grown in medium containing leukemia inhibitory factor (LIF). The EF cells were not passaged more than 3 times and treated with Mitomycin C (Sigma) in EF cell medium 2 hours to prevent further proliferation, immediate before use for ES cell culture. The ES cells were passaged at a subconfluent stage to prevent differentiation. ES and EF cells were culture in ES cell medium and EF cell medium respectively, cells were frozen in 90% FCS 10% dimethylsulfoxid (DMSO) at –80°C, for long term storage cells were transferred to liquid nitrogen.

B.22.2. Transfection of Embyonic Stem Cells

The *itch* targeting vector was linearised by ClaI restriction enzyme digest, purified by phenol/chloroform treatment and washed with ethanol prior to solubilisation and transfection. For transfection $1x10^7$ of ES cells were mixed with 25 µg of linearised *itch* targeting vector in 0.7 ml transfection buffer (RPMI w/o phenol red w/o Glutamine (Gibco). Cells were transfected by electroporation (480 µF and 230 V, electroporator (Biorad), and plated on $2x10^6$ cells/10 cm culture dish. Positive selection of transfected cells harboring the neo^r gene started 48 hours after transfection by adding 350 µg/ml active G418 to the medium. 72 h after transfection cells harbouring randomly integrated targeting vector containing the Herpes Simplex Virus thymidine kinase (HSV-tk) gene, were negatively selected by adding 10^{-5} M gancyclovir to the medium. Single clones were picked at day 8-10 after transfection and expanded in 96 well plates. The clones were frozen in the 96 well plates in –80°C. For freezing, the cells were trypsinized with 50 µl 1x trypsin and thereafter 50 µl 20% DMSO in FCS was added. On top 100 µl of mineral oil (Sigma) was added to prevent evaporation, described in (Pasparakis and Kollias, 1995).

B.22.3. Generation of mutant mice

The in vitro mutated ES cells are injected into blastocysts and contribute to the development of somatic tissue and germ cells. The chimeric mice generated from these blastocysts can thereby give rise to mice in the next generation, harbouring the mutation in all cells. Procedures concerning the injection of ES cells into blastocyst for the generation of chimeric mice was preformed as described (Torres and Kühn, 1997). Briefly, chimeric mice were generated by injection of neo deleted homologous recombinant ES cells into blastocysts (10-15 ES cells/blastocyst) isolated from pregnant CB.20 mice. Injected blastocysts were tranferred into the ampulla of the uterin horn of pseudo-pregnant foster mothers (F1 C57BL/6 x Balb/C) to achieve inplantation of the embyos. Chimeric mice were identified by coat color and crossed to the ES cell donor strain (C57BL/6). Germeline transmission was identified by coat color and coat color positiv (black) mice were analysed by southern blot analysis for harbouring the mutated allele.

B. 23. Generation of Itch polyclonal antibodies

Polyclonal Itch antibodies were generated against the Itch N-terminal peptide SAKLKENKKNWFGPC and the Itch C-terminal peptide CLFAIEETEGFGQE and affinity purified against the peptide by Eurogentec on our request.

B.24. Preparation of single cell suspensions from mouse organs

Mice were sacrified by CO_2. Peritoneal cavity cells were prepared by injecting 10 ml RPMI medium in the peritoneal cavity, cells were collected with the medium using a 10 ml syringe and a 26G needle. Bone marrow cells were prepared by flushing femur with RPMI medium using a 20G needle and 10 ml syringe. Single cell suspension of spleen, thymus, inguinal lymph nodes and meseteric lymph nodes were prepared by carefully squeezing the organs with a bit of medium between the ruff side of two object glasses. Erytrocytes were lysed in bone marrow and spleen single cell suspensions by incubating cells in erytrocyte lysis buffer (0.75% NH_4Cl, 100 mM Tris-HCl pH 7.65) 2 minutes at RT. The lysis was stopped by addition of 10 ml medium.

B.25. Cytofluorometric analysis and cell sorting

Fluorescence stainings was preformed as descriebed (Texido et al., 2000). Briefly, 10^6 cells were washed in PBS/1%BSA/0.01% sodium azid. (PBA) and incubated with biotinylated antibody at saturated concentrations in 30 µl P µ for 20 min on ice. After washing with 2x 200-500 µl of PBA, cells were incubated in 30 µl PBA containing fluorochrom conjugated antibodies and strepavidin for 20 min. Following a final washing step the cells were resuspended in 150-250 µl of PBA and analysed on a FACScan or flow cytometer (Becton Dickinson), or sorted on a FACStar cell sorter (Becton Dickinson). For exclusion of dead cells during analysis, propidium iodide (Sigma) or TO-PRO-3 iodide (Molecular Probes) was added to the cell suspension before acquisition.

Table 6. Antibodies used in this study

Specificity	Antibody (clone)
B220	RA3-6B2
IgM	R33-18-10
CD43	S7
CD19	1D3
CD90 (Thy-1.2)	53-2.1
CD3	145-2C11
CD4	RM4-5
CD8	53-6.7
CD69	H1.2F3
CD62L	MEL-14
CD24 (HSA)	M1/69
CD25	7D4
murine IL-4	11B11
murine IFN	XMG1.2
Gr-1	Ly-6G
CD11b (Mac-1)	M1/70
F4/80	F4/80

B.26. Intracellular staining of Cytokines

To block cytokine secretion, cultures were incubated for 2-4 h with BrefeldinA (Sigma) in medium prior to fixation. Cells were washed with PBS and fixed in 4% paraformaldehyde (PFA) 20 min at RT, thereafter washed twice with PBS and stained with antibodies against intracellular proteins in saponin buffer (PBS/BSA/Azid, 0.05%Saponin) for 15 min at RT. Cells were then washed twice with Saponin buffer and once with PBS/BSA/Azid before staining of extracellular proteins, see Cytofluorometric analysis and cell sorting.

B.27. Purification of lymphocyte subpopulations using magnetic cell sorting (MACS)

Cells were purified from single cell suspentions by magnetic cell sorting (MACS) using depletion or enrichment protocols according to the manufacture's instructions (Miltenyi Biotech). For in vitro stimulation of peripheral T cells, non T cells were depleted from spleen and lymph nodes by incubation of biotinylated anti-CD24, anti-B220, anti-Mac-1 anti-Gr-1. Following a washing step, cells were incubated with strepavidin conjugated MACS-beads. For purification of T cells for RT-PCR, Western Blot or Southern blot analysis cells were purified by enrichment using anti-CD4 or anti-CD8 magnetic beads. Thymocytes were pre-purified with anti-CD4 or anti-CD8, before sorting DN, DP, CD4 SP and CD8 SP cells. Anti-CD43, Anti-CD19, Strepavidin, Anti-CD62L and Anti-CD4 MACS beads (Miltinyi Biotech) were used.

B.28. Th1 versus Th2 polarization in vitro

Naïve CD4+CD62L+ T lymphocytes, were enriched using anti-CD4 FITC isomer 1, multisort kit and CD62L magnetic beads (Miltiniy biotech). Purified cells were stimulated for 12 h with plate bound anti-CD3□(10 □g/ml), anti-CD3□(10 □g/ml) + anti-CD28 (5 □g/ml) or PMA (5 ng/ml)+ ionophor A23187 (100 ng/ml) in the presence of either recombinant mouse IL-12 (3.5 ng/ml, TEBU), anti IL-4 (2 □g/ml, clone 11B1, home made) and recombinant mouse IL-2 (20 U/ml, TEBU) for polarization to Th1 effector cells and with mouse recombinant IL-4 (1000 U/ml, R&D), anti-IFN□(2 □g/ml, Pharmingen), anti-IL12 (0.5 □g/ml, Pharmingen) and IL-2

(20 U/ml) for polarization to Th2 effector cells. As control cells were also stimulated without polarizing cytokines. On day 7 after the primary stimulation, the cells were restimulated 12 h in RPMI medium only. The supernatant was collected and the IFN and IL-4 concentrations were measured by ELISA. For intracellular staining the cultures were restimulated for 3 h in the presence of Brefeldin A (10 g/ml, Sigma).

C. Results

C.1. Analysis of itch mRNA expression in lymphocyte populations

The discovery of Itch and its initial isolation and characterization revealed that Itch is ubiquitously expressed and is also abundant in lymphoid tissues (Perry et al., 1998). Nevertheless we wanted to analyze in more detail the itch expression levels in the different subpopulations of B and T lymphocytes. Therefore, the level of itch mRNA expression in lymphocytes at different developmental stages was analyzed by reverse transcriptase polymerase chain reaction (RT-PCR) in real time using a Light Cycler. After enrichment by magnetic cell sorting (MACS) with the B cell specific marker protein CD19, bone marrow pro- B cells, pre-B cells, immature and re-circulating B cells were sorted (Fig. 4a). From the thymus double negative (DN), double positive (DP), CD4 single positive (CD4 SP) and CD8 single positive (CD8 SP) were sorted (Fig. 4a). To accelerate the sorting of CD8 SP and DN thymocytes, CD4+ cells were depleted prior to sorting. Splenocytes were enriched for B or T cells by CD43 depletion and enrichment respectively. Peripheral CD4 SP and CD8 SP T lymphocytes were sorted from spleen and lymph nodes and IgM^{high} IgD^{low}, $IgM^{+}IgD^{+}$ and $IgM^{low}IgD^{high}$ B lymphocytes were sorted from spleen of C57BL/6 wild-type mice (Fig. 4a). The analysis of itch transcripts is depicted as the ratio between itch mRNA and hypoxanthine phosphoribosyl-transferase (hprt) mRNA copies (Fig. 4b). I chose hprt because it is known to be expressed at low levels (Stout and Caskey, 1985). The expression of itch mRNA seems to increase during B lymphocyte development. The expression of itch mRNA in B cells is approximately doubled in recirculating B cells and in peripheral B cells compared to the pro-, pre- and immature cells in the bone marrow. The expression is then downregulated in peripheral $IgM^{low}IgD^{high}$ lymphocytes. For T lymphocytes an increase of the itch expression can only be seen in CD8 SP peripheral T lymphocytes (Fig.4b). The comparison of itch expression to hprt expression indicates that itch is expressed at low levels, since the expression of hprt is known to be expressed at low levels and the itch expression is at the most three times more.

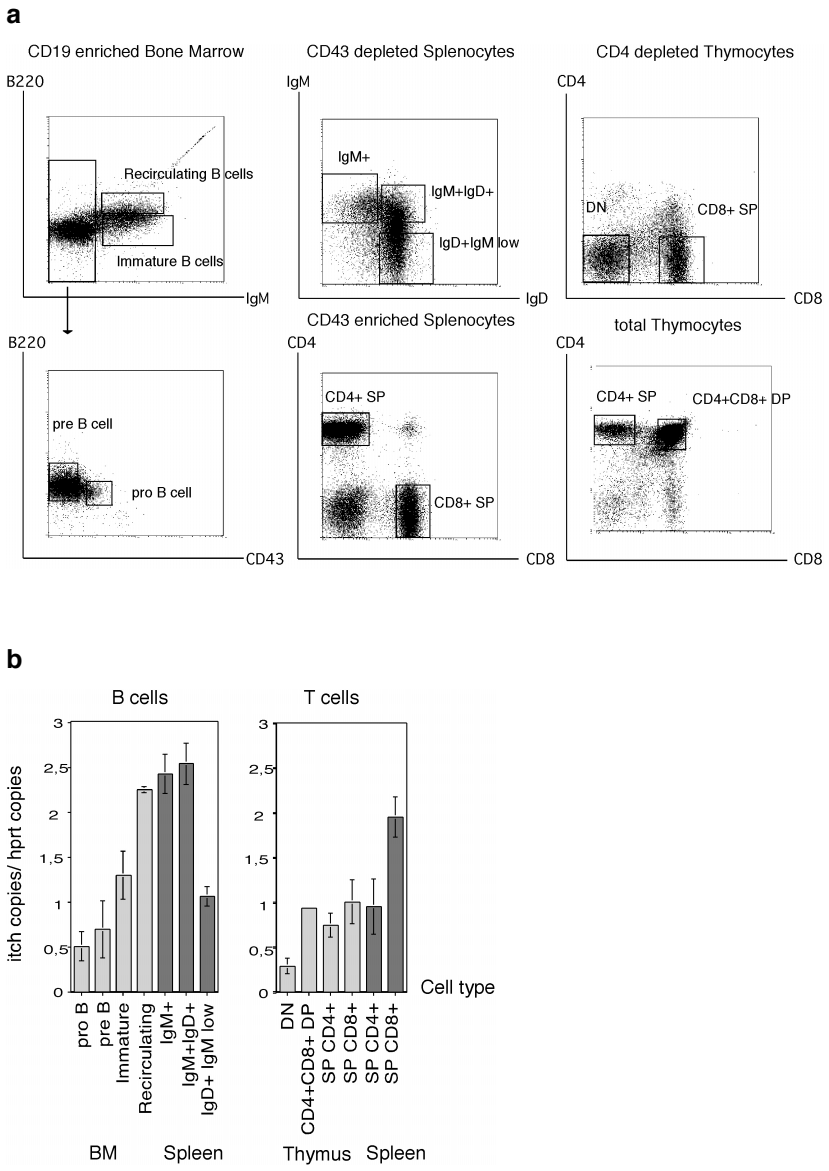


Figure 4. Itch mRNA expression in different lymphocyte populations.

a) Shows cell populations sorted for mRNA expression analysis. The sorted populations are indicated by gates. In order to enrich for B cells, the cells from bone marrow and spleen, were enriched for CD19 positive cells by MACS prior to sorting. To sort DN and CD8 SP thymocytes, thymocytes were depleted for CD4 positive cells prior to sorting. **b)** Shows the mRNA expression of itch compared to the expression of hprt in B and T cells at different developmental stages measured by RT-PCR in real time using the Light Cycler. The values indicated are the average of three independent experiments,

except for DP thymocytes were only one value was measured. The standard deviation is indicated. Peripheral cells from spleen are indicated in dark grey and cells from bone marrow and thymus are depicted in light grey.

C.2. Generation of mice harboring a loxP-flanked itch gene

C.2.1. Polymorphism in the *itchy* locus

a^{18H} mice lack the Itch protein due to a large inversion affecting the *itchy* locus and develop an autoimmune like disease on a C57BL/6 genetic background (Perry et al., 1998). The disease development of the mice seems to be strain dependent, since the phenotype is different in another genetic background (JU/Ct) analyzed. These mice also develop a severe disease, but other organs are affected (Perry et al., 1998). To analyze the involvement of Itch in the development of autoimmune disease, it is therefore important to generate *itch* mutant mice on a C57BL/6 genetic background. For this reason, we decided to use Bruce4 embryonic stem (ES) cells, which are derived from C57BL/6 mice. Since the efficiency of homologous recombination in ES cells usually decreases with increasing divergences between the source DNA, used to construct the targeting vector, and the ES cell DNA, we checked for polymorphism in the *itchy* locus by comparing restriction enzyme digest pattern of different mouse strains. Figure 5 shows the *itchy* genomic locus and the position of the restriction sites for BamHI and HindIII in C57BL/6 genomic DNA are indicated as well as the position of the probes used for identification by Southern blot analysis. For each mouse strain and genomic probe tested in the *itchy* locus, different size fragments were obtained by Southern blot analysis (Fig. 6), indicating a high polymorphism in the *itchy* locus. Therefore, the excisting *itch* clone derived from 129/OLA could not be used for our purpose and a genomic clone containing the *itchy* locus had to be isolated from the C57BL/6 mouse strain.

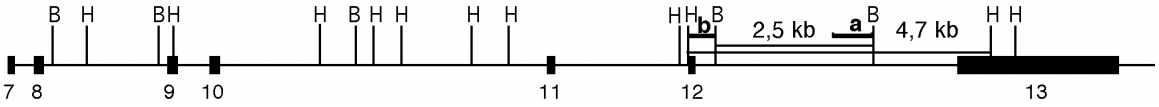


Figure 5. Restriction map of *itchy* genomic locus

Indicated are the positions of probe a and b in the itchy C57BL/6 genomic locus. B indicates BamHI restriction sites and H indicates HindIII restriction sites.

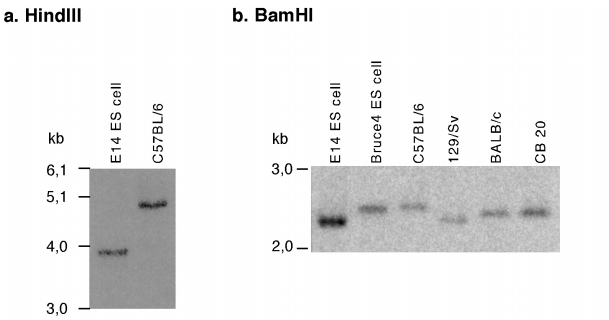


Figure 6. Southern blot analysis of genomic DNA derived from different mouse strains

The different mouse strains analyzed are indicated on top, the embryonic stem cell (ES) line E14 is derived from the 129/OLA mouse strain and the ES cell line Bruce 4 is derived from the C57BL/6 mouse strain. **a)** Genomic DNA digested with restriction enzyme HindIII and hybridized with probe a. **b)** DNA were digested with restriction enzyme BamHI and hybridized with probe b. The positions of the probes are depicted in Figure 5.

C.2.2. Sub-cloning and sequencing of the *itchy* genomic locus

A C57BL/6 BAC library from Genome Systems (BAC 9451 library) was screened with an *itch* cDNA probe containing most of the *itch* coding sequence. We used a probe obtained from the *itch* cDNA vector (a kind gift from Prof. N. Copeland), by a NsiI-SacI restriction enzyme digest resulting in a fragment of 4.2 kb containing the *itch* cDNA sequence from bp 26-4265.

BAC clone 23760 was identified and obtained from Genome Systems and was shown by Southern blot analysis to contain the *itchy* locus (data not shown). The BAC 23760 clone was digested with SpeI and two fragments of 7.2 kb and 7.8 kb, respectively, were cloned into pBSKS- vector. Both inserts were sequenced and the intron-exon boundaries of the *itch* gene were identified. The 7.2 kb fragment was shown to contain the last exon (13) of the *itch* gene and the 7.8 kb fragment was shown to contain exon 10 to 12. In addition, two fragments of 5.2 and 8.2 kb were purified after a SacI digest and cloned into the pBSKS- vector. The 8.2 kb fragment was shown to overlap the SpeI restriction site between the two SpeI fragments and contained exon 11, 12 and part of exon 13. The 5.5 kb fragment was shown to contain the exons 9 and 10 (data not shown).

C.2.3. Construction of the *itch* targeting vector for homologous recombination in ES cells

A targeting vector for conditional Itch inactivation was designed to introduce two *loxP* sites flanking exon 12, which encodes the part of the hect domain containing the conserved cysteine essential for ubiquitin binding and ligase activity (Fig. 7a) (Fang et al., 2002; Qiu et al., 2000). A splicing event of the itch mRNA from exon 11 to 13, which may occur after deletion of exon 12, would result in an out of frame message with the usage of a premature termination codon during mRNA translation. This is expected to result in an inactive protein of 812 aa of approximately 108 kDa, since a point mutation of the conserved cysteine at position 822 results in an inactive ubiquitin protein ligase (Fang et al., 2002; Qiu et al., 2000). The *itch* targeting vector was constructed by introducing the *itch* genomic sequence by blunt end ligation of all fragments into the pEasyflox vector (Alimzhanov, M. unpublished) (Fig.7a.). The pEasyflox vector contains the selection marker neomycin phosphotransferase gene (neo^r) driven by the phosphoglycerate kinase promoter (PGK- neo^r), that is used for positive selection of transfected clones with G418. The vector also contains a Herpes Simplex Virus-1 thymidine kinase gene (HSV-tk), used for negative selection by addition of Gancyclovir. First an additional EcoRV restriction site was introduced by blunt end ligation of an EcoRV linker into the pEasyflox vector adjacent to the third *loxP* site to allow identification of clones which had co-integrated the third *loxP* site. Secondly, a 7.6 kb HaeII-SpeI fragment,

harboring exon 10 and 11, was cloned into the XhoI restriction site by blunt end ligation between the third *loxP* site and the HSV-tk in the pEasyflox vector. This was followed by insertion of a 2.5 kb NheI-HaeII fragment harboring exon 12, into the XbaI restriction site, resulting in this region being flanked by two *loxP* sites. Finally, a 3.6 kb SacI-NheI fragment harbouring part of the last exon 13, was cloned into the NotI restriction site prior to the *loxP* flanked PGK- neo^r selection marker.

C.2.4. Transfection of ES cells and identification of homologous recombinant clones

The *itch* targeting vector was linearized by ClaI restriction enzyme digest prior to ES cell transfection by electroporation. The external probe d was used for identification of homologous recombinant clones and the internal probe e was used for identification of recombinant clones, which had co-integrated the third *loxP* site (indicated in Fig.7a). Southern blot analysis of genomic DNA using EcoRV digest and probe d results in a 9.9 kb fragment for the endogenous *itchy* locus, a 6.0 kb fragment for the homologous recombined locus, a 6.8 kb fragment after Cre-mediated deletion of the neo^r cassette and a 7.4 kb fragment for complete excision of the *loxP* flanked region (Fig. 7b and c). Usage of probe e after EcoRV digest, to identify co-integration of the third *loxP* site results in a 9.9 kb endogenous fragment, a 6.0 kb fragment for non co-integration and a 3.1 kb fragment for co-integration of the third *loxP* site (Fig. 7b). Results from the transfection of ES cells with the *itch* targeting vector are depicted in Table 1. The ratio of homologous recombinant clones was 1 in 8 picked clones and of these 40% had co-transmitted the third *loxP* site. To excise the neo^r cassette, 2 of the homologous recombinant clones were transiently transfected with 20 □g of a vector expressing Cre recombinase under the control of the HSV-tk promoter and enhancer (pIC-Cre) (Gu et al., 1993). After transient transfection with the Cre expressing vector, approximately 10% of the picked clones were G418 sensitive and of these 10% were partially deleted (deletion of the neo^r cassette only) and 90% had deleted the complete *loxP* flanked region. Genomic DNA of G418 sensitive clones was analysed for deletion of the neo^r cassette by Southern blot analysis. Deletion of the neo^r cassette resulted in a 6.8 kb fragment after digest with EcoRV and hybridization with probe d, whereas complete deletion of the neo^r and the *loxP* flanked (*floxed*) *itch* genomic sequence resulted in a fragment of 7.4 kb (Fig. 7c).

Two independent homologous recombinant clones carrying the *floxed itch* allele with the neo^{r} cassette removed only, were injected into CB20 blastocysts, which were then implanted into pseudo-pregnant (F1 C57BL/6 x Balb/C) foster mothers to generate chimeric mice. A total of 16 chimeras were generated of which 10 were bred to C57BL/6 mice. Of these, 3 gave 100% germ-line transmission by coat color, one gave partly germ-line transmission and two did not give any offspring. These results are summarized in Table 2.

Mice heterozygous for the *itch floxed* (*itch* $^{fl/+}$) allele were intercrossed to generate mice homozygous for the mutation. The homozygous *itch*$^{fl/fl}$ mice were viable, fertile and indistinguishable from wild-type (wt) littermates with regard to surface expression of common lymphocyte markers (data not shown).

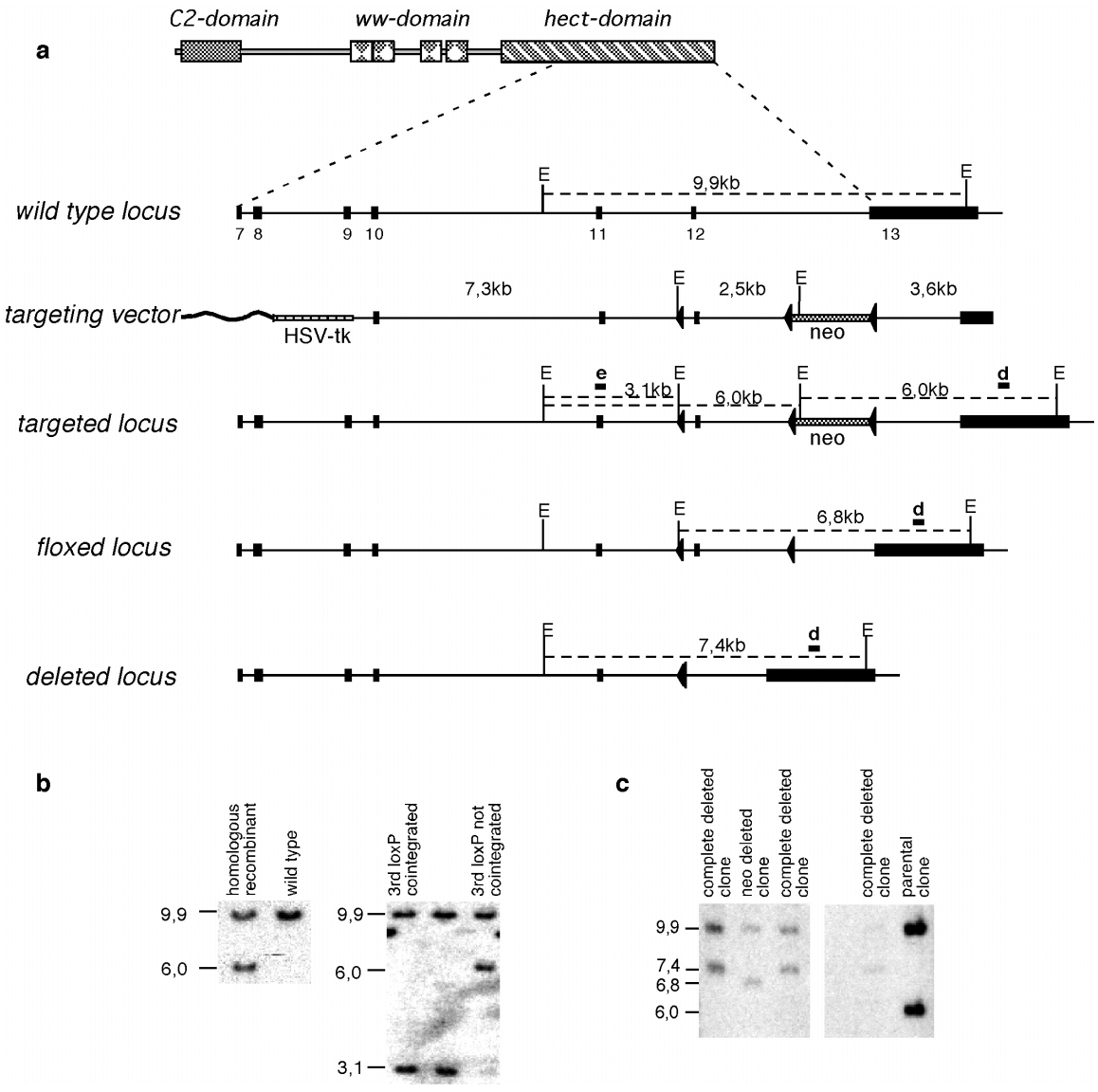


Figure 7. Generation of ES cells with targeted mutation in the *itchy* locus

a) The Itch protein and genomic structure. Indicated are the C2-domain, ww -domains and the hect-domain. The sequenced area of the *itch* gene harboring part of exon 7 to the last exon 13, is depicted and corresponds to approximately two thirds of the hect domain. A map of the targeting vector pBSitchflox and the targeted *itchy* locus before and after Cre mediated recombination are shown. Exons are indicated as filled boxes and introns as thin lines. The *loxP* sites are indicated as filled triangles also indicating their direction. The position of the neo^r and the HSV-tk genes are indicated on

the targeting vector. **b)** Identification of homologous recombinant ES cell clones by Southern blot analysis. Blot on the left hand side shows genomic DNA from two ES cell clones digested with restriction enzyme EcoRV and hybridized with probe d. Bands corresponding to the wt (9.9 kb) and the targeted allele (6.0 kb) are indicated. The blot on the right hand side shows homologous recombinant ES cell clones digested with restriction enzyme EcoRV and hybridized with probe e for identification of clones that have co-integrated the 3rd *loxP* site. Bands corresponding to the wt allele (9.9 kb), the allele with (3.1 kb) or without (6.0 kb) the third *loxP* site co-integrated are indicated. **c)** Southern blot analysis of Cre/*loxP* mediated recombination at the *itchy* locus in homologous recombinant ES cell clones to achieve deletion of the *loxP* flanked neo^r gene. Genomic DNA from homologous recombinant ES cells transfected with the Cre expression vector pIC-Cre as well as the parental clone were digested with EcoRV and hybridized with probe d. Bands corresponding to the wt (9.9 kb), homologous recombinant (6.0 kb), complete deleted (7.4 kb) and the partial deleted allele (6.8 kb) are indicated.

Table 7. Transfection efficiency

ES cell transfection with	Number of picked clones	Number of homologous recombinants	Homologous recombinant clones with third *loxP* site cointegrated	Clones transiently transfected with pIC-Cre (20□g)	Number of picked clones	G418 sensitive clones	Partially deleted clones
pBSitchflox	466	60	25	1B12	192	18	2
				2D4U	192	22	2

Table 8. Germ-line transmission

ES clone	Blastocysts injected	Born pups	Chimeras	Chimera in breeding	Chimeras giving 100% germ-line transmission	Off-spring	Germ-line transmission by coat color	Offspring with modified *itch* allele
1B12H	43	9	5	3	1	31	18	12
2D4U	44	19	11	7	2	81	48	23

C.3. Generation of mouse strains with ubiquitous or cell type specific inactivation of the itch gene

C.3.1. Generation of mice with ubiquitous deletion of *itch*

To generate mice with a ubiquitous Itch mutation, the *itch* $^{fl/+}$ mice were bred to the Cre-deleter strain (Schwenk et al., 1995), which expresses Cre in germ cells and in early blastocyst stage. Heterozygous mice for the deleted *itch* allele (*itch*$^{\Delta/+}$), were intercrossed to generate *itch*$^{\Delta/\Delta}$ mice.

C.3.2. Cell type specific inactivation of the *itch* gene

To achieve T cell specific inactivation of Itch, *itch* $^{fl/fl}$ mice were crossed to Cre-transgenic mice expressing Cre under the control of the CD4 promoter (CD4-Cre transgenic mice) (Wolfer et al., 2001). The CD4-Cre transgenic mice have previously been shown to delete *loxP* site flanked (*floxed*) DNA segments with high efficiency starting at the double negative (DN) stage of thymocyte development (Wolfer et al., 2001). The deletion efficiency for the *itchy* locus in *itch* $^{fl/+}$ CD4Cre+/- and *itch* $^{fl/fl}$ CD4Cre+/- mice was determined by Southern blot analysis of DNA from MACS purified thymocytes and peripheral T cells (Fig. 8). The intensity of the bands on the Southern blot was measured by phosphoimager and the deletion efficiency was estimated to be 90% in the thymus and 100% in the periphery. The absence of deletion in non T cells from spleen and lymph nodes shows that the deletion is specific for T cells.

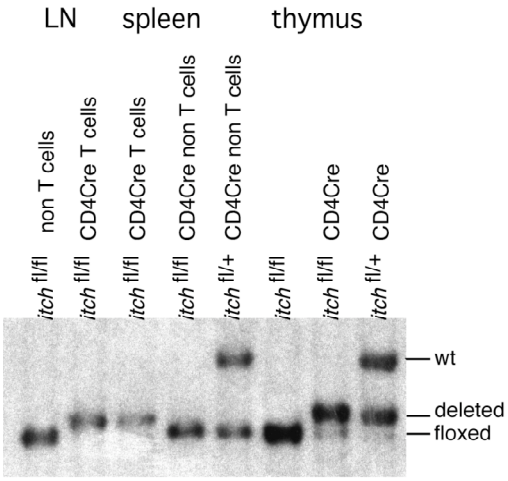


Figure 8. Deletion efficiency in *itch floxed* CD4-Cre mice

Genomic DNA from splenocytes or thymocytes was digested with EcoRV and hybridized with probe d. Lanes marked non T cells were enriched for B220, HSA (CD24), Gr-1 and Mac-1 by MACS technology, lanes marked T cells were purified by depletion for the same markers.

To achieve B cell specific deletion of the *itch* gene, *itch* $^{fl/fl}$ mice were crossed to CD19-Cre mice (Rickert et al., 1997). The CD19-Cre mouse express Cre under the transcriptional control of the B cell lineage restricted CD19-gene and was obtained by insertion of the Cre gene in the CD19 locus. This results in a disruption of the CD19 coding sequence, leading to a CD19 deficiency in the homozygous situation. However, mice heterozygous for the *cre* insertion, are phenotypically normal. The deletion efficiency for the *itchy* locus in *itch* $^{fl/+}$ CD19Cre+/- and *itch* $^{fl/fl}$ CD19Cre+/- mice was estimated to 80-90% in peripheral splenic B cells (Fig. 9). The purity of CD19+ cells was 97-99%. The non B cells represent the CD19 negative fraction and reached a purity of 70-90%. The absence of deletion in thymocytes indicates that the deletion is specific for B cells. In splenic non B cells there is a faint band for the deleted allele, which is probably due to a contamination of B cells since the purity of B cells reaches 70-90%.

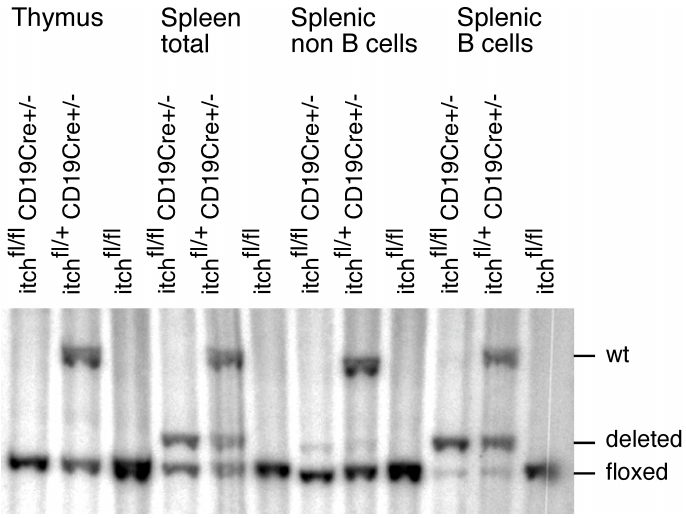


Figure 9. Deletion efficiency in *itch floxed* CD19-Cre mice

Genomic DNA from lymph nodes, splenocytes and thymocytes were digested with EcoRV and hybridized with probe d. Lanes marked B cells were enriched for CD19 positive cells and non B cells were depleted for the same marker using MACS technology.

C.4. Expression of Itch protein in itch mutant mice

C.4.1. Generation of anti-Itch polyclonal antibodies

Since no anti-Itch antibody was commercially available, we decided to generate rabbit polyclonal antibodies, in order to study expression of Itch protein. Because a truncated Itch protein could potentially be expressed from the disrupted *itchy* locus, both an antibody directed against a C-terminal Itch peptide as well as an N-terminal Itch peptide were generated. The antibody directed against the N-terminal peptide should detect any Itch protein truncated at the C-terminus, whereas it would not be recognized by an antibody against the C terminal peptide. The position of the peptides used to immunize rabbits for generation of the Itch antibodies are indicated in Fig.12a. The binding specificity of the anti Itch antibodies was tested by enzyme-linked immunosorbent assay (ELISA). Plates were coated with the peptides used for immunization or with recombinant GST-Itch fusion protein expressed and purified

from E.coli. Figure 10 shows the purity of the GST- Itch fusion protein used for the ELISA. I could show that both anti N-terminal and anti-C-terminal Itch antibodies specifically recognize the corresponding peptide as well as the recombinant GST-Itch fusion protein (Fig.11a-d), but not GST only (Fig. 11e-f).

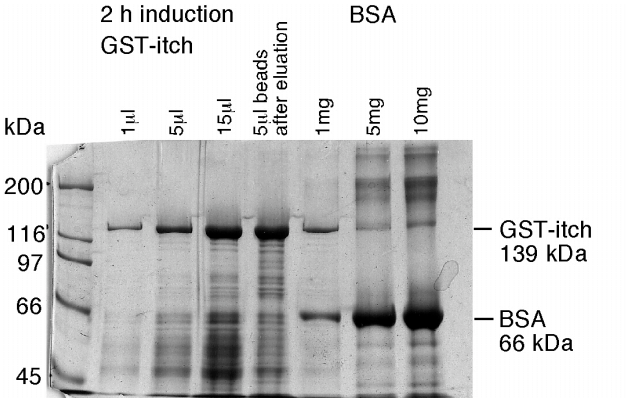


Figure 10. Expression of GST-Itch recombinant protein

Purity of GST-Itch recombinant protein. GST-Itch fusion protein was expressed in E.coli and purified on a Gluthathione Sepharose matrix. Eluate was loaded on an 8% SDS-polyacrylamid gel and stained with Coomassie. Different amounts of BSA were loaded to estimate protein concentration of GST-Itch. In the lanes loaded with BSA there is also a protein running at the same height as GST-Itch, this is due to unpurity in the BSA preparation.

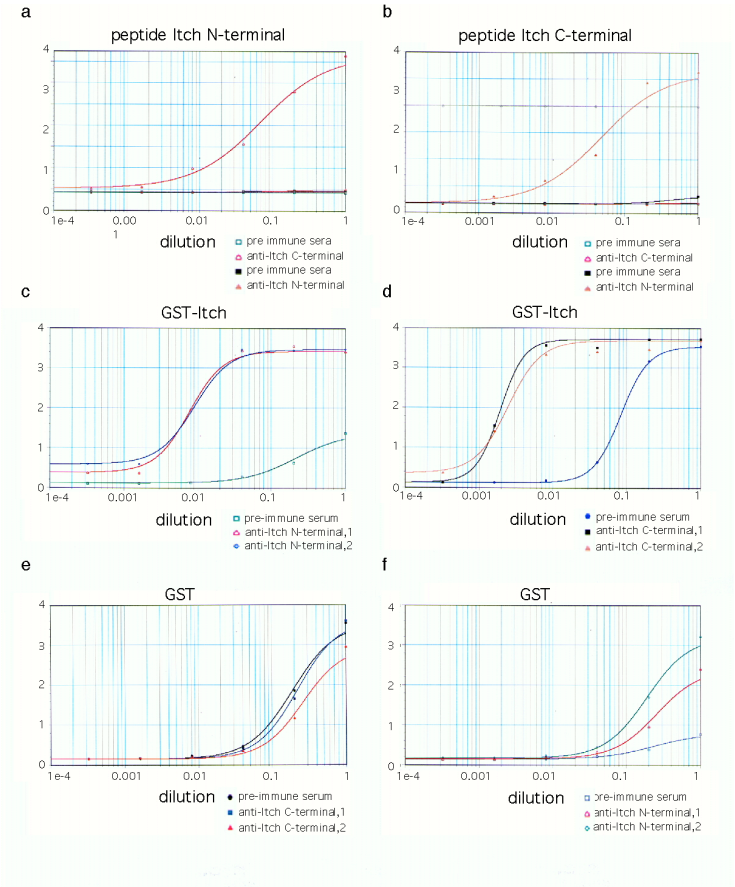


Figure 11. Specificity of rabbit anti mouse Itch polyclonal antibodies

ELISA assay for binding specificity of rabbit anti mouse Itch antibodies. ELISA plates were coated with the specific peptides used for immunization, **a)** N-terminal peptide **b)** C-terminal peptide, **c)** and **d)** recombinant GST-itch fusion protein or **e)** and **f)** GST only. Pre immune sera and anti-Itch immune sera directed against the C-terminal peptide or the N-terminal peptide were tested for specific binding. Anti-Itch 1 and 2 correspond to immune sera from the first and second bleeding, respectively.

C.4.2. Expression of Itch protein in *itch* mutant mice

The purpose of conditional gene targeting is the inducible or cell specific inactivation of the target gene. A prerequisite to this is that the locus should remain intact after introduction of two *loxP* sites. It is therefore necessary to confirm that the expression of the Itch protein remains unchanged in the $itch^{fl/fl}$ mice compared to wild-type mice. In addition, it should be confirmed that the genetic modification of the *itchy* locus indeed results in a deletion of active Itch protein after Cre mediated recombination. The targeting strategy used, aiming at deletion of exon 12, possibly results in the expression of an inactive truncated Itch protein. To check whether this was the case, I performed Western blot analysis using both the anti Itch N-terminal and the anti Itch C-terminal antibodies. Fig. 12b shows that the Itch expression was indeed unaltered in brain, spleen and thymus of $itch^{fl/+}$ and $itch^{fl/fl}$ compared to wild-type mice. This suggests that the *loxP* sites introduced in the intron12 and 13 of the *itch* gene have no effect on the expression of the Itch protein. Similarly, the expression of the Itch protein was determined in brain, liver and spleen lysates of $itch^{\Delta/\Delta}$ mice compared to $itch^{+/\Delta}$ and $itch^{+/+}$ (Fig.12c), and in thymocytes, peripheral T cells and non T cells of $itch^{fl/fl}$ CD4Cre+/- mice (Fig.12d). Expression of Tubulin was determined in order to control the amount of protein extract loaded on the gel. The T cells were purified by MACS technique by depletion of B220, CD24, Mac-1 and Gr-1 positive cells and reached a purity of 60% T cells determined by staining cells for the T cell marker CD90. The Itch antibody directed against the C-terminal peptide was unable to detect any protein in lysates from $itch^{\Delta/\Delta}$ mice, while the Itch protein could still be detected using the anti Itch N-terminal antibody, indicating that a truncated Itch protein is expressed. In the $itch^{fl/fl}$ CD4Cre+/- mice the expression of full length Itch was lost in the peripheral T cells, whereas in thymocytes, Itch expression could still be detected. Since the deletion of the floxed *itch* gene is almost complete in thymocytes from $itch^{fl/fl}$ CD4Cre+/- mice (Fig.8), the remaining expression of full-length Itch in this cell population could be explained by a relatively long half-life of this protein, which would delay the disappearance of full length Itch from the cells. A faint band of a slower migrating protein than Itch could be

detected in the *itch*$^{-/-}$ spleen lysate and in the peripheral splenic T cells of *itch*$^{fl/fl}$ CD4Cre+/- mice. This is most probably due to a cross-reactivity of the antibody towards a protein other than Itch, since in the *itch*$^{-/-}$ mice, the Itch gene is expected to be inactivated in all tissues. From this assay, we can conclude that no active Itch is expressed in *itch*$^{-/-}$ mice and in T lymphocytes from *itch*$^{fl/fl}$ CD4Cre+/- mice.

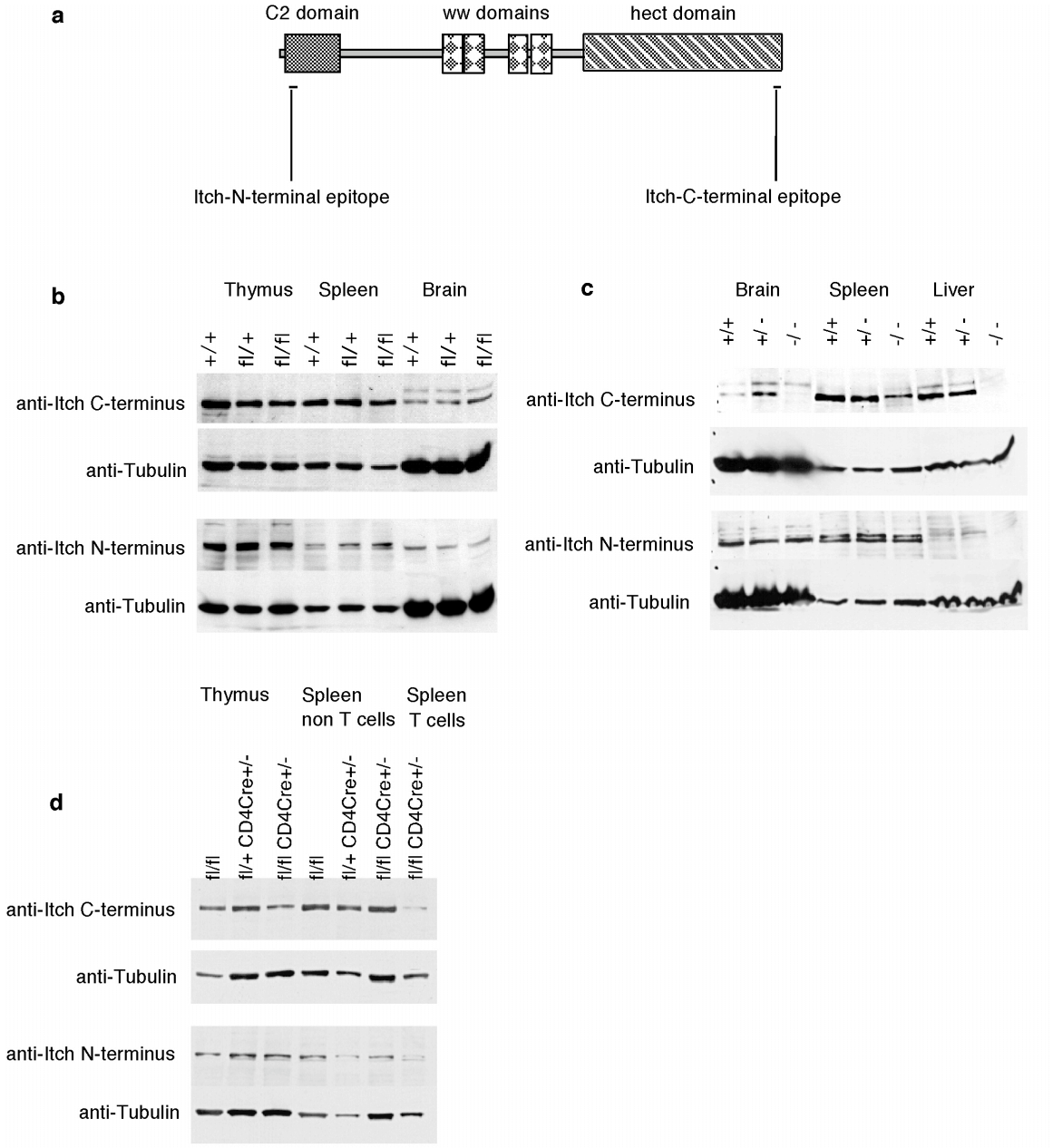


Figure 12. Expression of the Itch protein in *itch* mutant mice.

a) The Itch protein structure. The position of the peptides which were used for immunization of rabbits to generate the polyclonal Itch antibodies are indicated. **b)** Western blot analysis of thymus, spleen and brain lysates from witd type (*wt)*, *itch* $^{fl/+}$, and *itch* $^{fl/fl}$ mice. **c)** Western blot analysis of lysates from spleen, brain and liver from wt, *itch* $^{-/+}$ and *itch* $^{-/-}$.**d)** Shows Western blot analysis of lysates from

splenic T cells and non T cells of the *itch* $^{fl/fl}$, *itch* $^{fl/+}$ CD4Cre+/- mice and *itch* $^{fl/fl}$ CD4Cre+/- mice. The Itch protein was detected with either anti-Itch C-terminus or anti-Itch N-terminus antibodies and thereafter with anti-Tubulin for verification of equal protein loading.

C.5. Phenotypical analysis of itch mutant mice

C.5.1. Systemic inflammation in *itch* mutant mice

The *itch*$^{fl/fl}$ CD4Cre+/- mice as well as *itch*$^{\Delta/\Delta}$ mice, show splenomegaly at the age of 8 weeks (Fig.13). With age both of these mice develop a systemic inflammatory disease, at 16 weeks of age the mice were severely diseased and died at the age of 27-30 weeks. To describe the observed disease in more details, a pathological analysis was performed on old (6 and 7 month) *itch*$^{fl/fl}$ CD4Cre+/- mice. This analysis of the *itch*$^{fl/fl}$ CD4Cre+/- mice revealed pneumonia of the lungs, with large parts where the alveoli were filled with macrophages containing eosinophilic cytoplasma, as well as perivascular infiltration of lymphocytes and plasma cells. The liver showed hepatitis with central necrosis of hepatocytes, granoloma and clusters of lymphocytes and plasmocytes. Hyperemia of the spleen, which was infiltrated with macrophages containing crystalloid inclusions and basophilic multinuclear cells and a large increase of plasma cells. Kidney was changed to a cysterna, where the fibrotic chronical inflammation was evident. Parts of the kidney tissue were atrophic with evident inflammatory infiltration, the pelvis was extremely distended and the tissue of the medulla was very decreased. The stomach showed gastritis where focal clusters of lymphoid and polymorphonuclear cells were localized. Inflammatory processes were also observed in the duodenum, jejunum and ileum. The tail suffered from dermatitis, where clusters of polymorphonuclear and lymphoid cells surrounded some hair sheets and the surrounding tissue. The inflammatory infiltration was also found in the musculature of the tail. Dermatitis was also seen on the skin and ears (Fig.14). The pathological signs were more severe in females compared to males. Observed was also a loss of weight with age of *itch*$^{fl/fl}$ CD4Cre+/- compared to wild-type littermates and a thickening of the blood leading to a fast coagulation after bleeding. In the *itch*$^{fl/+}$ CD4Cre+/- and *itch*$^{\Delta/+}$ as well as in the *itch* $^{fl/fl}$ CD19Cre+/- and *itch*$^{fl/+}$ CD19Cre+/- mice, no development of disease or abnormalities could

be observed. We therefore assumed that the induction of the disease is dependent on loss of active Itch in T cells, and decided to focus our analysis on mice with a T cell specific deletion of active Itch.

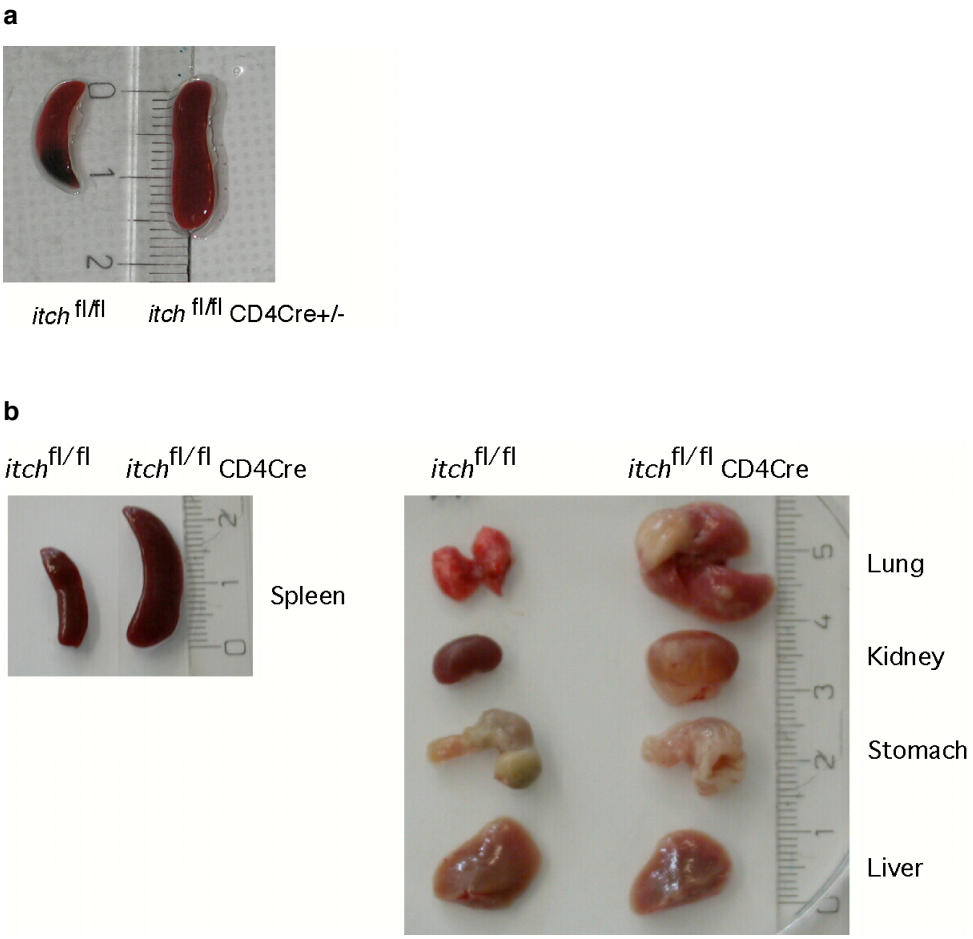


Figure 13. Organs of mice with a T cell specific deletion of Itch

a) Spleen from 8 week old itch $^{fl/fl}$ mice and itch $^{fl/fl}$ CD4Cre+/-. **b)** Organs from 24 week old diseased itch $^{fl/fl}$ CD4Cre+/- mice compared to organs of littermate itch $^{fl/fl}$ mice. Depicted are spleen, lung, kidney, stomach and a piece of the liver.

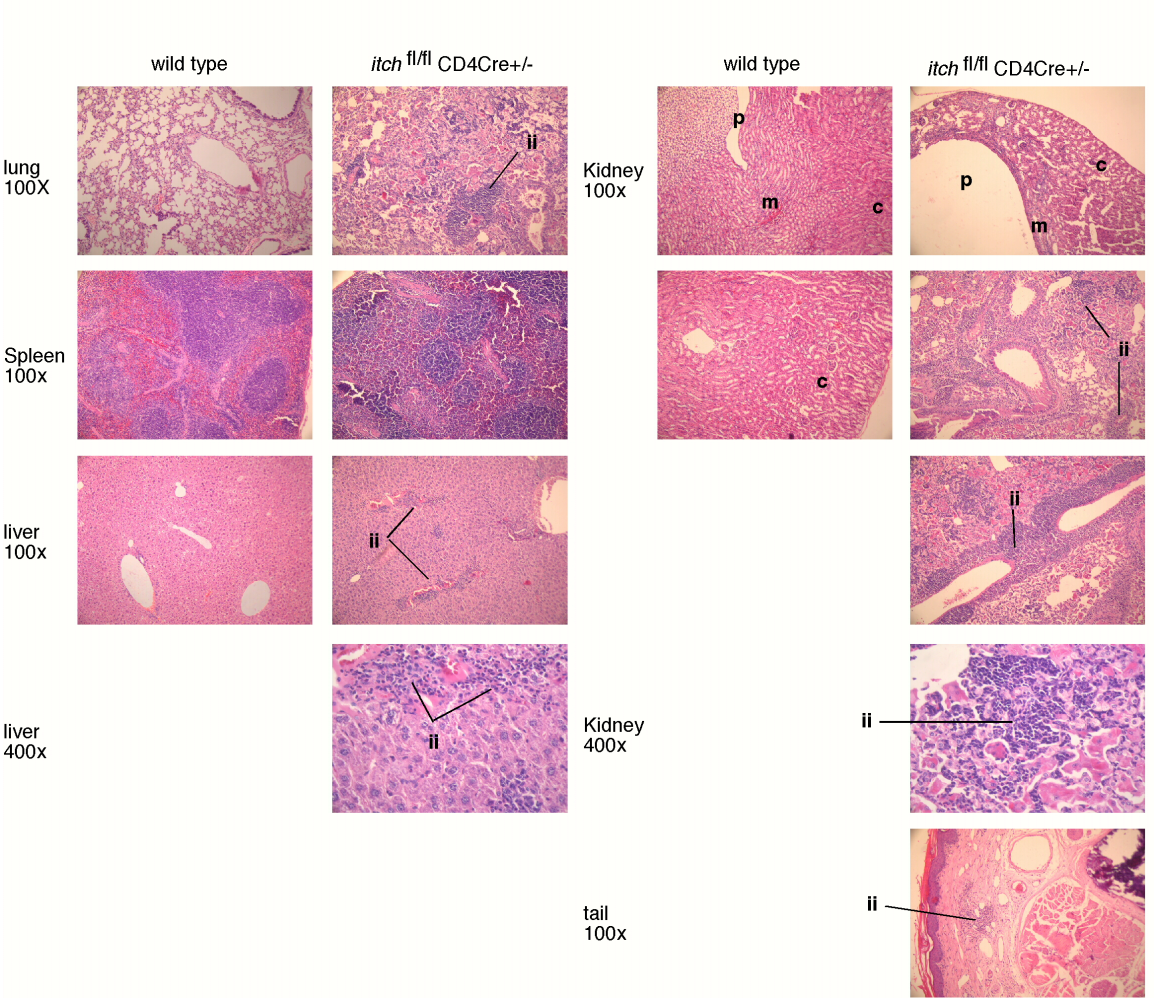


Figure 14. eosin/hematoxiline staining of sections from different organs of 20w old *itch* $^{fl/fl}$ CD4Cre+/- and wild type mice. Shows infiltration of inflammatory cells into several organs including organ destruction of the kidney, were the pelvis in extremely distended and the tissue of the medulla very reduced. Infiltration of inflammatory cells is observed in all organs. g=glomeruli, m=medulla, c= cortex, p= pelvis, ii= inflammatory infiltration, ib=intrahepatic billiary

In order to further identify the type of inflammatory cells infiltrating the affected organs, specific staining for neutrophils and macrophages as well as for B and T cells were performed. In older (20w) sick *itch* $^{fl/fl}$ CD4Cre+/- animals and controls, sections were performed of organs, as well as the ears and skin, the lung, liver and spleen. The sections were stained for macrophages (anti-F4/80) and neutrophils (anti-Ly-6G (anti-Gr-1)) (Fig.15). F4/80 is a marker, which is specific for macrophages. Mac-1 is expressed at varying levels on granulocytes, macrophages, dendritic cells and natural killer cells and Ly-6G is a specific marker for neutrophils in the periphery. In the *itch* $^{fl/fl}$ CD4Cre+/- mice, infiltration of neutrophils and macrophages of the ears, but also an infiltration of the lung was clearly apparent. In addition, an infiltration of neutrophils in the liver was observed. In contrast, no increase of the amount of liver macrophages (Kuppfer cells) could be identified in the *itch* $^{fl/fl}$ CD4Cre+/- mice. In addition, we used FACS analysis to detect these cell types in the peritoneal cavity exudates and in the spleen. An increase of the percentage of neutrophils and macrophages was also determined in the spleen and peritoneal cavity exudates of old *itch* $^{fl/fl}$ CD4Cre+/- mice (Fig.17). Fig.17 shows an increase of the Gr-1 and Mac-1 double positive cells in the *itch* $^{fl/fl}$ CD4Cre+/- spleen, which correspond to activated neutrophils, since Mac-1 is rapidly upregulated on neutrophils after activation (Kishimoto et al., 1989). These data indicate that there are ongoing inflammatory processes in several organs and the data support the concept of systemic inflammatory disease in these mice.

To clarify whether B and T cell are also infiltrating the affected organs, which would indicate a direct role of these cells in this process, staining for T and B cells of frozen sections of lung, ear, stomach, skin, kidney and liver were performed. CD3+ T cells were infiltrating the lung, parts of the stomach, at the border of the distended pelvis of the kidney and the area around some blood vessels in the liver. Infiltration of CD19+ B cells was found in the lung, ear and skin, and in the kidney (Fig.16).

The structure of the B cell and T cell areas in the lymphoid organs as spleen, lymph node and thymus were analyzed by double staining for the B cell marker CD19 and the T cell marker CD3(Fig.16). The normal structure of B and T cell area in the spleen was disrupted in *itch* $^{fl/fl}$

CD4Cre+/- mice (Fig.16a). In the *itch* $^{fl/fl}$ CD4Cre+/- mice, the T cell areas (red) were smaller and more dispersed. In addition, the areas of non B and non T cells seemed increased, possibly indicating an aberrant T-B cell interaction. The thymus of these animals had developed a structure, which reminded of secondary lymphoid organs (Fig. 16b). The thymus was also smaller and the tissue consistency was harder. In the mesenteric lymph nodes the structure of the T and B cell areas seemed similar to wild type mice but slightly larger (Fig. 16c).

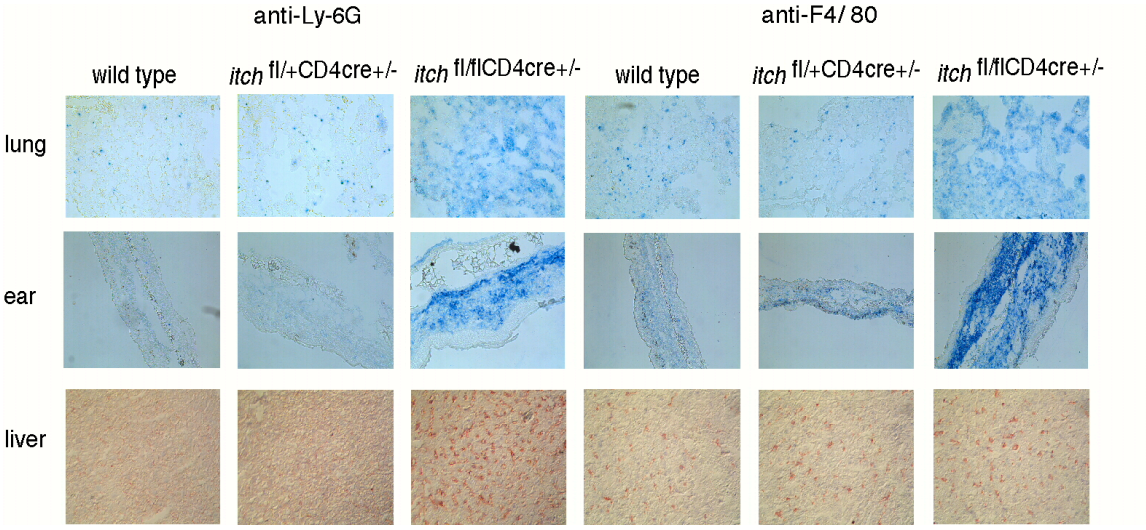


Figure 15. Infiltration of inflammatory cells into several organs of *itch* floxed CD4Cre+/- mice. Frozen sections of lung, ear and liver and spleen of 20 weeks old mice stained for neutrophils (anti-Ly-6G) and macrophages (anti-F4/80). The antibody used is indicated on the top. Magnification is 100x. In lung and ear anti-Ly-6G and anti-F4/80staining in blue and in liver staining in red.

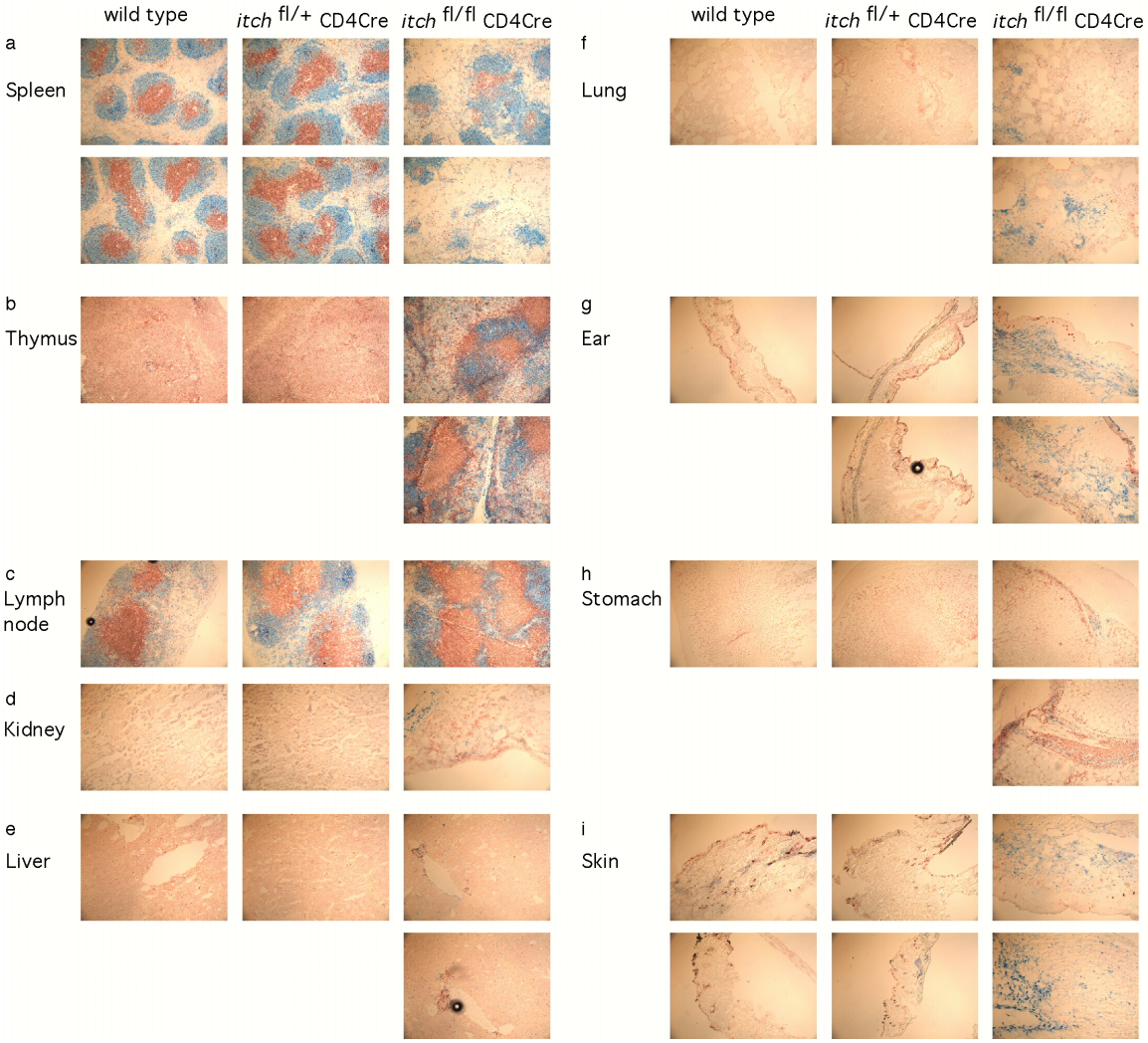


Figure 16. B and T cells in different organs of *itch* mutant mice

Frozen sections of spleen, thymus, lymph node, kidney, liver, lung, ear, stomach and skin of 20 week old mice stained for CD19+ B cells (blue) and CD3 positive T cells (red). The magnification is 100 x.

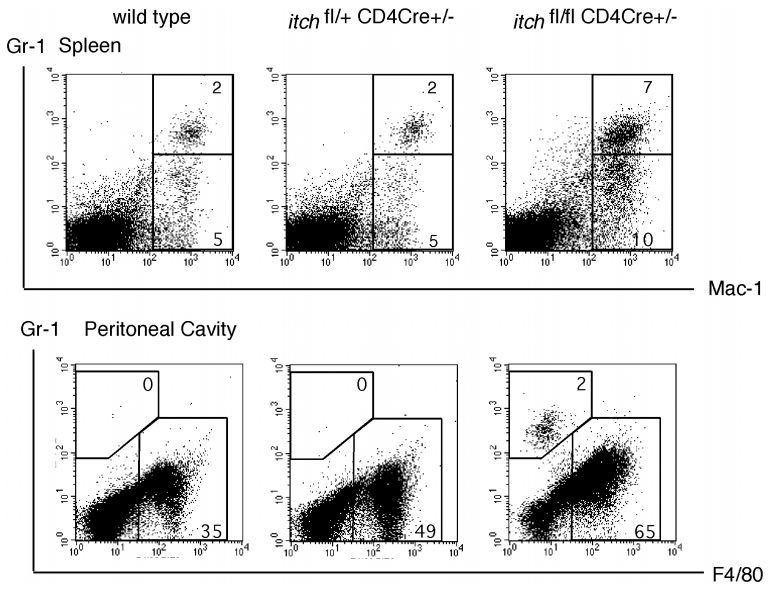


Figure 17. Analysis of neutrophils and macrophages in Spleen and Peritoneal Cavity in 17 week old *itch* $^{fl/fl}$ CD4Cre+/- mice.

The numbers correspond to the percentage of cells in the specific gate. Spleen and Peritoneal Cavity cells stained for neutrophils Gr-1 (Ly-6G) and macrophages (Mac-1) and (F4/80).

C.5.2. Elevated levels of blood eosinophils and basophils and signs of anemia in itch $^{fl/fl}$ CD4 Cre+/- mice

To better understand which factors could be involved in the development of the systemic inflammatory disease seen in *itch* $^{fl/fl}$ CD4 Cre+/- mice and *itch* $^{-/-}$, blood from these mice was analyzed in respect to leukocyte composition as well as erythrocytes and hemoglobin levels. In 8 week old *itch* $^{fl/fl}$ CD4 Cre+/- animals the only difference compared to wild type and heterozygous animals detected, was an increase of the percentage of eosinophils in the blood (data not shown). In older (16-23 weeks) *itch* $^{fl/fl}$ CD4 Cre+/- and *itch* $^{-/-}$ animals, there was in addition to the increase of eosinophils also a decrease of the erythrocytes number and the hemoglobin levels as well as a decrease of the hematokrit value (Fig. 18a and b). Reduced

levels of erythrocytes and hemoglobin are a typical indication of anemia. In addition, a low hematokrit value indicates a decreased amount of erythrocytes/liter blood, data which confirm the erythrocyte and hemoglobin data. Observed was also a slight increase of the percentage of basophils. Both eoinophils and basophils are typical mediators of allergic inflammatory reactions, reviewed in (Walsh, 2001). In addition, eosinophil differentiation and activation is promoted by cytokines secreted by Th2 polarized cells (Rothenberg, 1998).

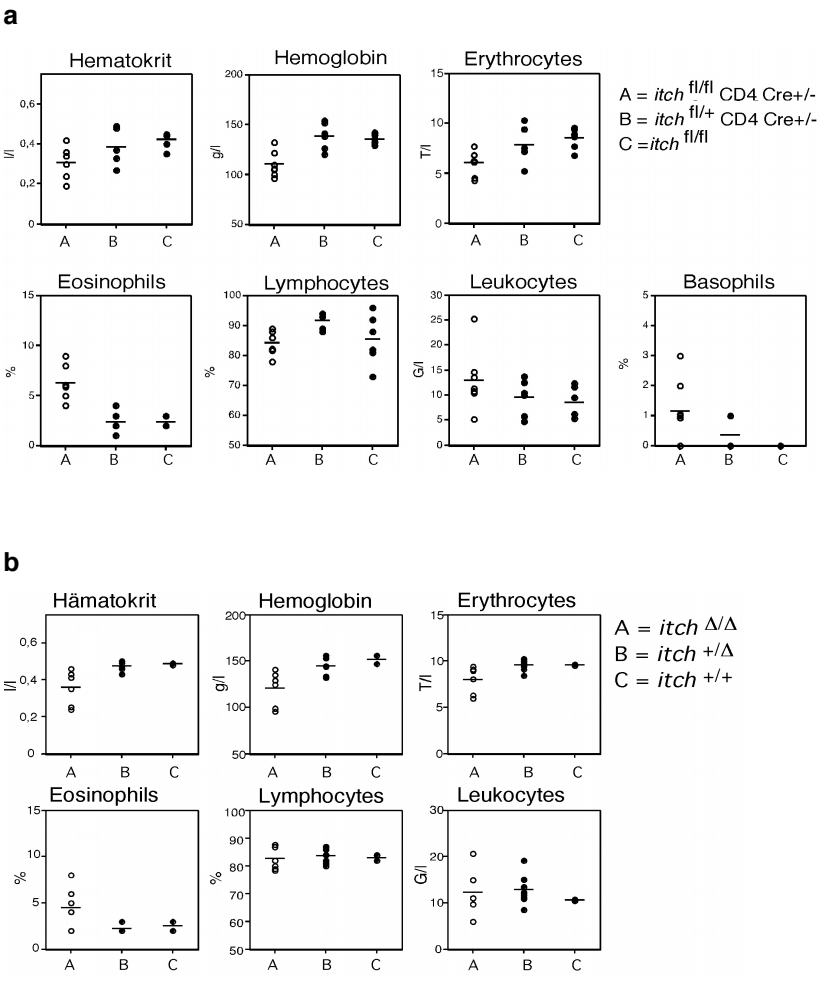


Figure 18. Blood analysis of *itch* mutant mice

a) Blood from 6 *itch* $^{fl/fl}$ CD4Cre+/-, 6 *itch* $^{fl/+}$ CD4Cre+/- and 7 wild type 17-23 weeks old mice and **b)** from 6 *itch* $^{\Delta/\Delta}$, 8 *itch* $^{\Delta/+}$,and 2 wild type animals were analyzed for erythrocyte, eosinophil, lymphocyte, leukocyte and basophil cell counts, hematokrit and hemoglobin. Hematokrit is measured as liter

erythrocytes/liter blood. Hemoglobin is measured in gram/liter blood. Eosinophils, basophils and lymphocytes are measured as percentage of total leukocyte number. Erythrocytes are measured as 10^{12} cells/liter blood (T/l). The average values are indicated as horizontal lines.

C.5.3 Cell numbers in lymphoid organs of itch mutants

Number of cells in lymphoid organs of 8 weeks old *itch* $^{fl/fl}$ CD4Cre+/- was compared to wild type mice. In the spleen, mesenteric lymph nodes and in the peritoneal cavity the total numbers of cells are increased in *itch* $^{fl/fl}$ CD4Cre+/- mice compared to wild type mice. Lymphocytes were identified by size and density, and could be gated for in a Fluorescence-activated cell sorter (FACS) forward scatter. The number of lymphocytes versus non lymphocytes were evaluated by comparing the percentage of cells in the lymphocyte gate to the total number of cells counted. In spleen and lymph nodes the total increase of cells is due to an increase of both lymphocytes and non lymphocytes, whereas in the peritoneal cavity the increase seems to be mainly due to an increase of non lymphocytes. The total cell number in the bone marrow is similar to wild type, but there seems to be an increase in the proportion of non lymphocytes compared to lymphocytes. In the thymus of *itch* $^{fl/fl}$ CD4Cre+/- mice the cell number is decreased, due to a reduction of the absolute number of lymphocytes (Fig.19). The cell numbers in the two *itch* $^{-/-}$ analysed was similar to the *itch* $^{fl/fl}$ CD4Cre+/- mutants (data not shown). For the heterozygous *itch* $^{fl/+}$ CD4Cre mice as well as homozygous and heterozygous *itch* floxed CD19Cre+/- mice (2-3 mice/genotype analyzed, data not shown) the cell number in these organs is comparable to wild type mice.

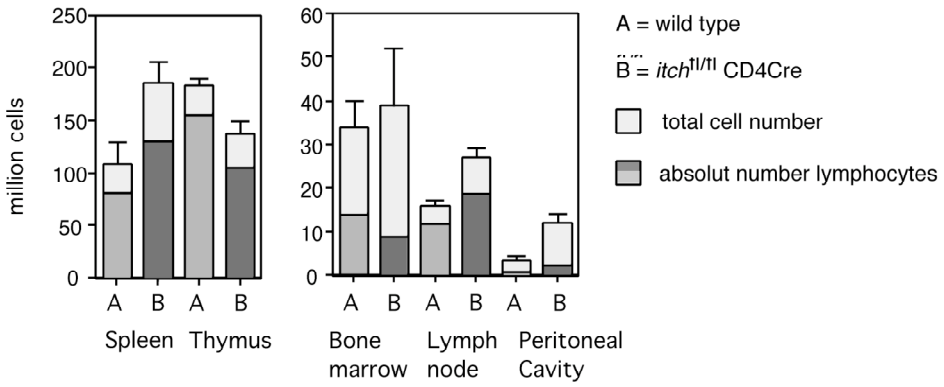


Figure 19. Absolute number of cells in lymphoid organs

Column height represent the total number of cells, dark areas represent the absolute number of lymphocytes and light areas the non lymphocyte population, depicted is the average from 3-6, 8 weeks old mice analyzed. The standard deviation is indicated. Lymph node represents the mesenteric lymph nodes.

C.5.4. No impairment of the T cell development or B and T cell ratio in itch $^{fl/fl}$ CD4 Cre+/- mice

In order to analyze the effect of *itch* deletion on the development of lymphocytes, flow cytometric analysis was performed on cells from lymphoid tissues. In young (8-9 week) mice with a T cell specific deletion of Itch there was no significant difference in the ratio of peripheral B and T cells and the development of T cells in these mice appeared normal. An upregulation of the activation marker CD69 was observed in *itch* $^{fl/fl}$ CD4 Cre+/- thymocytes, especially pronounced in the DP stage of thymocyte development, implicating that these cells are activated in the *itch* $^{fl/fl}$ CD4 Cre+/- mice (Fig.20).

We then further asked if there is an aberrant increase or decrease in different T cell subsets in the *itch* $^{fl/fl}$ CD4 Cre+/- mice, which could explain the development of autoimmune disease in these mice. We first analyzed if these mice have a normal ratio of naïve versus activated/memory T cells. These populations could be discriminated by the surface markers CD4, CD44 and CD62L (Fig.21). We found that *itch* $^{fl/fl}$ CD4 Cre+/- mice had a higher

percentage of activated/memory splenic T cells which in absolute cell numbers represents an increase of approximately two times. CD4+ CD25+ regulatory T cells is a T cell population, which has been shown to play a regulatory role in autoimmune diseases. It was shown that CD4+ CD25+ regulatory T cells inhibit organ specific autoimmune diseases induced by CD4+ CD25- T cells and that they can suppress the latter T cells in vitro (Shevach, 2000). Stainings for CD4+ CD25+ regulatory T cells in *itch* $^{fl/fl}$ CD4 Cre+/- mice revealed no divergence from wild type mice (Fig. 21).

NKT cells is another T cell population, which has been implicated to play a role in autoimmune disease by contributing to suppression of immune responses and prevention of autoimmunity (Mars et al., 2002) for review see (Hammond and Godfrey, 2002). This population can be discriminated from conventional $\alpha\beta$T cells by the NK1.1 expression. Surprisingly, we observed a large population (3%) of NKT cells in the thymus of *itch* $^{fl/fl}$ CD4 Cre+/- mice, which is not present in wild type mice. This corresponds to a 10 fold increase in absolute numbers, even though the absolute number of thymocyte in these *itch* $^{fl/fl}$ CD4 Cre+/- mice were reduced and corresponded to 75% of wild type numbers. To test if this increase of NKT cells could also be identified in the periphery, splenic T cells were stained for the same cell-surface marker, however no such difference could be detected.

In the innate immune response NK cells play an important role, in particular because they provide the early production of IFNγ necessary to control certain bacterial, parasitic and viral infections (for review see (Biron, 1997). For the NK subpopulation identified as TCR$\alpha\beta$ negative NK1.1 positive cells, we could not find any obvious difference in the spleen or thymus of *itch* $^{fl/fl}$ CD4 Cre+/- mice compared to wild type mice.

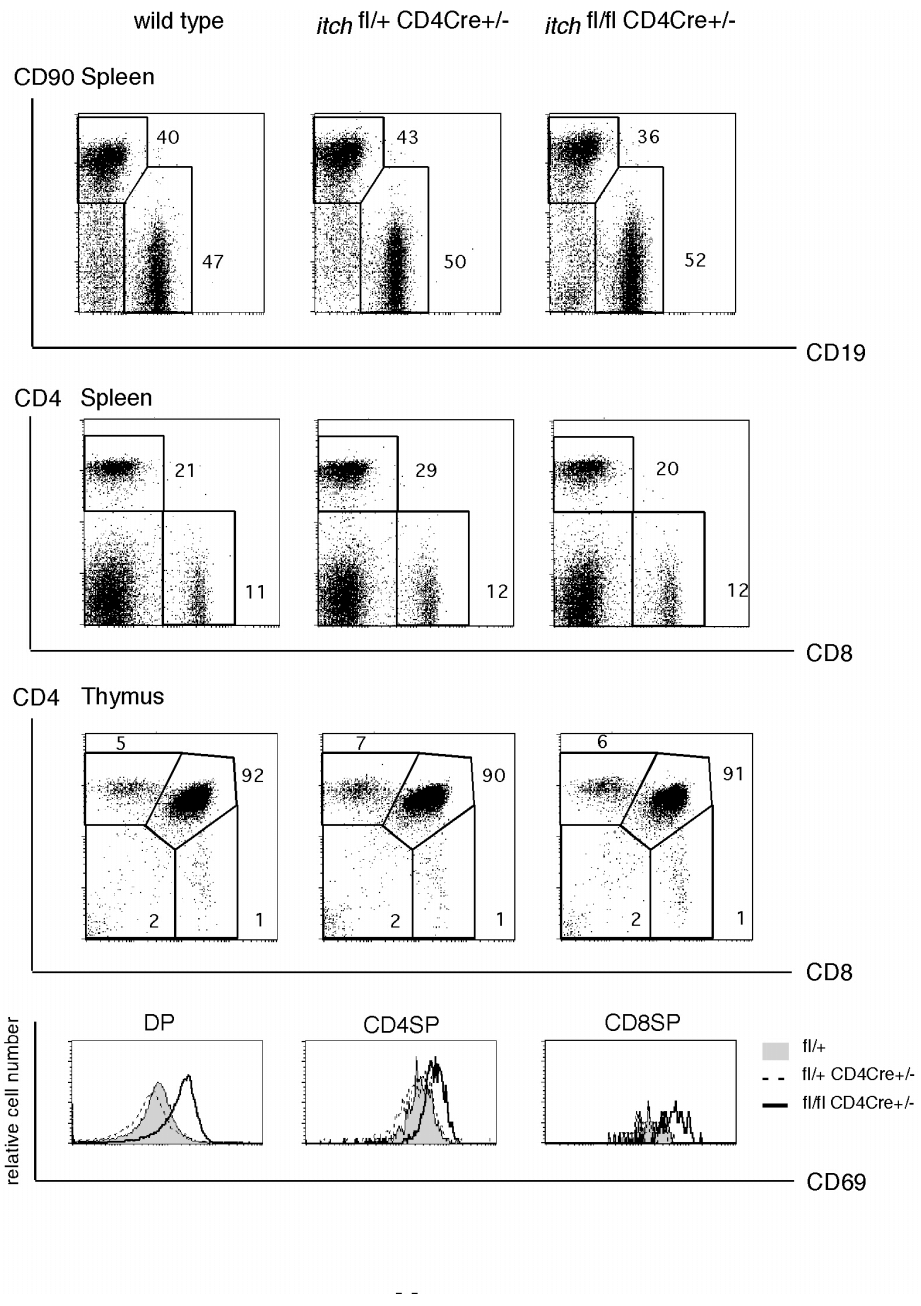


Figure 20. Analysis of T cell development in *itch* $^{fl/fl}$ CD4Cre+/- mice.

FACS analysis of 9 week old *itch* $^{fl/fl}$ CD4Cre+/- mice. Stainings of spleen and thymus using the T cell specific marker CD90 and the B cell specific marker CD19. Staining of T cell with CD4 and CD8 and the activation marker CD69. Numbers correspond to the percentage of cells in each specific gate.

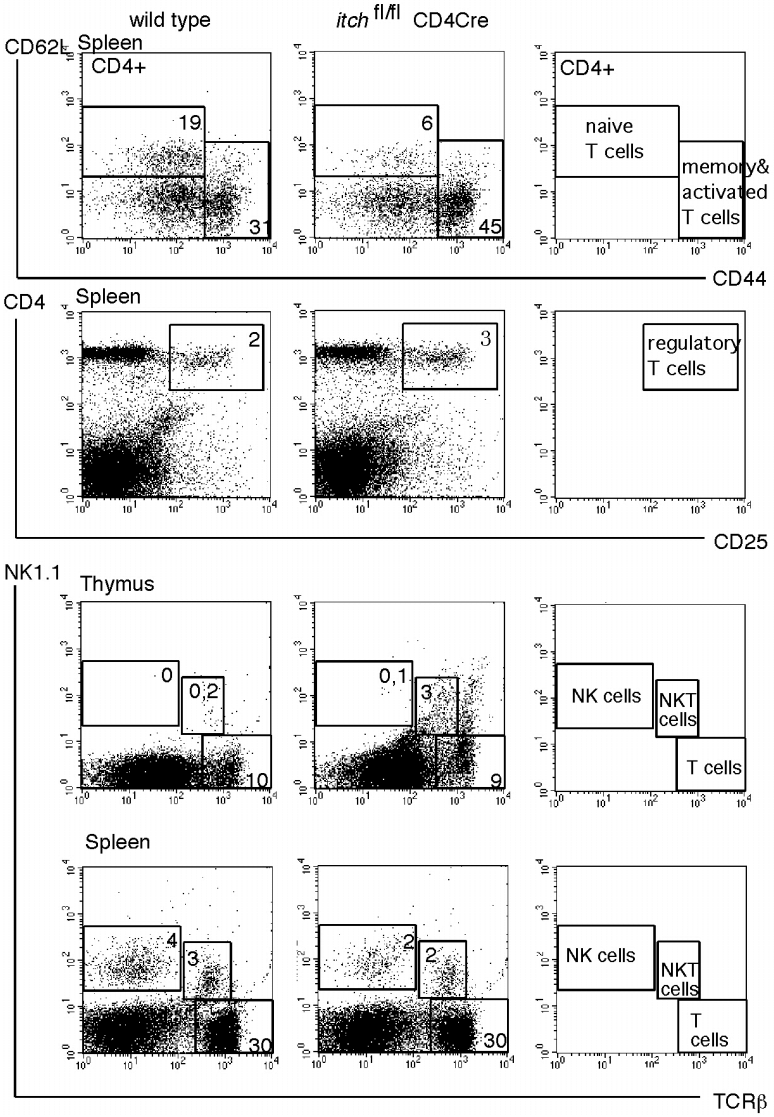
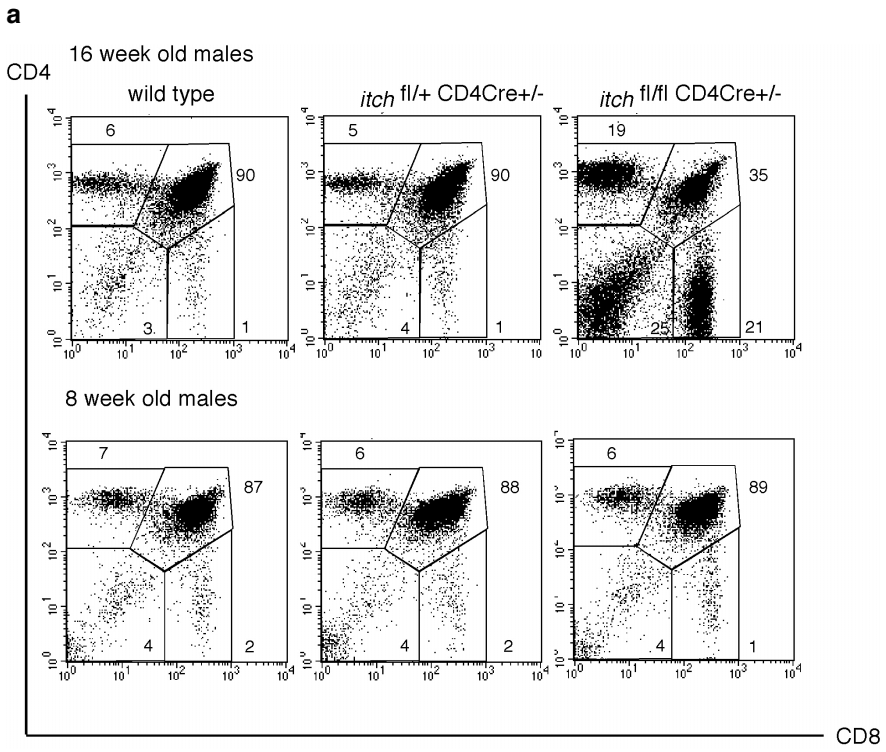


Figure 21. FACS staining for different T cells populations, NKT and NK cells in 8 week old *itch* $^{fl/fl}$ CD4 Cre mice

FACS analysis of T cell populations and NK cells in thymus and spleen of 8 week old *itch* $^{fl/fl}$ CD4 Cre+/- and wild type littermates. Numbers correspond to the percentage of cells in the specific gate. All windows are gated on living lymphocytes except for the upper panel, which is gated on CD4+ living lymphocytes.

C.5.5. Changed ratios of thymocyte subsets in old itch-CD4Cre mutants

In 8 weeks old *itch* $^{fl/fl}$ CD4 Cre+/- mice, no difference of thymocyte subpopulations could be identified compared to wild type mice (Fig. 22a). However in 16 week old animals, which showed clear signs of disease, the total thymocyte was approximately two fold reduced (Fig. 22a). The absolute number of DP cells was reduced by about 10 times, while the number of CD8 SP and DN cells were increased 10 and 5 fold respectively. In contrast, the absolute number of CD4SP cells was unchanged (Fig.22b). This phenotype is seen in older sick mice, indicating that this is an effect possibly due to the secretion of inflammatory cytokines by activated Itch deficient T cells.



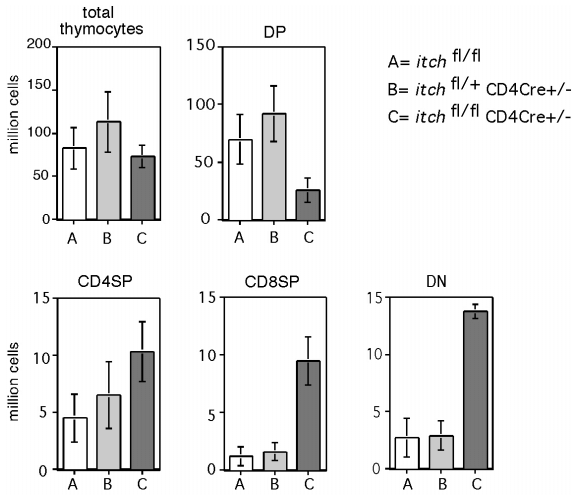


Figure 22. Changed ratios of thymocyte subsets of old *itch* $^{fl/fl}$ CD4 Cre+/- mutants.

a) Numbers represent percentage of cells in the specific gate. The lower FACS dot plots are gated on double positive thymocytes from 16 week old mice of the dot plots above. **b)** Absolute numbers of thymocyte population. Double positive (DP), CD4 single positive (CD4SP), CD8 single positive (CD8SP) and double negative (DN) thymocytes. The absolute numbers are based on 3 *itch* $^{fl/fl}$, 3 *itch* $^{fl/f+}$ CD4Cre+/- and 2 *itch* $^{fl/fl}$ CD4Cre+/- mice at the age of 15-16 weeks.

C.5.6. Serum titers of different immunoglobulin isotypes in mice with T-cell specific and ubiquitous deletion of itch

The pathological analysis of *itch* $^{fl/fl}$ CD4Cre+/- mice revealed an infiltration of plasma cell into several organs in particular the spleen but also the lungs, indicating a possible increase in the production of immunoglobulins (see section 5.1 Systemic inflammation in *itch* mutant mice). Thus immunoglobulin isotype titers were measured in the serum of *itch* mutant mice by ELISA. Increased levels of IgA and IgM and a slight increase of IgG1 and IgE was found in *itch* $^{-/-}$ mice and *itch* $^{fl/fl}$ CD4Cre+/- mice at the age of 8 weeks (Fig.23a). In older *itch* $^{fl/fl}$ CD4 Cre+/- mice (16-23 weeks) this increase is even more pronounced with a 10-100 fold increase of IgM, IgE and 10 fold increase of IgG1 levels (Fig.23b). The elevated levels of IgG1 and IgE are indicative of a Th2 response in these mice, since Th2 secreted cytokine IL-4 provide help for antibody responses including IgE and IgG1 isotype switching (DeKruyff et al., 1993; Shimoda et al., 1996; Stevens et al., 1988). In contrast, B cell help for IgM synthesis can be provided both by Th1 and Th2 cells. To test if these immunoglobulins were autoreactive, an ELISA for anti single stranded (ss) and anti double stranded (ds) DNA was performed (Fig.24). The titers of anti DNA IgG1, IgG2a and IgG2b antibodies are depicted as EC50 of the titer of a serum from a MRL/lpr mouse (see Matrial & Methods), which is a mouse mutant with very high titers of anti DNA antibody (Andrews et al., 1978). No significant increase of anti-DNA antibodies could be detected in the mutant animals. In addition, no anti-nuclear antibody (ANA) IgG was detected (data not shown). This indicates that the increase of IgG1 in these animals is not due to an increase of auto-reactive antibodies.

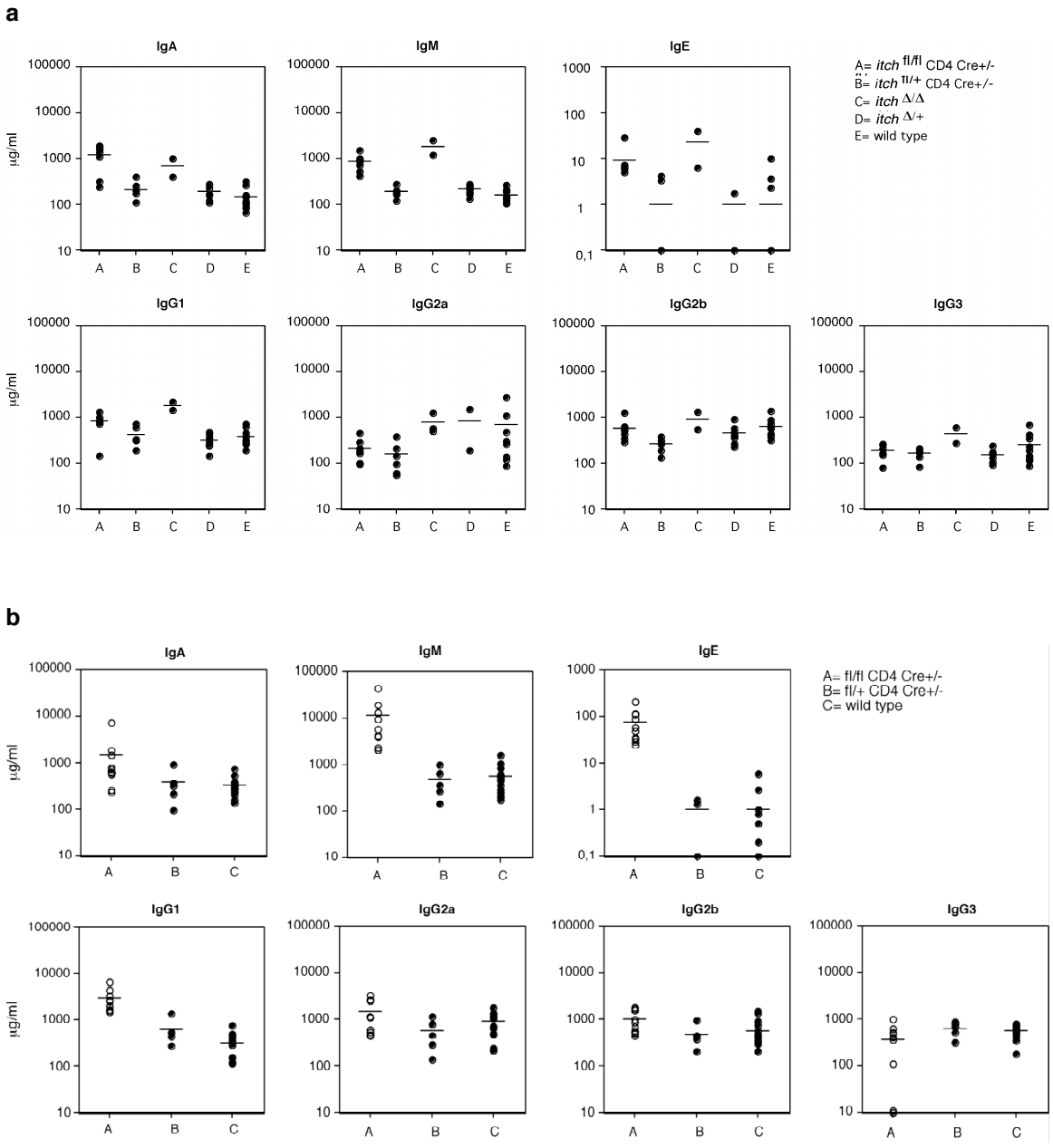


Figure 23. Serum immunoglobulin titers in *itch* mutant mice.

a) Serum immunoglobulin titers of *itch* $^{fl/fl}$ CD4 Cre+/- (7 mice) and *itch* $^{fl/+}$ CD4 Cre+/- (6 mice), *itch* $^{\Delta/\Delta}$ (2 mice) and *itch* $^{\Delta/+}$ (9 mice) and wild type (10 mice) mice at 8-9 weeks of age. Each dot represents one mouse, the homozygous mutants are depicted as open circles and heterozygous mutants as well as wild type mice are indicated as filled dots. **b)** Serum immunoglobulin titers of 16-23 week old homozygous (9 mice) and heterozygous (5 mice) itch-CD4 Cre mice compared to homozygous floxed (15mice). The immunoglobulin serum titers are depicted as µg/ml serum in a logarithmic scale. The average values are indicated as horizontal lines.

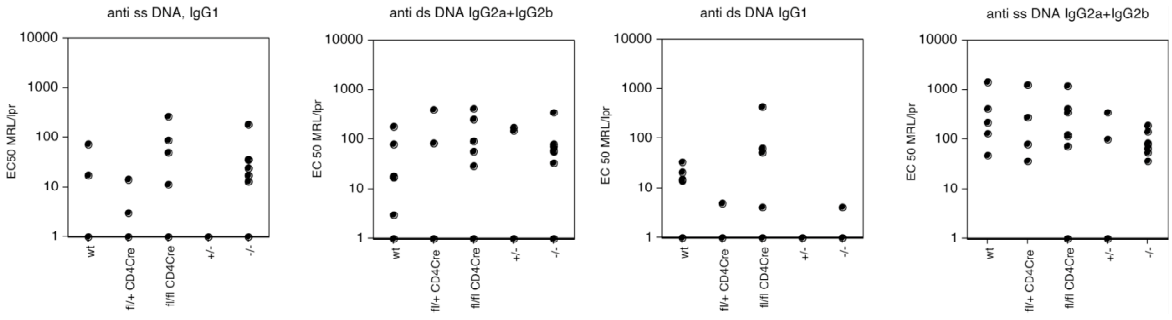


Figure 24. No increase of anti DNA antibodies in *itch* mutant mice

The levels of anti-ss-DNA and anti-ds-DNA antibodies in the serum were measured by ELISA. The levels are depicted as EC50 binding of serum from MRL-lpr/lpr mice. Each dot represents serum from one mouse. Sera from *itch* $^{fl/fl}$ CD4 Cre+/- (8 mice), *itch* $^{fl/+}$ CD4 Cre+/- (5 mice), *itch* $^{-/-}$ (8 mice), *itch* $^{-/+}$ (5 mice) mice and wt mice (8 mice) in the age of 14-20 weeks were measured.

C.5.7. In vitro Th1-Th2 differentiation of *itch* deleted naïve CD4 single positive peripheral T cells

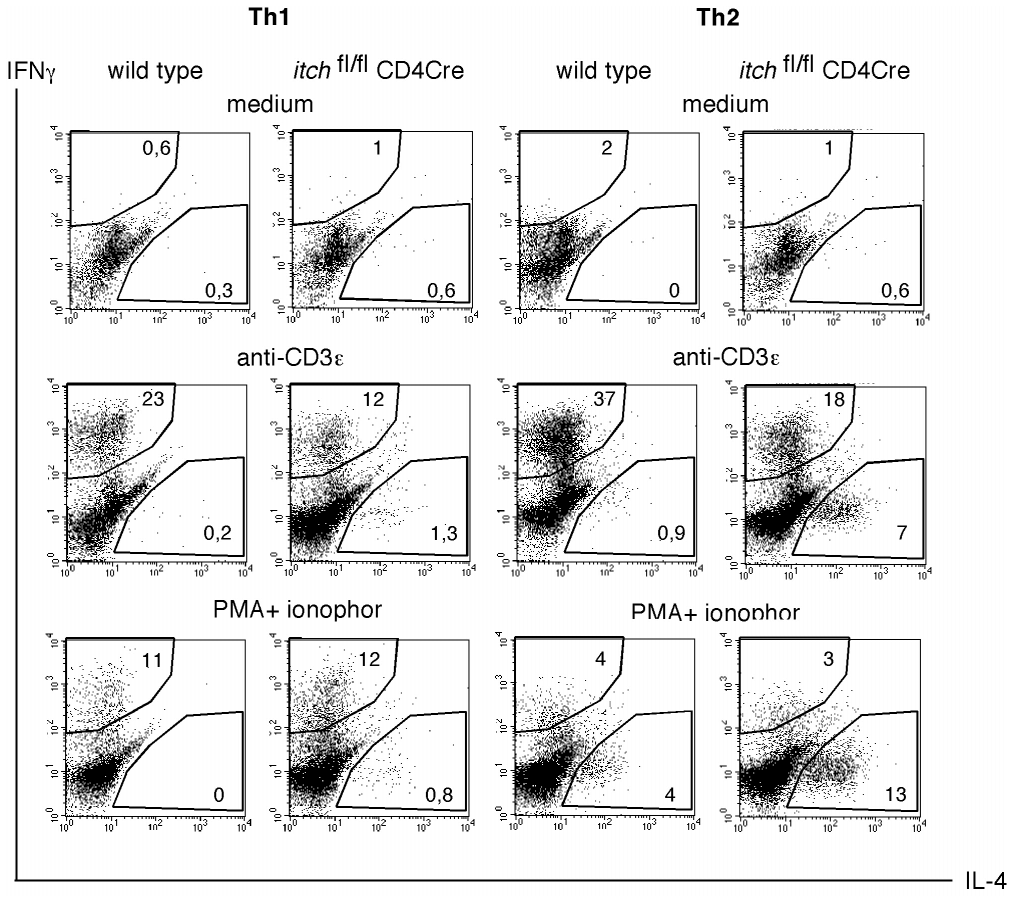
In the *itch* $^{fl/fl}$ CD4Cre+/- mice, harboring a T cell specific deletion of Itch, the development of the systemic inflammatory disease is likely to be due to the action of the mutated T cells in these mice. A shift of the Th1/Th2 balance of the immune response seems to play an important role in the development of several autoimmune diseases. In an attempt to explain the complex phenotype observed in *itch* $^{fl/fl}$ CD4Cre+/- mice, we hypothesized that the systemic inflammatory disease might be a result of a dysregulation in the polarization towards a Th1 or a Th2 response.

To clarify if the T cells from *itch* $^{fl/fl}$ CD4Cre+/- mice are more prone to become either Th1 or Th2 effector cells, in vitro cultures for polarization towards Th1 and Th2 using purified naïve CD4+ CD62L high T cells from spleen of 8 week old littermates were performed. CD4+ CD62L high T cells from spleen were purified by MACS. The purity reached 97% of CD4+CD62L high cells. The naïve T cells from mutants and wild type controls were stimulated for 12 hours. The stimuli were removed and the cells were cultured 7 more days

under either Th1 or Th2 polarizing conditions, before they were restimulated with the same stimuli in medium without polarizing cytokines. IFN or IL-4 producing cells were identified by intracellular staining with antibodies against the two cytokines followed by FACS analysis (Fig.25a). T cells from the C57BL/6 strain are prone to become Th1 cells and are usually more difficult to polarize to Th2 cells. We observed an increased percentage of IL-4 producing cells in the cultures with *itch* $^{fl/fl}$ CD4Cre+/- compared to wild type cells after Th2 polarization and stimulation with either plate bound anti-CD3 antibodies or with PMA plus the ionophor A23187. Anti-CD3 antibodies activate T cells by cross linking the T cell receptor whereas PMA and the ionophor A23187 act more downstream on the signaling pathway by activating protein kinase C (PKC) and mobilization of the intracellular Ca2+ ions, respectively. In the non stimulated cultures with polarizing cytokines and in stimulated cultures without polarizing cytokines, neither wild type nor mutant cells were polarized as expected.

In addition, the levels of IL-4 were measured in the supernatant of the restimulated cells polarized to Th2 by ELISA. The levels of IL-4 in the supernatant in the Th2 polarized cultures are depicted in Figure 22b. As already observed in the FACS analysis the *itch* $^{fl/fl}$ CD4Cre+/- T cells produce very elevated levels of IL-4 after Th2 polarizing conditions compared to wild type littermates. The amount of IL-4 after stimulation with anti CD3 was below detection limit. The numbers of living cells were equivalent in the cultures with the same stimuli. This data implicate that the *itch* $^{fl/fl}$ CD4Cre+/- T cells have a bias to become Th2 cells.

a


Th1
Th2
IFNγ
wild type
itch fl/fl CD4Cre
wild type
itch fl/fl CD4Cre
medium
0,6
0,3
1
0,6
2
0
1
0,6
anti-CD3ε
23
0,2
12
1,3
37
0,9
18
7
PMA+ ionophor
11
0
12
0,8
4
4
3
13
IL-4

b

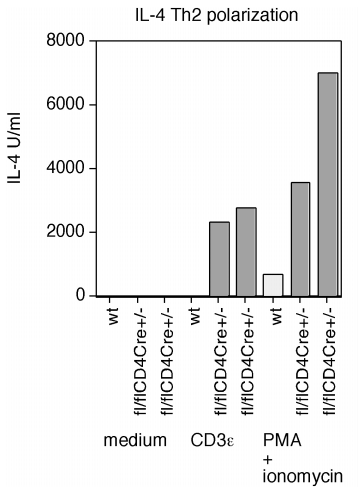


Figure 25. In vitro polarization of CD4+ CD62+ naïve splenic T cells

CD4+ CD62L high naïve splenic T cells from 8 week old itch $^{fl/fl}$ CD4 Cre or wild type (wt) mice were stimulated for 12 h and cultured for one week with cytokines for polarization to Th1 or Th2 cells and then restimulated without cytokines (see Material & Methods). **a)** IFN and IL-4 producing cells were detected by FACS analysis after intracellular staining with anti-IFN and anti-IL-4 antibodies after activation with anti- CD3 or PMA plus ionophor. **b)** In the supernatant of the Th2 cultures the levels IL-4 were measured by ELISA. Dark grey filled columns represent the titers measured in the supernatant of cells from *itch* $^{fl/fl}$ CD4Cre+/- and white *itch* $^{fl/fl}$ littermates. Each column represent cells from one mouse.

D. Discussion

D.1. The role of Itch in the development of systemic inflammatory disease of a^{18H} mice

A^{18H} mice contain an inversion of a DNA segment involving the *agouti* and the *itchy* loci (Perry et al., 1998). These mice develop an inflammatory disease and hyperplasia of secondary lymphoid organs. The purpose of this thesis was to identify the possible function of Itch in the development of systemic inflammatory disease seen in a^{18H} mice and to elucidate which cell type is involved in the initiation of disease.

D.2. Expression of Itch is increased in mature B cells and T cells

The information about Itch expression in lymphocytes was important in order to identify the possible cell type in which the absence of Itch may induce the systemic inflammatory disease in the mutant mice. Real time RT-PCR provided an easy assay to investigate the expression profile of Itch in purified subpopulations of B and T lymphocytes. Indeed the data in (Fig. 4) were consistent with previous observations that Itch is ubiquitously expressed in lymphoid tissue. However, this analysis revealed that its expression was at a low level. The expression of Itch seems to increase during B cell development in that the expression level increases approximately two times from the stage of pro- to immature B cells to the stage of recirculating mature B cells in the bone marrow and peripheral B cells. In T cells the expression was elevated only in CD8 SP mature T cells. However, the expression of Itch in B cells remains elusive as no function for Itch could be detected in the frame of this thesis.

D.3. Polymorphism in the itchy locus

The disease development of a^{18H} mice seemed to be strain dependent, since the phenotype is different on C57BL/6J and JU/Ct genetic background (Hustad et al., 1995). Therefore it was important to generate *itch* mutant mice on the C57BL/6J background. Studies of the level of polymorphism in the *itchy* locus (Fig. 6) reveled a high polymorphism, which prevented the possibility to use strain divergent genomic DNA for the generation of *itch* mutant mice.

D.4. Generation of mouse strains with ubiquitous or cell type specific inactivation of the itch gene

To investigate whether the inactivation of the *itch* gene is responsible for and which cell types are involved in the development of the disease observed in a^{18H} mice, we took advantage of the Cre/*loxP* recombination technology to generate germline and B- or T-cell specific mutation/inactivation of the *itch* gene in the mouse. By crossing *itch flox* mice to CD4-Cre or CD19-Cre transgenic mice, efficient (80-100%) and specific deletion of the *floxed* DNA segment could be achieved in peripheral T cells and B cells, respectively (Fig. 8 and 9). In addition, there was no difference in the deletion efficiency in heterozygous compared to homozygous animals harbouring the deletion, indicating that selection for or against *itch* mutated cells did not occur.

D.5. The T cells, but not B cells, in Itch mutant mice are responsible for the systemic inflammatory disease

Mice harbouring a ubiquitous or T cell specific inactivation of the *itch* gene developed a systemic inflammation, similar to the one observed in a^{18H} mice (see below). This strongly indicates that inactivation of the *itch* gene is the cause of the immunological disease observed in a^{18H} mice and that T cells are key players in the development of this disease. In contrast, we could not detect any phenotypical change in the $itch^{fl/fl}$ CD19 Cre mice, with B-cell specific inactivation of *itch*, compared to wild-type mice neither by immunohistochemistry staining for B and T cells, nor by antibody titers analysed by ELISA. In addition, the $itch^{fl/fl}$ CD19 Cre mice were indistinguishable from wild-type littermates with respect to surface expression of common lymphocyte markers including markers for B cell development and activation (data not shown). Since most B cells in the $itch^{fl/fl}$ CD19 Cre actually carried the deleted allele, the lack of phenotype therefore suggests that B cells do not play any major role in the initiation of the systemic inflammatory disease observed in a^{18H} mice. The *itch floxed* mice could also be used to study the function of the ubiquitously expressed *itch* gene in other cell types. However, this was beyond the scope of this study.

D.6. No dominant negative effect of the mutated Itch protein

The *itch flox* mice were generated so that exon 12, which encodes the part of the hect domain containing a conserved cysteine essential for the enzymatic function of the protein, was deleted upon Cre-mediated recombination (Fig. 7). Exon 12 is the second last exon of the *itch* gene and a splicing event from exon 11 to exon 13 would lead to an almost full-length even though inactive protein.

Analysis of protein expression by Western blot (Fig. 12) using an antibody recognising the N-terminal part of Itch revealed that a truncated Itch protein is expressed in the homozygous *itch* mutant mice. This protein is lacking the epitope located in the C-terminal part of Itch recognised by the C-terminal directed antibody and should therefore be inactive as an ubiquitin protein ligase. An Itch protein mutated in its active site may be expected to have a dominant negative effect on Itch function. If this was the case, the heterozygous animals should display a similar phenotype as the one observed in animals carrying homozygous itch mutation. However, in all assays performed, the $itch^{fl/+}$ CD4 Cre animals were comparable to wild-type animals, suggesting that the inactive truncated Itch protein does not act as a dominant negative protein.

One possible explanation for not seeing a dominat negative effect of the truncated Itch protein, could be that the truncated Itch binds the substrate and since no ubiqiutination can take place the substrate stays bound to the truncated Itch protein and its further action is prevented. We know that the expression of Itch is low (Fig. 4b). Therefore, the binding of a few substrate molecules to the truncated Itch might not have a real effect on the whole reaction in the heterozygous situation, but the level of Itch protein coded from the non mutated allele can then take care of the ubiquitination of the excess substrate. In the case of the homozygous mutants the scenario would then be that the truncated Itch molecules are bound to the substrate, which thereby prevents it to act as a dominant negative and the large excess of substrate can not be ubiquitinated and down regulated.

D.7 Inactivation of the itch gene in T cells induces inflammation in mice

Mice with germline or T cell-specific inactivation of the *itch* gene develop a systemic inflammatory disease with age. In young (8 weeks) *itch* $^{fl/fl}$ CD4 Cre mice we could detect splenomegaly. In addition, signs of inflammation were evident in several organs at the age of 16 –20 weeks.

We typically observed infiltration of inflammatory cells in *itch* $^{fl/fl}$ CD4 Cre, such as neutrophils, polynuclear macrophages, into several organs, indicating ongoing inflammatory processes in these organs. This is seen both in the hematoxylin and eosin stained section and in the immunohistochemistry sections stained for specific neutrophil and macrophage markers as well as FACS stainings using the same markers (Fig. 14 and Fig. 15). In addition, an increased percentage of Gr-1 and Mac-1 double positive cells could be identified in the spleen (Fig. 17), indicative of the presence of activated neutrophils. In addition, we could observe destruction of organ tissue in these mice.

D.8. Splenomegaly and lymphodenopathy in itch $^{fl/fl}$ CD4 Cre+/- mice is due to an increase of lymphocytes as well as other cell types

In the *itch* $^{fl/fl}$ CD4 Cre mice, we also detected an increase of the total number of cells in the spleen, mesenteric lymph nodes and peritoneal cavity. This was partly due to an increase in the number of the peripheral lymphoid cells but also to a large extent due to an increase in the number of the non lymphoid cells (Fig. 19). Among these cells are macrophages, neutrophils and possibly also eosinophils. Increase of the number of these cells was confirmed using FACS analysis and whole blood analysis (Fig. 15 and Fig. 18). This implies, that the *itch* mutated T cells directly or indirectly induce the infiltration, activation and proliferation of the inflammatory cells and to some extent also lymphocytes.

D.9. Mutant Itch mice have increased levels of serum immunoglobulin IgM, IgG1, IgA and IgE

We found an increased amount of plasma cells in the lung and spleen of old *itch* $^{fl/fl}$ CD4 Cre animals (detected by E&H stainings, data not shown), which correlates well to the increased serum levels of IgM, IgG1, IgA and IgE seen in these animals (Fig. 18). This suggest, that the *itch* mutated T cells also promote differentiation of B cells into plasma cells.

D.10. Disrupted splenic architecture in itch $^{fl/fl}$ CD4 Cre mice

The *itch* $^{fl/fl}$ CD4 Cre animals also show disrupted splenic architecture with severe B and T cell disorganization in the follicles (Fig. 16). This might indicate a defect in the interaction between B and T cells in secondary lymphoid organs. Similar disturbed splenic architecture is also observed in mutant mice showing an autoimmune like phenotype as the MRL-lpr/lpr mice, which have a mutation in the Fas gene involved in apoptosis (Mandik-Nayak et al., 1999).

In order to analyse if the disruption of the splenic architecture indeed influence the T and B cell interaction, we immunised mice with a T cell dependent (TD) antigen. Preliminary results, obtained by challenging *itch* $^{fl/fl}$ CD4 Cre mice with nitrophenol (NP)–haptenated chicken □globulin, revealed a defect in T-cell dependent immune response of these mice (data not shown). The titers of IgA, IgM, IgG1 and IgE in the serum were, already elevated in mice before immunization as compared to wild-type animals. These isotypes also increased after immunization, however there were no or little generation of NP specific antibodies. This indicate, that the *itch* mutated T cells activate B cells to produce antibodies, but are not able to give specific T cell help.

It would be interesting to investigate whether *itch* $^{fl/fl}$ CD4 Cre mice are able to form germinal centers (GC) during the course of an immune response. GC are T cell dependent antigen-induced microenvironment formed within the lymphoid follicles, where B cells mature to

become high affinity antibody secreting plasma cells or memory B cells, for review see (Kosco-Vilbois et al., 1997). An absence of antibody affinity maturation and selection of high-affinity antibodies expressing B cells to become plasma cells or memory B cells, may indicate a dysfunction of the T and B cell interaction necessary for GC formation.

D.11. Itch is not required for normal lymphocyte development

The cell-surface expression of CD4 and CD8 in *itch* $^{fl/fl}$ CD4 Cre thymocytes and mature T cells from spleen and lymph nodes were indistinguishable from wild-type cells (Fig. 20). These results suggest that Itch is not involved in conventional T cell development in the thymus and peripheral T cells.

D.12. Alterations of cell populations in the thymus and activated phenotype of T cells

In young (8 week) mice the *itch* deleted T cells have a relatively inconspicuous phenotype when analysed for extracellular markers by FACS analysis. The increased CD69 expression of *itch* mutant T cells on both thymocytes and peripheral cells compared to wild-type, indicate that they are in an activated state. Moreover the increased number of activated/memory cells further points in the direction that the *itch* mutation may lead to an increased or prolonged activation of the T cells. One could therefore speculate that Itch regulates a signaling molecule acting in a signaling cascade promoting T cell activation.

Analysis of the fraction of NKT cells revealed a 10-fold increase of the absolute number of this population in the thymus of 8 week old *itch* $^{fl/fl}$ CD4 Cre mice compared to wild-type mice. In the spleen, however, such an increase was not observed. Further experiments have to verify, whether the increase of NKT cells in the thymus is confined CD1 restricted NKT cells. These can be identified either by their reactivity to α-Galactosyl Cerimide or their preferential expression of a TCR encoded by Vα14Jα281 (Lantz and Bendelac, 1994; Makino et al., 1995; Sidobre and Kronenberg, 2002). If these cells constitute the main part of the enlarged

population, the *itch* deficiency seems to influence either generation or survival of these cells during thymic development. In contrast, if these cells express MHCII restricted receptors of polyclonal specificities, it is possible that *itch* deficiency somehow influences the expression of the NK1.1 molecule. To test whether these cells then are functionally equivalent to the above described NKT cells, the cytokine profile activation characteristic of NKT cells should be analysed. The reason for NKT cell levels not also beeing increased in the spleens of *itch* $^{fl/fl}$ CD4 Cre mice is unclear. These could be the result of hemeostatic regulation, thus reducing the life time of the cells in order to retain the optimal population size. In addition, thymic export may be diminished or these NKT cells possibly undergo apoptosis in the thymus in *itch* $^{fl/fl}$ CD4 Cre mice.

Old *itch* $^{fl/fl}$ CD4 Cre mice had a 2-fold reduced cell number in the thymus compared to wild-type animals. However, we observed a mayor increase in number of CD8 SP cells. Moreover an increased amount of B cells, macrophages and neutrophils were found in the thymus of these mice. Interestingly, these cell populations are usually not present in the thymus. It remains unclear if these cells migrated to the thymus or whether they developed there. This constellation of the thymus reminds of the structure of a secondary lymphoid organ. The effect on the thymus in the mutant animals is most likely secondary since it is only observed in older animals.

Other mutant mice also showing a similar phenotype are motheaten mice, which have reduced number of thymocytes and overall a small thymi. Motheaten mice develop an autoimmune disorder due to its defect in expression of a functional protein-tyrosine phosphatase SHP-1. SHP-1 is involved in the regulation of several cytokine- and antigen receptor-mediated signaling pathways (Healy and Goodnow, 1998). The similarities of the *itch* $^{fl/fl}$ CD4 Cre and motheaten mutant mice implicate that Itch could also be involved in the same SHP-1-mediated signaling pathways.

One can speculate about the reasons for a diminished thymus, which could be an enhanced output of mature T cells or killing of lymphocytes by for example increased levels of

glucocorticoids due to stress caused by the inflammatory response, for review see (Ashwell et al., 2000).

The *itch* deleted T cells have a relatively inconspicuous phenotype when analysed for extracellular markers by FACS analysis. The increased CD69 expression of *itch* mutant T cells on both thymocytes and peripheral cells compared to wild-type, indicate that they are in an activated state. Moreover the increased number of activated/memory cells further points in the direction that the *itch* mutation may lead to an increased or prolonged activation of the T cells. One could therefore speculate that Itch regulates a signaling molecule acting in a signaling cascade promoting T cell activation.

D.13. Th2 versus Th1 biased immune response in itch $^{fl/fl}$ CD4Cre mice

The high levels of the IgG1 and IgE immunoglobulin titers, which are found in the *itch* $^{fl/fl}$ CD4Cre mice indicate a Th2 response in these mice (Coffman and Carty, 1986; Sideras et al., 1985; Vitetta et al., 1985). It has indeed been shown that class switch to IgE is dependent on cytokines expressed by Th2 (Coffman and Carty, 1986; Paul, 1991). Therefore the itch mutated T cells in *itch*$^{fl/fl}$ CD4Cre mice seem to activate B cells for immunoglobulin secretion and antibody class switching either through direct interaction or indirectly by cytokine secretion.

Other data suggesting a Th2 biased immune response in these mice is, the increased number of eosinophils in the blood already in young mice (Fig.18), since eosinophils are activated by IgE and Th2 secreted cytokine (Garlisi et al., 1995), for review see (Rothenberg, 1998). In addition, the in vitro polarization assay of naïve T cells from young (8 weeks) towards Th1 or Th2 cells, indicated that the *itch*$^{fl/fl}$ CD4Cre naïve T cells were indeed more prone than wild-type cells to polarize to Th2 effector cells (Fig. 25).

D.14. Old itch fl/fl CD4 Cre mice show signs of anemia

Older animals also show signs of anemia indicated by reduced number of erythrocytes as well as reduced hemoglobin and hematokrit levels. Factors that can result in a defect of erythropoetin production, leading to reduced erythrocyte production and anemia are for example kidney damage, since erythropoetin is produced in the Kidney (Jacobson et al., 2000) as well as inflammatory cytokines, as tumor necrosis factor (TNF) and interleukin-1 (IL-1) (Faquin et al., 1992). Both of these factors could in fact play a role in the anemia of *itch* $^{fl/fl}$ CD4 Cre mice. These animals have an obvious destruction of the kidney tissue, which probably leads to a defect in kidney function. In addition, these animals have infiltrations of inflammatory cells as macrophages and neutrophils, both cell types able to secrete the inflammatory cytokines TNF and IL-1 (Cassatella, 1999; Furmanski and Johnson, 1990).

D.15. The phenotype of itch $^{fl/fl}$ CD4Cre mice resembles that of a^{18H} mice on C57BL/6 genetic background

The pathological analysis of the *itch* $^{fl/fl}$ CD4Cre mice revealed a high similarity to the phenotype described for a^{18H} mice on C57BL/6 background. For example, the phenotype of the lung showing pneumonia with accumulation of macrophages with eosinophilic cytoplasma in the alveoli, infiltration of lymphocytes and plasma cells and some polynucleated macrophages containing large crystals. The skin ulcers and inflammatory processes as well as Splenomegaly, and slight infiltration of inflammatory cells in the stomach mucosa were seen in both mice strains (Hustad et al., 1995). However, the kidney phenotype, with distended pelvis and chronical inflammation, as well as dermatitis of the tail and the slight infiltration of inflammatory cells in the liver of *itch* $^{fl/fl}$ CD4Cre mice (Fig. 14) was not described for the a^{18H} mice. In addition, hyperplasia of the thymus was described for a^{18H} mice (Perry et al., 1998). This was not seen in *itch* $^{fl/fl}$ CD4Cre mice, where on the contrary a decrease in thymic cell number was observed (Fig. 19). The similarities in these phenotypes strongly suggest that the autoimmune/inflammatory disease seen in a^{18H} mice is mainly due to the loss of Itch expression while the differences could possibly be explained by the differences of the

mutation. It has formally not been excluded that the expression of other genes in the inverted gene segment in a^{18H} mice is also affected and that this would contribute partly to the observed phenotype. In addition, the a^{18H} mice have a complete loss of the Itch expression, whereas in *itch* $^{fl/fl}$ CD4Cre mice there is expression of a truncated Itch protein. One can speculate that Itch have other functions beside ubiquitination, as for example transport of interacting proteins to certain cell compartments. In that case this possible function is predicted to be retained in the almost full length truncated Itch protein and could contribute to the differences in phenotype of in *itch* $^{fl/fl}$ CD4Cre and a^{18H} mice. For the organs showing a phenotype in *itch* $^{fl/fl}$ CD4Cre mice, which was not described for a^{18H} mice, it is not clear if these organs were analysed or not at all affected.

D.16. The possible role of infectious agents or pathogens in the induction of disease

All the animals analysed during this study were kept in a non pathogen free facility. In the pathological analysis of these animals, which was carried out by Anlab (Prague), an extensive health screen and evaluation by eosin/hematoxilin staining where 4 animals were tested, the pathogen *Pasturella pneumotropica* was found in the vagina/prepuce of both of the wild-type animals. In one of the *itch* $^{fl/fl}$ CD4 Cre mice, this pathogen was found in the vagina/prepuce, the lung and in the colon, in the second *itch* $^{fl/fl}$ CD4 Cre mice the pathogen was not found at all. The non-pathogenic infectious agent *Flagellata tritrichomonas muris* was found in all animals in the ileum and colon. Only one of the mutant animals harboured *Pasturella pneumotropica*, but both of the animals developed a systemic inflammatory disease. It is, therefore, unlikely that the development of this disease is initiated by this specific pathogen. The analysed a^{18H} and itch $^{fl/fl}$ CD4 Cre mice strains come from different non-SPF facilities. Therefore an additional possible explanation for the differences in phenotype seen in these two strains, is that they harbour different infectious agents or pathogens, which might have an influence on the organs affected. Preliminary data from mice kept in a pathogen free facility (SPF) (although harbouring the infectious agent *Staphylococcus aureus*) show that these mice also spontaneously develop the disease (data not shown), this indicates that the type of infectious agent or pathogen in the non SPF facility might not be important for the

development of the disease. Further analysis of these *itch* $^{fl/fl}$ CD4 Cre mice is however needed to confirm that the disease development is identical in these mice compared to mice from the non-SPF facility. This data however, does not exclude the possibility that infectious agents play an important role in promoting initial activation of T cells during the initiating phase of disease.

D.17. Potential mechanism(s) underlying the development of the systemic inflammation disease in itch $^{fl/fl}$ CD4Cre mice

In mice with T cell specific inactivation of the *itch* gene, T cells display phenotypic features of activation. This is expected to be the result of a lack of accurate regulation and degradation of Itch substrate/s. The activated Itch deficient T cells are likely to constitute the primary cause of the disease observed in *itch*$^{fl/fl}$ CD4Cre mice. The question arizes how activated T cells influence inflammatory cells as neutrophils and macrophages to infiltrate the effected organs, eosinophils to proliferate and B cells to differentiate into plasma cells. It is of course possible, that at least some of these effects are indirect consequences. However, T cells in the normal immune response can influence these effector cells either by direct interaction as for B cells and eosinophils, as well as via secretion of soluble factors such as different cytokines.

Eosinophils usually play a role in the immune response involved in allergic diseases of the lung, skin and gastrointestinal tract (Gleich et al., 1993; Rothenberg, 1998) and have the ability to secrete several cytokines, which can function as both proinflammatory but also as growth factors stimulants or chemoattractants as IL-2, IL-4, IL-5, IL-10, IFN-□, and IL-12 (Bjerke et al., 1996; Bosse et al., 1996; Grewe et al., 1998; Lamkhioued et al., 1996; Levi-Schaffer et al., 1996; Moller et al., 1996; Moller et al., 1996; Nonaka et al., 1995). In addition, eosinophils express molecules as MHC class II and co-stimulatory molecules as CD28, CD40, CD80 and CD86, which implicate their interaction with helper and cytotoxic T cells to regulate immune response (Gauchat et al., 1995; Tamura et al., 1996; Woerly et al., 1999). Therefore, one could speculate that Itch mutant T cells with an activated phenotype directly interact with eosinophils to induce proliferation and activation of these cells leading to inflammatory responses by secretion of inflammatory cytokines. The Th2 secreated cytokines

IL-4 and IL-5, as well as crosslinking of eosinophil expressed Fc□RII receptor (CD23) by IgE are important for eosinophils activation, differentiation and migration (Coyle et al., 1996; Garlisi et al., 1995; Viola et al., 1998). Considering these data together with the bias towards a Th2 immune response, the disease developed by *itch* $^{fl/fl}$ CD4Cre mice shows many similarities to an IgE mediated type I response.

In an IgE-mediated type I Hypersensitivity reaction or allergic inflammatory reaction, eosinophils expressing Fc receptors for IgG and IgE isotypes, bind directly to antibody-coated allergen. This activates the eosinophils leading to their granulation and release of inflammatory mediators, which contribute to extensive tissue damage in the late phase of the reaction, reviewed in (Kuby, 1997). Neutrophils are then attracted to the area of a type I reaction by neutrophil chemotactic factor secreted by degranulating mast cells. In addition, the levels of Th1 and Th2 subsets play a key role in regulating type I hypersensitive responses. Th1 cells reduce the response, whereas Th2 cells enhance the response. The *itch* $^{fl/fl}$ CD4Cre mice have high IgE titers and elevated levels of blood eosinophils as well as infiltration of neutrophils in the affected tissues and a bias to Th2 polarization, all indications of a IgE-mediated type I hypersensitivity reaction.

The classical features of a type III immune complex mediated hypersensitivity is antigen-antibody complexes deposited in various tissues, which induce complement activation and an ensuing inflammatory response. Large amount of immune complexes can lead to tissue damage type III reactions. The immune complexes activate the complement system and its activation causes mast cell degranulation and increase in local vascular permeability. The complement split products are in turn chemotactic factors for neutrophils, which can accumulate in large numbers at the site of immune complex deposit. The tissue damage seen in type III hypersensitivity reactions stems to a large extent from lytic enzymes released by neutrophils as they attempt to phagocytose immune complexes. Typical clinical manifestations of a type III hypersensitivity reaction are serum sickness, with symptoms as lymphodenopathy, arthritis and sometimes glomerulonephritis. Diseases involving type III hypersensitivity reactions are for example Rheumatoid arthritis and Systemic lupus erythematosus, reviewed in (Kuby, 1997). The phenotype described here for the itch $^{fl/fl}$ CD4

Cre mice correlates with the disease description in the sense of clinical manifestations as lympodenopathy and glumerulonephritis as well as infiltration of neutrophils in various tissues and phagocytosis of protein complexes, which are probably immune complexes by inflammatory cells in the lung.

However, from the data available, we cannot make a definite conclusion of the immune reaction involved in the disease development of *itch* $^{fl/fl}$ CD4 Cre mice, but most of the data presented within this thesis point in the direction of a type I Hypersensitivity reaction.

D.17. Possible signaling pathways affected by itch inactivation

Itch being a ubiquitin protein ligase, the phenotype observed in mice with T cell-specific inactivation of *itch* is likely to be correlated with the lack of degradation, and therefore accumulation, of one or more of the substrates of Itch.

Potential substrates identified for Itch are Notch-1 and JunB. Notch-1 is expressed in CD34+ hematopoetic precursor cells (Varnum-Finney et al., 1998). Expression of a constitutively activated form of Notch1 (Notch IC) in these bone marrow precursors leads to development of T cells in the bone marrow, implicating a role for Notch-1 in providing regulatory signals determining T versus B lymphoid lineage decisions (Pui et al., 1999). On the other hand expression of Notch IC in developing T cells of the mouse leads to both an increase in CD8 lineage T cells and a decrease in CD4 lineage T cells (Robey et al., 1996). Although the increase of CD8 single positive cells in *itch* $^{fl/fl}$ CD4Cre mice correlates to the increase of CD8 lineage T cells in the mice expressing Notch IC in T cells, systemic inflammation has not been reported for the latter mice. In addition, the phenotype in mice expressing Notch IC in BM precursors show a different phenotype compared to mice expressing Notch IC in T cells, where as *itch* $^{-/-}$ and *itch* $^{fl/fl}$ CD4Cre mice show a similar phenotype. Therefore, Notch-1 is rather unlikely to be the substrate of Itch, which accumulation could explain the phenotype seen in *itch* $^{fl/fl}$ CD4Cre and *itch* $^{-/-}$ mice.

Another potential substrate for Itch is JunB, a component of the AP-1 transcription factor (Wagner, 2001). JunB was shown to upregulate Th2 cytokines in T cell specific transgenic

mice and to synergize with c-Maf to activate the IL-4 promoter (Li et al., 1999). The transgenic expression of JunB do not show any overt effect on lymphocyte development (Grigoriadis et al., 1993), which is similar to what we see in itch $^{fl/fl}$ CD4Cre mice. On the other hand, it has not been reported that these mice develop a systemic inflammatory disease (Passegue et al., 2002; Schorpp et al., 1996), which would be expected if JunB is indeed the substrate inducing this phenotype in itch $^{fl/fl}$ CD4Cre mice.

No other substrate for Itch has been identified so far. However, other molecules, which overexpression could result in a phenotype similar to that seen in *itch* $^{fl/fl}$ CD4Cre mice, are several co-stimulatory molecules involved in TCR signaling as for exampleCD40L, ICOS and OX40. In T cell-dependent antibody responses, switching of plasma cells to produce other isotypes than IgM is highly dependent on CD40 ligation (Ferlin et al., 1996; Kawabe et al., 1994; Renshaw et al., 1994; Xu et al., 1994). The direct interaction between the B cell CD40 molecule and its ligand CD40L on activated T cells is required for the generation of thymus dependent (TD) humoral immune responses. Studies of both CD40L knock-out mice and transgenic mice have helped elucidating the role of this protein in T cell dependent immune responses. Studies using antibodies disrupting the CD40-CD40L interaction have shown a primary role of this interaction in the regulation of B cell proliferation, production of immunoglobulins (Ig) and Ig class switching (Clark et al., 1996; Foy et al., 1996). In addition, CD40L knock-out mice show defects in priming of CD4 T cells to protein antigens (Grewal et al., 1995) and T cells from antigen immunized CD40L knock-out mice proliferate poorly and produce little or no IL-4 and IFN when challenged with priming antigen. T cell specific CD40L transgenic mice show inflammation in peripheral tissue as well as lymphoid tissue hypertrophy. The mice also have a reduced number of thymocytes, most evident for the double positive (DP) population (Clegg et al., 1997). By comparing the *itch* $^{fl/fl}$ CD4Cre mice to CD40L transgenic, many similarities in the phenotype are found and in fact we hypothesized that CD40L might be a substrate of Itch. This was tested in in vitro cultures measuring the expression of CD40L on Itch mutant CD4+ T cells after stimulation and withdrawal of stimulation using anti CD3 or PMA plus ionophor. In this assay it was found that the CD40L molecule can be downregulated as in wild-typeT cells, but the upregulation of

CD40L was diminished on the mutant T cells. Only approximately half as many cells upregulated CD40L as on wild-typecells and in addition the levels of CD40L were reduced (data not shown). This was especially pronounced after stimulation with PMA plus ionophor, which is known to be a stronger signal compared to anti CD3. These data argue against CD40L being a substrate of Itch, but might indicate that the *itch* mutated T cells compensate for a too strong signal by reducing the expression of CD40L implicating a disturbed signaling downstream of the T cell receptor or co-stimulatory molecules.

Another interesting candidate is the inducible co-stimulatory molecule (ICOS), which is upregulated on CD4+T cells after stimulation of the T cell receptor. It binds specifically to its ligand B7h on antigen presenting (APC) cells (Ling et al., 2000). ICOS has been suggested to be involved in the amplification and regulation of T cell responses and it is preferentially inducing IL-4 and IL-10 production (Coyle et al., 2000). Mice mutant for the ICOS molecule, show reduced level of IgG1, IgG2a and IgE and impaired germinal center formation. ICOS has therefore been proposed to play an important role in class switching (McAdam et al., 2001; Tafuri et al., 2001). In addition, ICOS deficient T cells primed with a specific antigen in vivo and restimulated in vitro produce very reduced levels of IL-4 but are fully competent to produce IFN (Tafuri et al., 2001). This is in line with previous data suggesting that ICOS stimulation enhances Th2 differentiation (Coyle et al., 2000; Hutloff et al., 1999).
One could imagine that an overexpression of ICOS would result in an opposite phenotype, with increased immunoglobulin levels as well as increased IL-4 production and thereby considerable similarities with the Itch mutant phenotype. Therefore ICOS and proteins involved in the ICOS signaling pathway are interesting candidates as Itch substrates. Experiments analysing the role of ICOS in *itch* deleted T cells were not undertaken due to the lack of commercially available anti-ICOS antibody.

OX40 is another co-stimulatory molecule and interesting candidate as an Itch substrate.Ligation of OX40 Ag, a member of the tumor necrosis factor receptor (TNF-R) family, increases IL-4 production by naive T cells and promotes their development into effector cells producing high levels of the Th2 cytokines IL-4, IL-5, and IL-13 (Ohshima et al., 1998). In addition, ligation of OX40 induces clonal expansion and survival of CD4 cells

during primary responses, and results in the accumulation of memory cells with time (Gramaglia et al., 2000). The OX40/OX40L interaction has also been suggested to be important in T cell-dependent antibody production by activated B cells (Stuber et al., 1995) (Stuber and Strober, 1996). It was shown that OX40 activated T cells preferentially accumulated in inflammatory sites associated with various diseases and disease models, including rheumatoid arthritis (RA) (Brugnoni et al., 1998), inflammatory skin diseases (ISD) (Matsumura et al., 1997), graft vs-host disease (GVHD) (Tittle et al., 1997), and experimental auto-immune encephalomyelitis (EAE) (Buenafe et al., 1996; Weinberg et al., 1996). OX40 being a molecule, which stimulation is involved in several mechanisms dysregulated in the *itch*$^{fl/fl}$ CD4 Cre mice as Th2 polarization, memory cell differentiation and involvement in inflammatory processes implicate OX40 as a potential substrate.

The IL-4 signaling pathway is another pathway, which dysregulation would be expected to result in a similar phenotype as the *itch* $^{fl/fl}$ CD4Cre mice. For example, transgenic mice overexpressing IL-4 develop allergy-like diseases and autoimmunity (Erb et al., 1997). This also includes factors involved in the regulation of IL-4, as for example Nuclear factor of activated T cells (NFAT), which is one of four related transcription factors implicated in cytokine and early response gene expression in activated lymphocytes. NFAT acts in synergy with AP-1 and Maf, where it transactivates a variety of cytokine and other activation genes including IL-2, IL-4, and GM-CSF (Cockerill et al., 1993; Ho et al., 1996; Hodge et al., 1996; Rooney et al., 1995). Mice lacking one of its members, NFATp, display modest splenomegaly, B and T cell hyperproliferation and elevated levels of IL-4 (Hodge et al., 1996; Oukka et al., 1998).

Further work is needed to test these proposed substrates and downstream signaling molecules in order to come closer to an elucidation of the molecular mechanism of how Itch inactivation induces a systemic inflammatory disease in mice. This list of potential substrates is not exhaustive, but a starting point for further analysis.

Further analysis of the functional properties of *itch* deficient T cells, as the proliferative response and degree of hyperresponsiveness can be studied in vitro by concentration and time

titrated stimulation assays as well as the immediate signaling response of *itch* mutant T cells by interacellular calcium flux studies. Such studies might give an insight into which signaling pathways are dysregulated. In addition, two dimensional gel analysis comparing activated and non activated T cells from Itch deficient and wilt type control cells, might give an indication of proteins not properly downregulated in Itch deficient cells.

All in all, the results of my experiments show that Itch is involved in the development of systemic inflammatory disease found in a^{18H} mice and that overall the T cells are involved in the initiation of the disease.

E. Abstract

Ubiquitination of substrate proteins takes place in an enzymatic reaction involving several enzymes. The specificity in this process is most probably determined by the ubiqiutin-protein ligases. In this work, we aimed to study the ubiquitin-protein ligase Itch. A mouse strain was generated, in which the *itch* gene could be conditionally inactivated. This was possible by taking use of Cre/*loxP* mediated recombination. Ubiquitous as well as a T cell specific deletion of Itch leads to the development of a systemic inflammatory disease. The characteristic feature of the disease is infiltration of inflammatory cell into several organs We could therefore show that *itch* mutated T cells are responsible for the initiation of systemic inflammatory disease in these mice. Furthermore, the *itch* mutated T cells displays an activated phenotype. In addition, high titers of serum IgG1 and IgE and increased number of blood eosiniophil implicates a Th2 polarisation of the immune reaction of these mice

F. Kurze Zusammenfassung

Die Polyubiquitinylierung von Substratproteinen führt in der Regel zu deren Degradation im Proteasom. Die Ubiquitinylierung ist eine Proteinmodifikation, bei der mehrere Enzyme involviert sind. Die Spezifität dieser Reaktion wird vermutlich durch die Familie der Protein-Ubiquitinligasen bestimmt. In der hier beschriebenen Arbeit wurde die hect-Domänen Ubiquitinligase *itch* in einem Mausmodell studiert. Es wurde ein Mausstamm generiert, in dem das *itch*-Gen konditional inaktiviert werden kann. Dies geschieht durch Cre/*loxP*-vermittelte Deletion.Eine ubiquitäre als auch eine T-Zell-spezifische Deletion von *itch* führt in der Maus zu einer systemischen Entzündungsreaktion. Wir können dadurch zeigen, daß die *itch*-mutierten T-Zellen für die Initiation der systemischen Entzündungsreaktion verantwortlich sind. Dabei führt die Deletion von *itch* nicht zu einer Hemmung der T-Zell-Entwicklung. Die mutierten Zellen weisen eher einen aktivierten Phänotyp auf, wie die Expression des Aktivierungsmarkers CD69 nahelegt. Ein weiterer Hinweis dafür ist die erhöhte Anzahl aktivierter memory-T-Zellen in diesen Mäusen. Die erhöhte Anzahl eosinophiler Zellen und der erhöhte Antikörper-Titer von IgG1- und IgE-Isotypen in den Mäusen mit einer T-Zell-spezifischen Deletion von *itch* weisen darauf hin, daß es sich vermutlich um eine Th2-polarisierte Immunantwort handelt.

G. Summary

Polyubiquitination of substrate proteins usually followed by their degradation in the proteasome. The pathway has the function of removing defect and miss folded proteins, as well as degrading signaling proteins and thereby regulate signal transduction. The ubiquitination of substrate proteins takes place in an enzymatic reaction involving several enzymes. The specificity in this process is most probably determined by the ubiqiutin-protein ligases. In this work, we aimed to study the ubiquitin-protein ligase Itch. A mouse strain was generated, in which the *itch* gene could be conditionally inactivated. This was possible by taking use of the Cre/*loxP* mediated deletion of an *itch* exon essential for Itch function. This enabled for the generation of mice with a ubiquitous as well as a cell specific deletion of Itch. Both ubiquitous as well as a T cell specific deletion of Itch leads to the development of a systemic inflammatory disease. The characteristic feature of the disease is infiltration of inflammatory cells including neutrophils, polynuclear macrophages and eosinophils in the affected organs. The most severely affected organs are the lung, kidney and spleen. In the spleen and lung there is additionally a large increase of antibody producing plasma cells. In contrast, mice with a B cell specific deletion of the *itch* gene do not develop any striking phenotype. We could therefore show that *itch* mutated T cells are responsible for the initiation of systemic inflammatory disease in these mice. The *itch* mutation in the T cells does not influence the T cell development, instead the T cells rather display an activated phenotype. This is further pronounced by the increased number of activated/memory T cells in these mice. Many autoimmune as well as inflammatory diseases show a polarization towards either a Th1 or a Th2 immune response. The increased amount of eosinophils and the increased antibody titers of IgE and IgG1 isotypes in mice with a T cell specific deletion of Itch implicates a Th2 polarized immune defense in these mice. Therefore, we have taken Itch deleted naïve T cells as well as wild type cells for polarization to Th1 and Th2 effector cells in vitro. Indeed, the Itch deleted T cells showed a bias to Th2 polarization compared to wild type control cells.

H. Zusammenfassung

Die Polyubiquitinierung von Substratproteinen fürt in der Regel zu deren Degradation im Proteasom. Durch diese Reaktion können z.B. falsch synthetisierte oder gefaltete Proteine aus der Zelle eliminiert werden oder Signalproteine abgebaut werden, wodurch Signalkettenreaktionen unterbrochen werden können. Die Ubiquitinylierung ist eine Proteinmodifikation, bei der mehrere Enzyme involviert sind. Die Spezifität dieser Reaktion wird vermutlich von der Familie der Protein-Ubiquitinligasen bestimmt. In der hier vorligende Arbeit wurde die hect-Domänen-Ubiquitin-Proteinligase Itch in einem Mausmodell studiert. Es wurde ein Mausstamm generiert, in dem das *itch* Gen konditional inaktiviert werden kann. Dazu wurde ein für die itch-Funktion essentielles Exon mit *loxP*-Stellen flankiert. Abhängig von der eingekreuzten Cre-Linie wurde eine ubiqutitäre oder zellspezifische Deletion von Itch erreicht. Sowohl die ubiquitäre als auch die T-zell-spezifische Deletion von *itch* führte zu einer systemischen Entzündungsreaktion. Charakteristisch für diese Entzündungsreaktion war die Infiltration inflammatorischer Zellen, wie neutrophile und eosinophile Granulocyten und polynukleären Makrophagen. Diese Infiltrationen traten hauptsächlich in der Lunge, Niere und Milz auf. Außerdem konnten in der Milz und der Lunge eine erhöhte Anzahl antikörperproduzierender Plasmazellen nachgewiesen werden. Da die Mäuse mit einer B-zellspezifischen Deletion von *itch* keinen auffälligen Phänotyp zeigten, vermuten wir, daß nur die Deletion des itch-Gens in T-Zellen für die Erkrankung verantwortlich ist. Dabei führt die Deletion von *itch* nicht zu einer Hemmung der T-Zell-Entwicklung. Die mutierten T-Zellen weisen eher einen aktivierten Phänotyp auf, wie die Ausprägung des Aktivierungsmarkers CD69 nahelegt. Ein weiterer Hinweis dafür ist die erhöhte Anzahl aktivierter memory T-Zellen in diesen Mäusen. Viele Autoimmun als auch inflammatorischen-Krankheiten zeigen eine Verschiebung zu einer Th1- oder Th2-Immunantwort. Die erhöhte Anzahl eosinophiler-Zellen und die erhöhten Antikörpertiter von IgE und IgG1 Isotypen weisen darauf hin, daß die T-zellspezifische Inaktivierung von *itch* hauptsächlich eine Th2-Immunantwort auslöst. Dies wurde durch in vitro Experimente unterstützt. Wildtyp- und *itch*-deletierte naive T-Zellen wurden in Kultur genommen und zu Th1 oder Th2- Effektorzellen differenziert. Es konnte gezeigt werden, daß im Vergleich zu den Wildtyp-Zellen in *itch*-deletierten Zellen das Gleichgewicht zugunsten der Th2-Effektorzellen verschoben war.

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J. Acknowledgement

The work presented was carried out at the Institute for Genetics, University of Cologne under the supervision of Prof. Dr. Alexander Tarakhovsky. The project was financed by the Deutsche Forschungs Gemeinshaft (DFG),Schwerpunkts programm “Struktur, Funktion und Regulation des 20S/26S ubiquitin-Proteasomsystems

First I would like to thank Prof. Alexander Tarakhovsky for giving me the opportunity to work independently on this very stimulating and interesting project. I would also like to thank Prof. Dr. Klaus Rajewsky for the forum of scientific discussion in the form of clubs and work shops, which were essential for the learning of how to attack scientific problems and for letting me finish my practical work in your department. I am very grateful to Prof. Thomas Langer for facilitate the finishing of my Ph.D. by financing my salary the two last months of work.

I would like to thank all the people from the 2nd and 6th floor, who supported me in this work in any kind of way. Especially I would like to thank Sigrid Irlenbusch, who helped me in so many ways from innumerable single cell suspensions, tail DNA preparations and ELISA assays but last but not least for your moral support. I also would like to thank Claudia Uthoff-Hachenberg for ELISA, Brigitte Hampel for sections, Christoph Göttlinger for cell sorting, Anke Leinhaas and Angela Egert for ES cell injections and to all the people in the animal facility for taking care of my mice.

I also would like to thank my friends in the lab Ingrid Mecklenbräuker for many well written protocols and for being such a great moral support also from abroad and I-hsin Su for teaching me ES cell culturing.

My special thanks:

To my friends and lunch mates Thomas Wunderlich and Teresa Corona, for many nice evenings also outside the lab and for always being so helpful and caring. To my lab partners Gloria Esposito and Natalie Uyttersprot, who has been such a great support in hard times with all your positive feedback. Manolis Pasparkis for good advice in science and life. Lily Pao for sharing your knowledge and your great expertise in theoretical and technical matters. Anke Leinhaas for being the sunshine of the lab spreading so much joy.

To Natalie Uyttersprot, Ingrid Mecklenbräuker and Ari Weissman for spending your time proofreading my thesis.

To Ewelina Piatek and Jennie Svallebo for taking so good care of me outside the lab during my time in Cologne.To my biggest supporter and best friend Anna Aurell for always saying the right things in the right moment.

To Jens Schlegel for the years you held out.

To my parents Annagreta and Börge Jönsson for your great support and for always believing in me.

L. Erklärung

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