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IL-23R^{+} γδ T cells: A population of effector cells that is pre-programmed in the embryonic thymus and enhances autoimmunity by restraining Foxp3^{+} regulatory T cells

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1 Summary

Interleukin (IL)-23 is considered to play a pivotal role in the establishment and maintenance of organ-specific autoimmunity and chronic inflammation as it serves as a growth and maturation factor for pathogenic IL-17 producing αβ T cells (Th17 cells). While αβ T cells up-regulate the IL-23 receptor (IL-23R) in response to inflammatory conditions, a subset of γδ T cells expresses the IL-23R constitutively under steady-state conditions. Upon stimulation with IL-23 these γδ T cells produce high amounts of the pro-inflammatory cytokines IL-17, IL-21, and IL-22.

By using an IL-23R reporter mouse approach, the development of IL-23R positive γδ T cells in the adult thymus, their maintenance in the periphery and their role during experimental autoimmune encephalomyelitis (EAE), a mouse model for multiple sclerosis, were analyzed.

The obtained results indicate that IL-23R expressing γδ T cells are mainly generated within the embryonic thymus. This finding suggests that those IL-23R$^+$ γδ T cells found in the peripheral lymphoid tissues of adult mice originate in large part from embryonically generated cells and are maintained in the periphery by self-renewal. In the peripheral immune compartment, IL-23R expressing γδ T cells become activated and expand under inflammatory conditions. Upon immunization with MOG_{35-55} emulsified in CFA, IL-23R$^+$ γδ T cells proliferate in the secondary lymphoid tissue and accumulate in the central nervous system (CNS) at the peak of disease. Interestingly, regulatory T cells (Treg cells) that are supposed to suppress antigen-specific αβ T cell responses show inverse population dynamics reaching their highest numbers in the CNS during the recovery phase of the disease supporting the hypothesis that γδ T cells might be able to antagonize Treg cell induction and/or function. In vitro, supernatant of γδ T cells stimulated polyclonally in the presence of IL-23 inhibits the TGF-β-driven conversion of naïve T cells into Treg cells and abrogates Treg cell-mediated suppression of effector T cells. Moreover, γδ T cells inhibit Treg cell responses in vivo and thus, a lack of γδ T cells, as in *Tcrd*$^{-/-}$ mice, results in exaggerated Treg cell responses.

In conclusion, the data presented in this thesis show that the embryonic thymus provides favorable conditions for the development of IL-23R$^+$ γδ T cells that populate particular niches. These γδ T cells, once activated via IL-23, promote inflammatory processes by inhibiting Treg cell responses and thus enhance antigen-specific αβ T cell responses. These results point to IL-

23-driven pathogenic properties of γδ T cells that might be particularly relevant during host defense at epithelial surfaces but also during deleterious autoimmune processes.

2 Zusammenfassung

In seiner Funktion als Wachstums- und Reifungsfaktor für Interleukin (IL)-17-produzierende αβ T Helferzellen (Th17 Zellen) spielt IL-23 eine entscheidende Rolle in der Entstehung und Aufrechterhaltung von organspezifischer Autoimmunität und chronischer Inflammation. Im Zuge entzündlicher Prozesse kommt es zur Hochregulation des IL-23-Rezeptors (IL-23R) auf αβ T Zellen. Eine Untergruppe von γδ T Zellen hingegen exprimiert den IL-23R bereits konstitutiv unter nicht-entzündlichen Bedingungen. Werden γδ T Zellen mit IL-23 stimuliert, produzieren sie große Mengen der pro-inflammatorischen Zytokine IL-17, IL-21 und IL-22.

Mit Hilfe eines IL-23R-Reportermausstammes wurde die Entwicklung von IL-23R positiven γδ T Zellen im adulten Thymus, der Fortbestand ihrer Population in der Peripherie und ihre Rolle während des Verlaufs der experimentellen autoimmunen Enzephalomyelitis (EAE), einem Mausmodel für Multiple Sklerose, untersucht.

Es konnte gezeigt werden, dass IL-23R-exprimierende γδ T Zellen vornehmlich im embryonalen Thymus entstehen. Somit ist zu vermuten, dass die in der Peripherie nachgewiesenen IL-23R$^+$ γδ T Zellen zum großen Teil durch Zellteilungen aus langlebigen IL-23R$^+$ γδ T Zellen, die bereits embryonal entstanden sind, hervorgegangen sind. Entzündliche Prozesse führen zur Aktivierung und Expansion von IL-23R-exprimierenden γδ T Zellen im peripheren Immunkompartiment. Nach Immunisierung mit MOG_{35-55}/CFA expandieren IL-23R$^+$ γδ T Zellen in den sekundären lymphatischen Organen und akkumulieren am Krankheitshöhepunkt im zentralen Nervensystem (ZNS). Interessanterweise erreicht die Population regulatorischer T Zellen (Treg-Zellen) im entzündlichen ZNS erst beim Abklingen der Erkrankung ihre maximale Größe. Treg-Zellen sind immunregulatorische Zellen, die in der Lage sind, antigenspezifische αβ T Zell-Antworten zu unterdrücken. Das Ergebnis, dass IL-23R$^+$ γδ T Zellen und Treg-Zellen sich im Verlauf der EAE populationsdynamisch invers verhalten, führte zu der Hypothese, dass γδ T Zellen unter Umständen befähigt sind, die Induktion und/oder Funktion von Treg-Zellen zu antagonisieren. γδ T Zellen wurden in vitro polyklonal unter Zugabe von IL-23 kultiviert. Es zeigte sich, dass die daraus resultierenden Zellkulturüberstände sowohl die TGF-β-induzierte Konversion von naiven T Zellen in Treg-Zellen als auch die Treg-Zell-vermittelte Suppression von Effektor-T-Zellen verhinderten. γδ T Zellen besitzen zusätzlich die Kapazität Treg-Zell-Antworten in vivo zu unterdrücken.

Dementsprechend konnten in *Tcrd*$^{-/-}$ Mäusen übersteigerte Treg-Zell-Antworten nachgewiesen werden.

Die Daten dieser Arbeit demonstrieren, dass der embryonale Thymus günstige Umgebungsbedingungen für die Entwicklung von IL-23R^{+} γδ T Zellen liefert, die daraufhin spezifische Nischen in der Peripherie besiedeln. Sobald sie durch IL-23 aktiviert werden, fördern diese γδ T Zellen inflammatorische Prozesse, indem sie Treg-Zell-Antworten unterdrücken und somit antigenspezifische αβ T Zell-Antworten unterstützen. Diese Ergebnisse deuten darauf hin, dass IL-23R^{+} γδ T Zellen einzigartige pathogene Eigenschaften besitzen, die sie zu relevanten Effektorzellen während der Immunabwehr aber auch während schädlicher Autoimmunprozesse machen.

3 Introduction

3.1 The immune system

The immune system protects the host from environmental agents such as microbes or toxins either by inhibiting their invasion or, if this fails, by destroying them within host tissues. The immune system has been divided into two major branches: the innate immune system and the adaptive immune system. The evolutionarily conserved innate immune system acts as a first-line defense against invading pathogens. Cells of the innate immune system detect pathogens such as bacteria via a set of receptors that recognize molecular structures on pathogens. These molecular structures, called pathogen-associated molecular patterns (PAMPs), are highly conserved soluble and membrane-bound proteins, DNA molecules, carbohydrates, and lipids that are unique to microbes and not present in mammalian cells. The recognition of PAMPs by pattern recognition receptors (PRRs) rapidly triggers an inflammatory response which includes the secretion of cytokines and the recruitment of phagocytic cells which finally results in the activation of the adaptive immune system. While innate immune responses are based on low-affinity receptors to achieve wide-ranging target recognition, adaptive responses rely on the recognition of pathogens by rearranged high-affinity receptors. The adaptive immune system includes humoral responses mediated by immunoglobulins (Ig) produced by plasma cells and cell-mediated responses which are based on antigen-specific T cells.

3.2 Autoimmunity

Immune cells recognize and remove foreign molecules while sparing molecules produced by the host. This non-reactivity to self-antigens is achieved by passive mechanisms such as the sequestration of self-antigens behind anatomical barriers with limited access by immune cells (e.g. blood-brain-barrier (BBB)) – a phenomenon, which is called immunologic ignorance. Active mechanisms of excluding self-antigens from adaptive immune responses comprise “central and peripheral tolerance”. Central tolerance refers to the deletion or inactivation of self-reactive immune cells during their development in the bone marrow (B cells) or the thymus (T cells). Since this process of elimination is incomplete, some auto-reactive T cells escape thymic deletion and can be found in the peripheral circulation of healthy individuals.

These mature T cells are kept in check by several additional mechanisms that are collectively referred to as peripheral tolerance. The presentation of self-antigens in the absence of co-stimulatory signals results in anergy, a status in which T cells are unable to proliferate and to attack their targets. Moreover, proliferation and function of conventional T cells can be suppressed by a distinct lineage of $CD4^+$ (cluster of differentiation) T cells that expresses the transcription factor forkhead box p3 (Foxp3), so-called regulatory T cells (Treg cells). Under certain circumstances, these tolerance mechanisms are breached and auto-reactive T cell responses are initiated resulting in systemic or organ-specific autoimmune pathology. The activation of auto-reactive T cells can be caused by several mechanisms. These include, for example, microbial molecular mimics of auto-antigens (sequence similarities between antigenic epitopes present in pathogens and host proteins)(Fujinami and Oldstone, 1985), by-stander activation during an ongoing immune response against an unrelated antigen (Horwitz et al., 1998), or true cross-reactivity (refers to the capability of a given T cell to recognize both, microbial and self-antigens) (Carrizosa et al., 1998).

3.3 Multiple sclerosis

Multiple sclerosis (MS) is a paradigmatic example of organ-specific autoimmunity. MS is the most common non-traumatic cause for disability affecting young adults in Northern America and Europe (Compston, 2005). The majority of MS patients experience their first symptoms between the ages of 20 and 40. MS is a chronic inflammatory disease of the central nervous system (CNS) that was first recognized as a distinct entity in 1868 by Jean Martin Charcot, professor of Neurology at the University of Paris. He succeeded in correlating clinical signs of the disease with pathological changes observed in post mortem CNS tissue (Charcot, 1877). Since any part of the brain or the spinal cord can be damaged in the course of MS, patients can suffer from a variety of symptoms, including vision problems, motor and sensory symptoms, and cognitive difficulties. Histopathologically, the hallmarks of MS are inflammation, demyelination, and axonal damage. Although MS has been considered to primarily be a disease of the CNS white matter, axons also become injured and degenerate, which leads to loss of neurological function and permanent disability (Trapp et al., 1998, Peterson et al., 2001).

The prevailing pathogenetic hypothesis of inflammatory demyelination and neurodegeneration in MS is that T cells that are reactive to myelin antigens migrate across the BBB, become

reactivated in the perivascular space, and invade the glia limitans, thus creating an inflammatory environment within the CNS parenchyma. This is then associated with the accumulation of myeloid phagocytic cells, B cells, and plasma cells. The secretion of myelin-toxic mediators such as tumor necrosis factor (TNF) or reactive oxygen species as well as effector functions of myelin specific autoantibodies finally mediate oligodendrocyte injury and secondary tissue damage. Tissue repair in the CNS is limited and remyelination after injury occurs slowly and in an incomplete manner. Finally, persistent inflammation and failure of remyelination under inflammatory conditions result in irreversible damage of the myelin sheath and in irreversible transection of axons, formation of scars, and persistent neurologic impairment of the patient. Although immunologic treatments of MS are available to prevent inflammatory bouts of the disease, MS cannot be cured yet and the therapeutic approach focuses on early control of tissue inflammation in order to minimize long term disability due to axonal damage and degenerative events secondary to inflammatory assaults.

3.4 Experimental autoimmune encephalomyelitis

To better understand the cellular and molecular mechanisms of MS pathology, several animal models have been developed. The most common model, experimental autoimmune encephalomyelitis (EAE), imitates specific features of the neurobiology of MS. EAE was first introduced in the 1930ies (Rivers and Schwentker, 1935) and much improved as an animal model for inflammatory demyelination in the CNS by the application of complete Freund's adjuvant (CFA) to boost the immune response during sensitization with auto-antigens (Kabat et al., 1947, Korn et al., 2010). Currently, EAE is mostly elicited by immunization with CNS myelin antigens, including myelin oligodendrocyte glycoprotein (MOG) (Linington et al., 1993, Amor et al., 1994) proteolipid protein (PLP) (Kuchroo et al., 1991) or myelin basic protein (MBP) (Ben-Nun et al., 1981, Fritz et al., 1983). To induce EAE, myelin antigens are emulsified with CFA and administered subcutaneously. CFA greatly enhances the immune response at the site of immunization and in the draining lymph nodes (dLN) probably by prolonging the presence of the antigen and by the activation of antigen-presenting cells (APCs) (Tsuji et al., 2000, Libbey and Fujinami, 2011). In C57BL/6 mice the immunization regimen also comprises administration of Pertussis toxin, which has additional adjuvant effects and might also contribute to activate the BBB to facilitate recruitment of inflammatory cells into

the CNS compartment (Amiel, 1976, Yong et al., 1993, Ben-Nun et al., 1997, Hofstetter et al., 2002).

Mice usually experience first motor symptoms 8-14 days after immunization with auto-antigen. The peak of disease (paraplegia) is reached three days after the onset of disease. Depending on which mouse strain and which myelin peptide are used, monophasic, relapsing, or progressive forms of EAE can be induced (Pollinger, 2012).

In contrast to active induction of EAE by immunization with myelin auto-antigens, EAE can also be induced by adoptive transfer of activated myelin-specific (e. g. MOG_{35-55} (MOG peptide 35-55)-specific (Bettelli et al., 2003)) T cells into naïve animals. In fact, by these adoptive transfer experiments, activated myelin-specific $CD4^+$ T helper cells were identified to be necessary and sufficient for the induction of EAE. Thus, EAE – and by analogy, MS – are considered as T cell-mediated organ-specific autoimmune diseases.

3.5 T helper cells

3.5.1 Activation of T helper cells in the periphery

Antigens are engulfed and transported to the dLNs by APCs, particularly dendritic cells (DCs). In the dLNs APCs present the digested antigen on their cell surface in association with major histocompatibility complex (MHC) class II molecules. Naïve T helper (Th) cells with the appropriate antigen-specific T cell receptor (TCR) recognize the antigen-MHC complex, become activated, and undergo clonal expansion. Full activation of Th cells requires the synergistic activation of co-stimulatory molecules (e.g. CD28) by specific ligands on the APC surface in addition to TCR engagement. Once activated, naïve Th cells differentiate into distinct effector subclasses, produce pro-inflammatory cytokines and recruit and activate other immune cells like macrophages and B cells. Some of the T and B cells involved in the inflammation develop into memory cells which are long-lived lymphocytes able to initiate a more rapid response against antigens they have previously encountered (Harrington et al., 2008, Lohning et al., 2008).

Th cell effector functions such as for example the production of pro-inflammatory cytokines and the activation of B cells with subsequent antibody production have evolved to help the host to fight against pathogens. However, the T cell repertoire of each individual contains mature Th cells with self-reactive T cell receptors that have escaped thymic deletion and populate the

peripheral immune compartment. Due to the failure of peripheral tolerance mechanisms, especially Treg cell-mediated suppression of auto-reactive T helper cells, auto-reactive T helper cells can become activated in peripheral immune compartment upon recognition of auto-antigens that drain from tissues and are presented by professional APCs in the context of MHC II molecules. Activated T cells expand and simultaneously initiate a developmental program that leads to the commitment of the T cell to a specific effector cell phenotype. Depending on the cytokines that are present in the environment, distinct T cell subsets arise that can be distinguished based on their cytokine profiles and transcriptional regulators (Petermann and Korn, 2011). These T cell subsets include Th1 cells, Th2 cells, and Th17 cells as effector cells and peripherally induced Foxp3^{+} Treg cells that are involved in immune regulation and suppression (Figure 3.1).

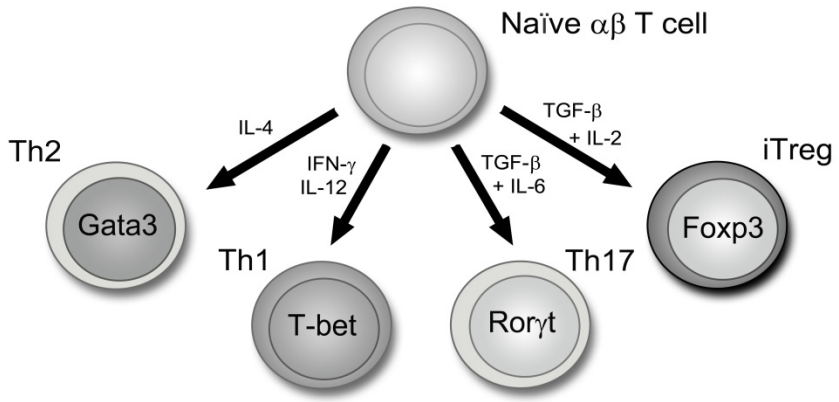


Figure 3.1: T cell subsets
Naïve T cells can differentiate into T helper type 1 (Th1), Th2, Th17, and Treg cells, depending on the cytokine environment at the time of their encounter of cognate antigen in secondary lymphoid tissues. The transcription factors Gata3, T-bet, Rorγt, and Foxp3 are, amongst others, required for the induction of Th2, Th1, Th17, and induced regulatory T cells (iTreg) cells, respectively.

3.5.2 Th1 cells

In 1986 Robert L. Coffman and Tim Mosmann identified two subsets of activated T cells that differed from each other by their cytokine profile and function (Mosmann et al., 1986). Based on this finding they formulated their so-called Th1-Th2 hypothesis, which proposes a categorization of T helper cells into IL-4 producing Th2 cells and Interferon (IFN)-γ producing Th1 cells. Th1 cytokines that include IFN-γ, TNF, and lymphotoxin, but not IL-4, activate macrophages. Therefore, Th1 cells serve as important mediators of phagocyte-dependent protective responses against intracellular pathogens. The commitment of naïve T cell

precursors into Th1 cells proceeds in two steps. First, TCR signaling together with sensing of small amounts of IFN-γ produced by innate immune cells up-regulate the transcription factor T-bet (T-box expressed in T cells) in a STAT1 (signal transducer and activator of transcription)-dependent manner (Afkarian et al., 2002). T-bet, in turn, induces IFN-γ and the specific IL-12 receptor (IL-12R) subunit beta 2, which is indispensable for IL-12 sensing (Afkarian et al., 2002). Then, IL-12, secreted by DCs and macrophages (Hsieh et al., 1993, Macatonia et al., 1995), further transactivates T-bet and thus, stabilizes the production of large amounts of IFN-γ, which determines the terminal commitment of naïve T cells to the Th1 lineage.

Since the histopathological and clinical features of EAE and MS, but also of other autoimmune diseases including psoriasis and rheumatoid arthritis, could be reconciled with Th1-type immune responses, Th1 cells were thought to be the major effector T cells in organ-specific autoimmunity. This conclusion was supported by the findings that mice deficient for T-bet or for the IL-12 subunit p40 were resistant to the induction of EAE (Becher et al., 2002, Bettelli et al., 2004, Nath et al., 2006). Accordingly, the adoptive transfer of myelin-specific Th1 cell clones was sufficient to promote EAE (Kuchroo et al., 1993). Furthermore IFN-γ, the key cytokine of Th1 cells, was identified in the lesions of MS patients and treatment of MS with IFN-γ exacerbated disease (Panitch et al., 1987). Together these results led to the conclusion that EAE and MS are predominantly driven by auto-reactive Th1 cells.

3.5.3 Th2 cells

Th2 cell responses are essential for the clearance of extracellular pathogens such as helminths but are also responsible for the development of asthma and allergy (Kay et al., 1991, Robinson et al., 1992). Th2 cells exert their functions through the production of various cytokines. Besides IL-4, Th2 cells secrete IL-5 and IL-13 but not IFN-γ. IL-4, the key cytokine secreted by Th2 cells, is also essential for Th2 commitment. The binding of IL-4 to its receptor results in an activation of STAT6, which in turn up-regulates GATA3 (GATA binding protein 3), the Th2 master transcription factor (Kurata et al., 1999). GATA3 transactivates Th2-specific effector cytokines such as IL-4, IL-5, and IL-13 (Zheng and Flavell, 1997).

Once committed to either the Th1 or Th2 lineage, Th cells mutually suppress one another via their characteristic cytokines, a process, designated as cross-regulation. This further supports the hypothesis that Th1 and Th2 cells are two distinct subsets of activated $CD4^+$ cells with

opposed functions. Accordingly, Th2 cells were found to exert suppressive effects during organ-specific autoimmunity. In 1995, Kuchroo et al. showed that the adoptive transfer of myelin-specific Th2 clones prevented EAE and even abrogated an established disease (Kuchroo et al., 1995). Moreover, the administration of IL-4 after the adoptive transfer of encephalitogenic T cells resulted in an alleviated course of EAE (Racke et al., 1994). The increased susceptibility and severity of EAE in *Il10*$^{-/-}$ and *Il4*$^{-/-}$ mice compared to wild type mice further strengthened the idea that Th2 cells have the capacity to suppress Th1-mediated inflammation during organ-specific autoimmunity (Bettelli et al., 1998).

In the therapy of MS, Glatiramer acetate (GA), a synthetic random polymer of the amino acids L-alanine, L-glutamate, L-lysine, and L-tyrosine, was shown to reduce the relapse frequency in MS patients and the appearance of new lesions (Mancardi et al., 1998, Johnson et al., 2000). It is believed that GA works at least partially by inducing a Th1 to Th2 shift which is accompanied by the production of IL-10 (Neuhaus et al., 2000, Oreja-Guevara et al., 2012).

3.5.4 Th17 cells

3.5.4.1 Discovery and effector functions during disease

Over several years IL-12 and Th1 cells had been considered as the archetypical inducers of autoimmune inflammation. However, this hypothesis was challenged as mice that were deficient for the Th1 hallmark cytokine (*Ifng*$^{-/-}$) or its receptor (*Ifngr*$^{-/-}$) were not resistant, but even more susceptible to the induction of EAE (Ferber et al., 1996, Willenborg et al., 1996) and other autoimmune diseases (Jones et al., 1997, Vermeire et al., 1997). Similarly surprising was the finding that EAE could easily be induced in mice lacking the p35 subunit of IL-12 (Gran et al., 2002) whereas mice lacking the p40 subunit of IL-12 were resistant to EAE (Becher et al., 2002). This problem was only addressed in 2003 when it was discovered that IL-23, which shares the p40 subunit with IL-12 but has a specific p19 subunit (Oppmann et al., 2000, Hunter, 2005)(see also Figure 3.2), and not IL-12 (which is a heterodimer consisting of p40 and p35) was required for the induction of EAE. In fact, *p19*$^{-/-}$ (and therefore *Il23*$^{-/-}$) and *Il23r*$^{-/-}$ mice were resistant to the induction of EAE (Cua et al., 2003, Awasthi et al., 2009). In a seminal study, Langrish et al. showed that IFN-γ producing CD4^{+} T cells that accumulated in the CNS of IL-23 deficient mice were not capable to induce EAE. However, *Il23p19*$^{-/-}$ mice lacked CD4^{+} T cells that produced IL-17 and the absence of IL-17 producing CD4^{+} T cells in these mice correlated with their resistance to EAE (Langrish et al., 2005). Additional

experiments in this study demonstrated, that the adoptive transfer of in vitro expanded myelin-specific IL-17 producing T cells into naïve recipients induced a severe EAE whereas recipients of Th1 cells showed no clinical signs of EAE. Based on these results the hypothesis was put forward that IL-17 producing T cells that developed under the influence of IL-23 were the major players in the induction of autoimmunity. Since IL-17 is not produced by Th1 or Th2 cells, the Th1/Th2 paradigm was extended by a third "lineage" of T helper cells, termed Th17 cells after their signature cytokine IL-17. Besides IL-17, Th17 cells also produce IL-17F (Starnes et al., 2001, Yang et al., 2008a), IL-21 (Korn et al., 2007a, Nurieva et al., 2007, Wei et al., 2007), and IL-22 (Liang et al., 2006).

IL-17 is the prototypic cytokine of the IL-17 family which contains IL-17, IL-17B, IL-17C, IL-17D, IL-17E, and IL-17F (Aggarwal et al., 2003, Moseley et al., 2003, Weaver et al., 2007). IL-17 and IL-17F are the best characterized family members and have distinct but overlapping (mostly pro-inflammatory) functions (Kolls and Linden, 2004). The receptor for IL-17 and IL-17F is a heterodimer composed of IL-17RA and IL-17RC (Yao et al., 1995, Toy et al., 2006, Kuestner et al., 2007) and is expressed on most parenchymal cells. Signaling through this receptor induces the expression of pro-inflammatory cytokines (such as IL-6 and TNF), chemokines (such as MCP-1 and MIP-2), and matrix metalloproteases in target cells (Yao et al., 1995, Fossiez et al., 1996, Park et al., 2005).

Th17 cells play a pivotal role in host defense at epithelial and mucosal surfaces mainly by recruiting and activating neutrophils (Yu et al., 2007). Particularly, the clearance of several extracellular pathogens such as Klebsiella pneumoniae (Ye et al., 2001) and Candida albicans (Huang et al., 2004) require Th17-mediated immunity.

Th17 cells have also been implicated in the pathogenesis of several autoimmune diseases both in humans and mice. In patients suffering from MS (Matusevicius et al., 1999), psoriasis (Teunissen et al., 1998), rheumatoid arthritis (Aarvak et al., 1999), or inflammatory bowel disease (Fujino et al., 2003) elevated levels of IL-17 were detected. IL-17 deficient mice experience an EAE with delayed onset and reduced severity (Komiyama et al., 2006) and are protected against collagen-induced arthritis (CIA) (Nakae et al., 2003). Furthermore, treating mice with an IL-17 neutralizing antibody attenuated the severity of EAE (Hofstetter et al., 2005).

In general, inflammatory conditions evoked by Th17 cells are characterized by chronicity and tissue destruction resulting in severe immunopathology. Thus, Th17-associated cytokines are

considered as promising therapeutic targets in autoimmunity and chronic inflammatory conditions.

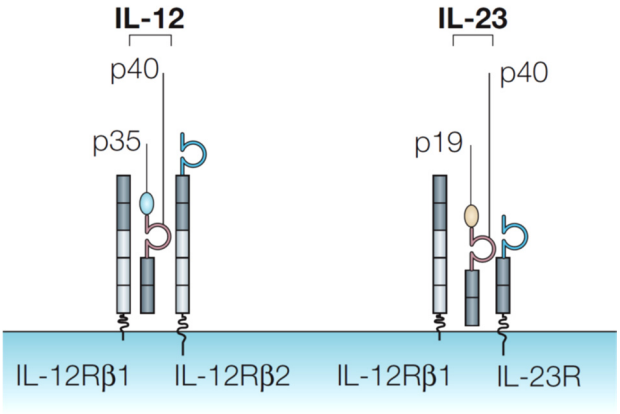


Figure 3.2: Structure of IL-23 and IL-12 and their respective receptors
(adapted from Hunter et al., 2005)

3.5.4.2 Generation of Th17 cells

Mice deficient for IL-23 have profound defects in the generation of IL-17 producing cells (Langrish et al., 2005). Therefore, it was initially suggested that IL-23 induces the differentiation of Th17 cells. However, IL-23R is not expressed on naïve T cells (Parham et al., 2002) and IL-23 itself cannot drive the differentiation of naïve T cells into Th17 cells (Bettelli et al., 2006, Mangan et al., 2006, Veldhoen et al., 2006). Instead, the combination of Transforming Growth Factor (TGF)-β and IL-6 induces high amounts of IL-17 in anti-CD3/anti-CD28 activated T cells (Bettelli et al., 2006, Mangan et al., 2006, Veldhoen et al., 2006). In the absence of IL-6, Th17 cells can also be generated by a combination of TGF-β and IL-21 (Korn et al., 2007a). Th17 cells themselves produce IL-21, which is believed to sustain Th17 lineage commitment in an autocrine manner (Korn et al., 2007a, Nurieva et al., 2007, Wei et al., 2007) similar to IL-4 and IFN-γ that promote the precursor frequency of Th2 and Th1 cells, respectively. Recent findings show that the amplification and stabilization of the Th17 phenotype is dependent on IFN regulatory factor 4 (IRF4) (Huber et al., 2008, Ciofani et al., 2012).

Th17 cells are considered to be a distinct lineage of T helper effector cells because they can be generated in the genetic absence of transcription factors required for Th1 and Th2 differentiation and are thus independent of the Th1 and Th2 transcriptional programs.

Moreover, Rorγt (Retinoic acid-related orphan receptor γt) has been identified as master transcription factor for Th17 cells (Ivanov et al., 2006). Besides Rorγt, other transcription factors such as Rorα and c-Maf are believed to be involved in Th17 commitment. Rorα deficiency impairs IL-17 production in vitro and in vivo. Double deficiency of Rorα and Rorγt even abolishes IL-17 production completely (Yang et al., 2008b). The transcription factor c-Maf is reported to participate in the development of Th17 cells, most likely by regulation of the IL-21 production (Bauquet et al., 2009).

In activated Th17 cells, IL-6 induces the expression of IL-23R and IL-1R1. IL-23 stabilizes and terminally differentiates Th17 cells (discussed below). IL-1 is described as a regulator of the transcription factors IRF4 and Rorγt (Chung et al., 2009). The finding that the generation of antigen-specific Th17 cells is abolished during EAE in IL-1R deficient mice further points to an important role of IL-1in the induction of Th17 cells (Sutton et al., 2006).

3.5.5 Role of IL-23 in promoting autoimmunity

IL-23 is a pivotal factor in the pathogenesis of organ-specific autoimmunity and chronic inflammation. IL-23 and IL-23R deficient mice are resistant to the induction of several autoimmune diseases, including EAE (Langrish et al., 2005, Awasthi et al., 2009). Accordingly, transgenic mice ubiquitously expressing IL-23p19 die from severe multi-organ inflammation (Wiekowski et al., 2001). Moreover, polymorphisms in the gene for IL-23R, *IL23RA*, are major susceptibility factors in humans to develop autoimmune diseases and chronic inflammatory conditions such as psoriasis (Smith et al., 2008) and inflammatory bowel disease (Duerr et al., 2006).

IL-23, which is secreted by APCs upon Toll-like receptor (TLR) stimulation in early stages of inflammation, was believed to be crucially involved in the induction of pro-inflammatory Th17 cells. But naïve T cells cannot sense IL-23 because they do not express IL-23R and naïve T cells stimulated with IL-23 alone do not differentiate into Th17 cells. IL-23 is clearly not required for initial induction of Th17 cells neither in vivo nor in vitro. However, mice deficient for IL-23 have profound defects in the generation of encephalitogenic IL-17 producing cells during EAE.

Several studies elucidated distinct IL-23-specific functions concerning Th17 stabilization and terminal differentiation. IL-17 producing myelin-specific $CD4^+$ T cells isolated from immunized mice expand in an ex vivo culture only when they are stimulated with IL-23

(Langrish et al., 2005). McGeachy et al. showed that the stimulation of T cells with TGF-β and IL-6 initiated Th17 differentiation but also induced IL-10 production which seemed to restrain the pathogenic potential of Th17 cells by a self-limiting feedback loop (McGeachy et al., 2007). In contrast, addition of IL-23 inhibited the production of IL-10 but promoted the expression of IL-17 and thus, conferred highly pathogenic properties to Th17 cells. Therefore, IL-23 is indispensable for full differentiation of T cells into pathogenic effector Th17 cells (McGeachy et al., 2009) and in the absence of IL-23, Th17 cells arrest at an early point of differentiation, fail to down-regulate IL-2R, and to maintain their IL-17 production.

Furthermore, IL-23 also induces the expression of other pro-inflammatory cytokines, such as IL-22 (Liang et al., 2006, Zheng et al., 2007) and GM-CSF (granulocyte-macrophage colony-stimulating factor) in Th17 cells (Codarri et al., 2011, El-Behi et al., 2011). In turn, GM-CSF induces the production of IL-23 by APCs, thus creating a positive feedback loop at sites of inflammation.

In summary, there is a general consensus that IL-23 is crucially involved in the maturation of Th17 cells. However, the role of IL-23 in vivo is still under debate and accumulating evidence suggests that the impact of IL-23 on the promotion of autoimmunity and chronic inflammation is partly mediated by innate immune cells. Innate immune cells were found to drive local intestinal inflammation in an IL-23-dependent manner even in the absence of T cells (Uhlig et al., 2006) and IL-23 responsive γδ T cells were sufficient to induce a psoriasis-like disease in mice (Cai et al., 2011). A variety of innate immune cells respond to IL-23 (Cua and Tato, 2010) and their role in promoting immunopathology in organ specific autoimmunity is incompletely understood. Therefore, a major focus of this thesis was to explore in more detail T helper cell extrinsic functions of IL-23.

3.6 Treg cells

3.6.1 Introduction

In 1982 Powell et al. first described a severe X-linked recessive disorder characterized by immunodysregulation, polyendocrinopathy and enteropathy (IPEX) leading to death within the first two years of life if untreated (Powell et al., 1982). Analogous symptoms were also observed in a spontaneous mouse mutant, known as “scurfy”. The characteristic phenotype of scurfy mice was found to be a result of mutations in the *Foxp3* gene (Brunkow et al., 2001)

which is located on the X chromosome. Accordingly, mutations in the human homolog, *FOXP3*, were identified to cause IPEX (Bennett et al., 2001). Foxp3 is the most specific marker of immunosuppressive T cells, called regulatory T (Treg) cells which do not develop in the absence of Foxp3. The pool of Treg cells in the periphery consists of two types of Treg cells: natural Treg (nTreg) cells and induced Treg (iTreg) cells, both expressing Foxp3 and high levels of the IL-2Rα subunit CD25. Phenotypically, iTreg cells are indistinguishable from thymic derived nTreg cells. However, it is believed that iTreg cells are particularly essential for the tolerance at mucosal surfaces, which are characterized by a permanent exposure to foreign antigens (Curotto de Lafaille and Lafaille, 2009). Nevertheless, it has to be assumed that iTreg cells are also generated in response to self-antigens and participate in maintaining self-tolerance.

In the periphery 5-10 % of all T cells are nTreg cells. Like conventional T cells, nTreg cells are generated in the thymus from progenitor cells originating from the bone marrow. iTreg cells differentiate in the periphery from naïve T cells. A combination of TGF-β and IL-2, concomitant with strong TCR signals and suboptimal co-stimulation induces Foxp3 expression in naïve $CD4^+$ $CD25^-$ $Foxp3^-$ T cells and thus, mediates a conversion of conventional T cells into Treg cells (Chen et al., 2003). The dependency of both, Th17 and Treg cell induction, on TGF-β is surprising because Th17 cells and iTreg cells are functional antagonists in host defense and autoimmunity. However, TGF-β alone induces Treg cells, while a combination of TGF-β and IL-6 inhibits the generation of Treg cells but promotes the generation of Th17 cells suggesting that IL-6 plays a crucial role in determining Th17/Treg cell balance (Bettelli et al., 2006). Similarly, IL-21 induces Th17 cells, but suppresses the generation of Treg cells (Korn et al., 2007a). In contrast, IL-2 serves as growth factor for Treg cells and promotes the generation of Treg cells, but inhibits the generation of Th17 cells (Laurence et al., 2007). These findings point to a reciprocal regulation of Th17 cells and iTreg cells during inflammatory processes.

3.6.2 Treg cells in autoimmunity

Autoimmunity is thought to arise when Treg cells fail to suppress effector mechanisms of auto-reactive T cells. It is proposed that this failure is caused by a reduced suppressive capacity of Treg cells. This assumption is supported by the findings that Treg cells of patients with MS (Viglietta et al., 2004, Haas et al., 2005), myasthenia gravis (Balandina et al., 2005), or rheumatoid arthritis (RA) (Ehrenstein et al., 2004) show impaired suppressive functions,

although their frequencies are not altered. Furthermore, activated auto-reactive T cells present at the site of inflammation are thought to be less susceptible to Treg cell-mediated suppression (van Amelsfort et al., 2004). By performing EAE experiments in *Foxp3gfp*.knock-in (KI) reporter mice Korn et al. provided an insight into the molecular mechanisms responsible for the failure of Treg cell-mediated suppression of effector T cells during autoimmunity (Korn et al., 2007b). They demonstrated that Treg cells accumulated in the CNS of immunized mice during the acute stage of EAE but were not able to suppress the proliferation and massive cytokine production of invading effector T cells. In a seminal experiment, they isolated effector T cells from the CNS and the spleen of immunized mice and performed in vitro suppression assays. They found that CNS-derived effector T cells were resistant to the Treg cell-mediated suppression whereas spleen-derived cells were not. Furthermore CNS-derived effector T cells secreted large amounts of IL-6 and TNF. The combination of IL-6 and TNF was found to completely abolish Treg cell-mediated suppression of effector T cells. This study emphasizes the role of the local inflammatory cytokine milieu in shaping regulatory and effector T cell functions in autoimmunity.

3.7 γδ T cells

3.7.1 γδ T cell development in the thymus

T cells develop from lymphoid progenitors originating from the extra-embryonic yolk sac and the fetal liver during embryogenesis (Huang and Auerbach, 1993, Sasaki and Sonoda, 2000). Later in gestation and after birth lymphoid progenitors are exclusively generated in the bone marrow (Bartlett, 1982). Committed T cell progenitors leave the bone marrow and migrate to the thymus for differentiation and maturation (Miller and Osoba, 1967). Immature T cell precursors entering the thymus do not express the co-receptors CD4 and CD8 and are therefore called double negative (DN) thymocytes (Fowlkes et al., 1985). During T cell ontogeny DN thymocytes undergo a developmental program which also includes TCR gene rearrangement (Godfrey et al., 1993). The TCR genes, called alpha (α), beta (β), gamma (γ), and delta (δ), consist of numerous, discontinuous coding segments that become rearranged during T cell ontogeny (somatic rearrangement) (Hozumi and Tonegawa, 1976). While most T cells express a TCR that is composed of a αβ heterodimer, a minor subset of T cells (γδ T cells) uses a TCR that consists of a γ and a δ chain. αβ T cells develop into CD4 expressing T helper cells or CD8

expressing T cytotoxic cells before exiting the thymus. In contrast, γδ T cells do not up-regulate neither CD4 nor CD8 but leave the thymus as DN cells.

Based on their γδ-TCR diversity and ontogeny, two major subsets of γδ T cells can be distinguished in mice: The first subset is a group of γδ T cells with invariant TCRs, named canonical γδ T cells. These cells are generated in the embryonic thymus during several distinct developmental waves. Canonical γδ T cells of the first wave populate the epidermis of the skin (skin resident dendritic epidermal T cells (DETCs)). DETCs express a TCR that is composed of a Vγ5 and Vδ1 chain in mice. Canonical γδ T cells of the second wave are later found in the vaginal epithelium and express Vγ6 and Vδ1 TCRs. γδ T cells of subsequent waves home to other tissues, such as lungs and gut. Thus, canonical γδ T cells are niche-specific effector cells with highly conserved invariant TCRs that respond fast and vigorously to environmental stimuli. In contrast, the second subset of γδ T cells has a broader TCR repertoire than canonical γδ T cells. These non-canonical γδ T cells appear in late phases of gestation and even postnatally and are found in secondary lymphoid organs (Carding and Egan, 2002). Similar to $CD4^+$ T helper cells, non-canonical γδ T cells can be defined on the basis of distinct cytokine profiles: IFN-γ producing (γδT1) and IL-17 producing γδ T (γδT17) cells constitute distinct functional phenotypes of γδ T cells. However, while T helper cells are committed to distinct T helper cell lineages upon antigen recognition in the peripheral immune compartment, non-canonical γδ T cells are instructed to adopt a distinct cytokine phenotype during their development in the thymus (Korn and Petermann, 2012). The development of functionally distinct non-canonical γδ T cell subsets as defined by distinct cytokine profiles is initiated as early as in the embryonic thymus, reaches a steady state around embryonic day 18 and continues postnatally.

3.7.2 Activation of γδ T cells in the peripheral immune compartment

In contrast to αβ T cells and B cells, but similarly to cells of the innate immune system, γδ T cells are equipped with a very limited repertoire of antigen receptors. While in the secondary lymphoid organs only 1-2 % of all T cells are γδ T cells, a substantial proportion of γδ T cells resides in epithelial tissues, particularly in the intestinal epithelium where they recognize invading pathogens but also self-molecules induced by inflammation (Girardi, 2006). γδ T cells, together with innate immune cells, act as first sensors of danger and dysregulation as they recognize molecules on the surface of cells that are only expressed under conditions of cellular

stress responses. This “altered self-recognition” might be part of a defense mechanism called lymphoid stress surveillance (Hayday, 2009) that permits immediate immune responses in early stages of infection. However, until now this hypothesis is not established in vivo as appropriate “altered self” ligands have been found in humans, but not in mice, which has yet precluded the generation of appropriate animal models.

γδ T cells are believed to recognize antigen without processing or presentation in the context of MHC molecules which is supported by the finding that γδ T cell function is not altered in MHC-deficient mice (Correa et al., 1992, Bigby et al., 1993). Thus, the manner of antigen recognition seems to be quite different from that of αβ T cells. γδ T cells are thought to recognize conserved structures of pathogens and self-molecules that are fixed to surfaces and denote cell stress or damage. However until now, only a few γδ TCR specificities have been described. Several mouse γδ T cells were found to be self-reactive to MHC molecules, or to MHC-related proteins, including the mouse MHC class II molecule I-E^k (Matis et al., 1989) and the non-peptide-binding MHC class Ib molecules H2-T10 and H2-T22 (Bonneville et al., 1989, Weintraub et al., 1994, Crowley et al., 1997). Furthermore, Herpes simplex virus glycoprotein I (Johnson et al., 1992) and Mycobacterium tuberculosis peptides (O'Brien et al., 1989) were identified as antigens of γδ T cells.

Importantly, murine γδ T cells were found to be activated directly by cytokine signals or TLR stimuli even in the absence of TCR ligation (Martin et al., 2009) which further highlights the innate-like phenotype of γδ T cells.

3.7.3 γδ T cell effector mechanisms

γδ T cells are activated in early stages of inflammation when αβ T cell responses are not yet initiated. Within hours upon infection, γδ T cells are able to produce large amounts of pro-inflammatory cytokines, chemokines, and cytotoxic molecules (Ferrick et al., 1995, Boismenu et al., 1996, Paget et al., 2012) and thus, to create an inflammatory milieu that serves to recruit other immune cells. Accordingly, γδ T cell deficient mice exhibit specific immune defects, particularly in immune responses that are dominated by neutrophils such as Klebsiella pneumonia and Nocardia asteroides (King et al., 1999, Tam et al., 2001). Surprisingly, γδ T cells, and not Th17 cells, were identified as a critical source of IL-17 in several infection models, including infections with Mycobacterium tuberculosis and Listeria monocytogenes. (Lockhart et al., 2006, Shibata et al., 2007). Interestingly, Awasthi et al. demonstrated that

peripheral immune compartments comprise a sizeable fraction of γδ T cells with constitutive expression of the IL-23 receptor and thereby, with the ability to sense IL-23 (Awasthi et al., 2009). Since T helper cell extrinsic functions of IL-23 were a main focus of this work, the subset of IL-23R$^+$ γδ T cells was a promising innate immune cell population to look at in greater detail in order to unravel functions of IL-23 beyond the stabilization of IL-17 production in T helper cells.

3.8 Aim of the study

Uncontrolled activation of T helper cells is a hallmark of many autoimmune diseases, such as EAE and MS. Organ-specific autoimmunity has long been considered as a classical Th1-mediated inflammatory process. However, in the last years IL-17 producing T helper cells, the so-called Th17 cells, have emerged as key players during autoimmunity. IL-23 was identified to be a crucial maturation factor of Th17 cells and promote autoimmunity in a T cell intrinsic manner. However, a subset of γδ T cells was identified to express the IL-23R as well. Thus, this thesis was designed to investigate the development of IL-23R$^+$ γδ T cells, to explore their role during the initiation of adaptive immune responses in the peripheral immune compartment, and to analyze their contribution to CNS inflammation in the course of EAE. Therefore, the underlying concept of this project referred to the idea to elucidate non-redundant T helper cell extrinsic functions of IL-23 during organ-specific autoimmunity that might be mediated through IL-23R$^+$ γδ T cells.

4 Materials and Methods

4.1 Materials

4.1.1 Reagents

7-AAD	BD Biosciences (Heidelberg, Germany)
10x DNA Loading buffer	Life Technologies (Carlsbad, USA)
Agarose	Sigma-Aldrich (St. Louis, USA)
Aqua	B.Braun (Melsungen, Germany)
Arlacel A (Mannide Monooleate)	GERBU (Wieblingen, Germany)
Baytril, 2.5 %	Boehringer Ingelheim (Ingelheim am Rhein, Germany)
Bovine serum albumin (BSA)	Sigma-Aldrich (St. Louis, USA)
DNA ladder (1kb and 100bp)	Life Technologies (Carlsbad, USA)
Dulbecco's modified Eagle medium (DMEM)	PAA (Pasching, Austria)
EDTA	Merck Millipore (Darmstadt, Germany)
Ethidium bromide solution	Sigma-Aldrich (St. Louis, USA)
Fetal calf serum (FCS)	Sigma-Aldrich (St. Louis, USA)
Fixation and permeabilization solution-BD Cytofix/Cytoperm®	BD Biosciences (Heidelberg, Germany)
H37RA Mycobacterium tuberculosis	BD Biosciences (Heidelberg, Germany)
Ionomycin calcium salt, from Streptomyces conglobatus	Sigma-Aldrich (St. Louis, USA)
Isofluran	Abbott (Chicago, USA)
L-Arginine	Sigma-Aldrich (St. Louis, USA)
L-Asparagine	Sigma-Aldrich (St. Louis, USA)
Liquid scintillation fluid, Betaplate Scint®	PerkinElmer (Waltham, USA)
MEM Non-Essential Amino Acids (100x)	Life Technologies (Carlsbad, USA)
MOG_{35-55}	Auspep (Tullamarine, Australia)
Nuclease free water	Life Technologies (Carlsbad, USA)
Paraffin oil	Sigma-Aldrich (St. Louis, USA)
Percoll	GE Healthcare (Munich, Germany)
Permeabilization/wash buffer-BD Perm/Wash	BD Biosciences (Heidelberg, Germany)
Penicillin/Streptomycin solution	Life Technologies (Carlsbad, USA)

Peroxidase substrate solution B	KPL (Gaithersburg, USA)
Pertussis toxin	Sigma-Aldrich (St. Louis, USA)
Phorbol 12-myristate 13-acetate (PMA)	Sigma-Aldrich (St. Louis, USA)
Phosphate-buffered saline (PBS)	PAA (Pasching, Austria)
Protein transport inhibitor (Containing Monensin)-GolgiStop®	BD Biosciences (Heidelberg, Germany)
Real-Time PCR reaction master mix	Life Technologies (Carlsbad, USA)
Red blood cell lysis buffer-PharmLyse®	BD Biosciences (Heidelberg, Germany)
RNase surface decontamination solution-RNaseZap®	Sigma-Aldrich (St. Louis, USA)
TMB peroxidase substrate	KPL (Gaithersburg, USA)
Trypan blue	Life Technologies (Carlsbad, USA)
Thymidine, [Methyl-^{3}H]-	PerkinElmer (Waltham, USA)
Vitamin solution, MEM Vitamin Solution (100x)	Life Technologies (Carlsbad, USA)

Any other, not itemized chemicals were purchased from Sigma-Aldrich (St. Louis, USA).

4.1.2 Enzymes

Collagenase D	Roche (Mannheim, Germany)
DNase I	Roche (Mannheim, Germany)
Green Taq	Thermo Fisher (Waltham, USA)
Platinum PCR Super Mix	Life Technologies (Carlsbad, USA)
Proteinase K	Sigma-Aldrich (St. Louis, USA)
RNase inhibitor 20 U/µl	Life Technologies (Carlsbad, USA)

4.1.3 Kits

ELISA mouse IFN-γ set	R&D (Minneapolis, USA)
ELISA mouse IL-6 set	R&D (Minneapolis, USA)
ELISA mouse IL-17 set	R&D (Minneapolis, USA)
ELISA mouse IL-21 set	R&D (Minneapolis, USA)
ELISA mouse IL-22 set	R&D (Minneapolis, USA)
Foxp3 staining buffer set	eBioscience (San Diego, USA)
Live/dead fixable cell stain kit	Life Technologies (Carlsbad, USA)
PKH26 kit	Sigma-Aldrich (St. Louis, USA)

Reverse transcription kit	Life Technologies (Carlsbad, USA)
Total RNA purification kit (RNeasy®)	Qiagen (Venlo, Netherlands)

4.1.4 Magnetic beads

CD4 (L3T4) Microbeads	Miltenyi Biotec (Bergisch Gladbach, Germany)
γδ T cell isolation kit	Miltenyi Biotec (Bergisch Gladbach, Germany)
$CD4^+CD62L^+$ T cell isolation kit II	Miltenyi Biotec (Bergisch Gladbach, Germany)
CD90.2 Microbeads	Miltenyi Biotec (Bergisch Gladbach, Germany)

4.1.5 Cytokines

human recombinant TGF-β	R&D (Minneapolis, USA)
mouse recombinant IL-1β	R&D (Minneapolis, USA)
mouse recombinant IL-6	R&D (Minneapolis, USA)
mouse recombinant IL-12	R&D (Minneapolis, USA)
mouse recombinant IL-21	R&D (Minneapolis, USA)
mouse recombinant IL-23	R&D (Minneapolis, USA)

4.1.6 Antibodies

Antigen *(clone)*	**Conjugate**	**Application**	**Manufacturer**
CD25 *(PC61)*	unconjugated	Neutralizing	BioXCell (West Lebanon, USA)
IL-6 *(polyclonal)*	unconjugated	Neutralizing	R&D (Minneapolis, USA)
IL-6R *(15A7)*	unconjugated	Neutralizing	R&D (Minneapolis, USA)
IL-21 *(polyclonal)*	unconjugated	Neutralizing	R&D (Minneapolis, USA)
Goat IgG	unconjugated	Neutralizing	R&D (Minneapolis, USA)
Rat IgG1	unconjugated	Neutralizing	BioXCell (West Lebanon, USA)
CD3 *(145-2C11)*	unconjugated	T cell culture	BioXCell (West Lebanon, USA)
CD28 *(PV-1)*	unconjugated	T cell culture	BioXCell (West Lebanon, USA)
IFN-γ *(R4-6A2)*	unconjugated	T cell culture	BioXCell (West Lebanon, USA)
IL-4 *(11B11)*	unconjugated	T cell culture	BioXCell (West Lebanon, USA)
IL-12 *(C17.8)*	unconjugated	T cell culture	BioXCell (West Lebanon, USA)

CD16/CD32- Fc block *(2.4G2)*	unconjugated	FACS	BD Biosciences (Heidelberg, Germany)
Vα3.2 *(RR3-16)*	FITC	FACS	BD Biosciences (Heidelberg, Germany)
IgG1	PE	FACS	eBioscience (San Diego, USA)
IgG1 α	PE	FACS	BD Biosciences (Heidelberg, Germany)
IgG2b κ	PE	FACS	BD Biosciences (Heidelberg, Germany)
IgG2 κ	PE	FACS	BD Biosciences (Heidelberg, Germany)
IgG2a	PE	FACS	eBioscience (San Diego, USA)
TCR β *(H57-597)*	PE	FACS	BD Biosciences (Heidelberg, Germany)
Vβ11 *(RR3-15)*	PE	FACS	BD Biosciences (Heidelberg, Germany)
TCR γδ *(GL-3)*	PE	FACS	BD Biosciences (Heidelberg, Germany)
CD25 *(PC61)*	PE	FACS	BD Biosciences (Heidelberg, Germany)
CCR6 *(140706)*	PE	FACS	R&D (Minneapolis, USA)
IFN-γ *(XMG1.2)*	PE	FACS	BD Biosciences (Heidelberg, Germany)
IL-17A *(TC11-18H10)*	PE	FACS	BD Biosciences (Heidelberg, Germany)
Foxp3 *(FJK-16s)*	PE	FACS	eBioscience (San Diego, USA)
RORγt *(AFKJS-9)*	PE	FACS	eBioscience (San Diego, USA)
CD3e *(145-2C11)*	PE-Cy7	FACS	eBioscience (San Diego, USA)
CD4 *(RM4-5)*	PE_CF594	FACS	eBioscience (San Diego, USA)
CD45.1 *(A20)*	PE-Cy7	FACS	eBioscience (San Diego, USA)
CD196 *(29-2L17)*	PE-Cy7	FACS	BioLegend (San Diego, USA)
IFN-γ *(XMG1.2)*	PE-Cy7	FACS	eBioscience (San Diego, USA)
CD3 *(145-2C11)*	PerCP	FACS	BD Biosciences (Heidelberg, Germany)
CD4 *(RM4-5)*	PerCP	FACS	BD Biosciences (Heidelberg, Germany)
CD8 *(53-6.7)*	PerCP	FACS	BD Biosciences (Heidelberg, Germany)
CD90.1 *(OX-7)*	PerCP	FACS	BD Biosciences (Heidelberg, Germany)
CD11b *(M1/70)*	PerCP-Cy5.5	FACS	BD Biosciences (Heidelberg, Germany)
IL-17A *(TC11-18H10)*	PerCP-Cy5.5	FACS	BD Biosciences (Heidelberg, Germany)
IgG2a κ	PcBl	FACS	BioLegend (San Diego, USA)
CD3 *(500A2)*	PcBl	FACS	BD Biosciences (Heidelberg, Germany)
CD4 *(RM4-5)*	PcBl	FACS	BD Biosciences (Heidelberg, Germany)
TCR γδ *(GL-3)*	Brill. Violet 421	FACS	BioLegend (San Diego, USA)
IgG	Brill. Violet 421	FACS	BioLegend (San Diego, USA)

IFN-γ *(XMG1.2)*	eFluor 450	FACS	eBioscience (San Diego, USA)
IL-17A *(TC11-18H10.1)*	PcBl	FACS	BioLegend (San Diego, USA)
IgG2a	PcO	FACS	Life Technologies (Carlsbad, USA)
CD4 *(RM4-5)*	PcO	FACS	Life Technologies (Carlsbad, USA)
IgG	APC	FACS	R&D (Minneapolis, USA)
IgG1 κ	APC	FACS	eBioscience (San Diego, USA)
IgG2a κ	APC	FACS	BD Biosciences (Heidelberg, Germany)
IgG2b	Alexa-eFluor 647	FACS	eBioscience (San Diego, USA)
CD44 *(IM7)*	APC	FACS	BD Biosciences (Heidelberg, Germany)
CD45 *(30-F11)*	APC	FACS	BD Biosciences (Heidelberg, Germany)
CD90.2 *(53-2.1)*	APC	FACS	BD Biosciences (Heidelberg, Germany)
NK-1.1 *(PK136)*	APC	FACS	eBioscience (San Diego, USA)
IFN-γ *(XMG1.2)*	APC	FACS	BD Biosciences (Heidelberg, Germany)
IL-10 *(JES5-16E3)*	APC	FACS	BD Biosciences (Heidelberg, Germany)
IL-17A *(TC11-18H10.1)*	APC	FACS	BioLegend (San Diego, USA)
Foxp3 *(FJK-16s)*	APC	FACS	eBioscience (San Diego, USA)
CD4 *(RM4-5)*	APC-eFluor 780	FACS	eBioscience (San Diego, USA)
CD4 *(GK1.5)*	APC-CY7	FACS	BD Biosciences (Heidelberg, Germany)
CD45.2 *(104)*	APC-eFluor 780	FACS	eBioscience (San Diego, USA)

4.1.7 TaqMan probes

Gene	Exon boundary	Amplicon length	Manufacturer
Actb	-	115	Life Technologies (Carlsbad, USA)
Ccr6	2-3	78	Life Technologies (Carlsbad, USA)
Ccr6	2-3	73	Life Technologies (Carlsbad, USA)
Ifng	1-2	101	Life Technologies (Carlsbad, USA)
Il6	2-3	75	Life Technologies (Carlsbad, USA)
Il17a	1-2	80	Life Technologies (Carlsbad, USA)
Il21	2-3	67	Life Technologies (Carlsbad, USA)
Il22	1-2	65	Life Technologies (Carlsbad, USA)

Il23, p19	2-3	61	Life Technologies (Carlsbad, USA)
Il1r1	5-6	82	Life Technologies (Carlsbad, USA)
Il6ra	6-7	98	Life Technologies (Carlsbad, USA)
Il21r	5-6	65	Life Technologies (Carlsbad, USA)
Il22ra1	5-6	153	Life Technologies (Carlsbad, USA)
Il23r	8-9	72	Life Technologies (Carlsbad, USA)
Il23r	10-11	113	Life Technologies (Carlsbad, USA)
Foxp3	7-8	72	Life Technologies (Carlsbad, USA)
Rorc	8-9	77	Life Technologies (Carlsbad, USA)
Tbx21	1-2	69	Life Technologies (Carlsbad, USA)

4.1.8 Buffers and solutions

Clone Medium:

DMEM, 10 % FCS, 50 U/ml Penicillin, 50 µg/ml Streptomycin, non-essential amino acids (MEM-NEAA, Life Technologies, final concentration: 1x), vitamins solution (MEM Vitamin Solution, Life Technologies, final concentration 1x), 0.66 mM L-Arginine, 0.27 mM L-Asparagine, 13.6 mM Folic acid, 0.2 mM β-Mercaptoethanol, and 0.1 mg/ml Gentamycin, sterile filtered

CNS digestion solution:

DMEM with 2.5 mg/ml Collagenase and 1 mg/ml DNase I

Complete Freund's Adjuvant:

17 ml Paraffin oil, 3 ml Arlacel A, and 100 mg Mycobacterium tuberculosis (pestled)

ELISA blocking solution:

PBS with 1 % BSA (w/v)

ELISA wash buffer:

PBS with 0.05 % Tween

FACS buffer:

PBS with 2 % FCS, sterile filtered

Lysis buffer for DNA extraction:

Aqua dest. with 25 mM NaOH and 2 mM EDTA

MACS buffer:

PBS with 0.5 % BSA (w/v) and 2 mM EDTA, pH 8.0, sterile filtered

Neutralization buffer:

Aqua dest. with 40 mM Trizma Base

Percoll mix solution:

90 ml PBS (10x) and 264 ml aqua dest., pH adjusted to 7.2 with HCl

Percoll, 70 %:

30 ml Percoll, and 18 ml Percoll mix solution

Percoll, 37 %:

5.2 ml 70 % Percoll and 4.8 ml PBS

Proteinase K digestion buffer:

Aqua dest. with 50 mM Tris-HCl, 100 mM NaCl, 5 mM EDTA, and 0,5 % SDS (w/v)

Restimulation medium:

Clone medium with 50 ng/ml PMA, 1 µg/ml Ionomycin, and 1 µl/ml GolgiStop

TAE, 50x:

242 g Tris, 57.1 ml Glacial acetic acid, and 0.5 mM EDTA, pH 8.0, ad 1 l aqua dest.

4.1.9 Laboratory equipment and consumables

Agarose gel electrophoresis systems-Sub-Cell GT	Bio-Rad (Hercules, USA)
Blood lancets	Megro (Wesel, Germany)
Cell culture plates (96-, 24-well flat bottom, 96-well round bottom)	Greiner Bio-One (Frickenhausen, Germany)
Cell harvester-MicroBeta FilterMate-96	PerkinElmer (Waltham, USA)
Cell sorter, FACSAria II	BD Biosciences (Heidelberg, Germany)

Cell sorter, high speed-MoFlo	BD Biosciences (Heidelberg, Germany)
Cell strainer	BD Biosciences (Heidelberg, Germany)
Centrifuge 5424	Eppendorf (Hamburg, Germany)
Centrifuge-Multifuge 3SR+	Thermo Scientific (Waltham, USA)
CO_2 Incubator-APT.line C 150	Binder (Tuttlingen, Germany)
Combined refrigerator-freezer	Bosch (Gerlingen, Germany)
Conical centrifuge tubes, 15 and 50 ml	Greiner Bio-One (Frickenhausen, Germany)
Counting chamber, Neubauer-improved	Sigma-Aldrich (St. Louis, USA)
Cryogenic storage vials	Greiner Bio-One (Frickenhausen, Germany)
Cryostorage system	Taylor-Wharton (Minneapolis, USA)
ELISA plates-Costa (96-well)	Sigma-Aldrich (St. Louis, USA)
ELISA reader-Genios	Tecan (Männedorf, Switzerland)
Filtermat A, printed, for counting in cell harvester	PerkinElmer (Waltham, USA)
Flow cytometry-CyAn ADP Analyzer	Beckman Coulter (Brea, USA)
Fridge (+4 °C)	Siemens (Munich, Germany)
Gel documentation-Felix 1010	Biotec-Fischer (Reiskirchen, Germany)
Irradiation cage	Workshop, Klinikum Rechts der Isar (München, Germany)
Irradiation unit	Gulmay (Buford, USA)
Laminar flow cabinet	Kojair Tech Oy (Vilppula, Finland)
Microscope-VistaVision	VWR (Radnor, USA)
Microwave-R-2V14	Sharp (Munich, Germany)
Mini centrifuge-Spectrafuge	Labnet international (Edison, USA)
Mouse ear tags and ear tag applier	Fine Science Tools (Heidelberg, Germany)
Nanodrop-NP-1000	PEQLAB (Erlangen, Germany)
Pipet Controllers -Accu-jet pro	Sigma-Aldrich (St. Louis, USA)
Pipettes (P10, P200, P1000)	Eppendorf (Hamburg, Germany)
Pipette tips	Eppendorf (Hamburg, Germany)
Power Supply-PowerPac Basic	Bio-Rad (Hercules, USA)
Quadro MACS® Multistand	Milteny Biotech (Bergisch Gladbach, Germany)
Reaction tubes, 0.2, 0.5, 1.5, and 2.0 ml	SARSTEDT (Nümbrecht, Germany)
Real-Time PCR reaction plates	Life Technologies (Carlsbad, USA)
Real-Time PCR Systems-StepOne	Life Technologies (Carlsbad, USA)
Sample bag, used for counting of filters in cell harvester	PerkinElmer (Waltham, USA)

Scalpel-Feather	Electron Microscopy Sciences (Hatfield, USA)
Scintillation counter-MicroBeta TriLux	PerkinElmer (Waltham, USA)
Sterile filter storage bottle-Rapid flow	Thermo Scientific (Waltham, USA)
Syringe filter units-Millex-GS	Merck Millipore (Darmstadt, Germany)
Syringes and needles of different sizes	B.Braun (Melsungen, Germany)
Thermal cycler-2720	Life Technologies (Carlsbad, USA)
Thermomixer-comfort	Eppendorf (Hamburg, Germany)
Ultra low temperature freezer-MDF-U73V	Panasonic (Osaka, Japan)
Vortex mixer	VWR (Radnor, USA)
Waterbath	Memmert (Schwabach, Germany)

4.1.10 Software

Adobe Illustrator	Adobe Systems Incorporated (San Jose, USA)
BLAST	NCBI (National Center for Biotechnology Information, Bethesda, USA)
Endnote	Thomson Reuters (New York, USA)
Excel	Microsoft (Redmond, USA)
Flowjo	Tree Star (Ashland, USA)
Magellan	Tecan (Männedorf, Switzerland)
Photoshop	Adobe Systems Incorporated (San Jose, USA)
Prism	GraphPad Software (San Diego, USA)

4.2 Methods

4.2.1 Experimental animals

4.2.1.1 Housing and breeding conditions

All mice were kept in individually ventilated cages at a temperature of 20-22 °C, humidity of 40-50 % and a 12/12-hour light/dark cycle. Food and water were provided *ad libitum*. Each cage was equipped with bedding, stripes of paper towel, and a plastic nest box.

For breeding two at least 8 weeks old female mice were housed together with one at least 6 weeks old male mouse (harem mating). 3-4 weeks after birth, pups were weaned and ear

tagged. Tissue for genetic analysis of mice was obtained by tail biopsy. If necessary, a blood sample was taken from the retrobulbar venous plexus, the cheek pouch or the lateral tail vein. All interventions and experiments were performed in accordance with the guidelines prescribed by the Bavarian state authorities (Az 55.2-1-54-2531-88-08).

4.2.1.2 Mouse strains

C57BL/6 (stock number (SN): 000664), *Il6*$^{-/-}$ (SN: 002650), *Rag1*$^{-/-}$ (SN: 002216), *Il1r1*$^{-/-}$ (SN: 003245), and *Tcrd*$^{-/-}$ (SN: 002120) mice and the congenic strains *Cd45.1* (SN: 002014) and *Cd90.1* (SN: 000406) were obtained from Jackson Laboratories. Congenic strains differ from a particular inbred strain at a single locus. CD45 and CD90 are surface proteins that exist in two isoforms, CD45.1 and CD45.2, and CD90.1 and CD90.2, respectively, that can be easily stained with commercially available antibodies. Except *Cd45.1* and *Cd90.1* mice all used mice expressed CD45.2 and CD90.2. By using congenic mice in cell transfer experiments a discrimination of donor and host cells was possible.

Foxp3gfp.KI mice (Bettelli et al., 2006), *Il23r.gfp*.KI mice (Awasthi et al., 2009), and 2D2 mice (Bettelli et al., 2003) were kindly provided by Prof. Vijay Kuchroo (Center for Neurologic Diseases, Brigham and Women's Hospital, Harvard Medical School, Boston, USA).

Foxp3gfp.KI mice, in which a bicistronic enhanced green fluorescent protein (EGFP) was introduced into the endogenous Foxp3 locus, were used as reporter mice to analyze Foxp3 expressing cells. To investigate the cellular targets of IL-23, *Il23rgfp*.KI mice, in which the exon 9 of *Il23r* was replaced by a GFP encoding cassette, were used. Heterozygous mice served as reporter mice, homozygous mice as knock-out mice due to two mutated alleles.

2D2 mice are TCR transgenic mice. Almost all T cells of 2D2 mice express the same MOG_{35-55}-specific TCR which is composed of the chains Vα3.2 and Vβ11.

4.2.1.3 Genotyping

For genotyping, DNA was extracted from mouse tail biopsies. For this purpose, biopsies were put in 1.5 ml reaction tubes and boiled for 30 min in 180 µl tail lysis buffer in a heat block. After the tubes cooled down, 180 µl neutralization buffer was added and the mixture was vortexed. If a higher DNA quality was required, biopsies were subjected to a Proteinase K digestion at 55 °C overnight, followed by a standard phenol/chloroform/isoamyl alcohol

extraction. Finally, the genotype was determined by performing PCRs with Green Taq or Platinum polymerase and locus specific primers and subsequent gel electrophoresis using TAE-agarose-gels. All mice obtained from Jackson Laboratories were genotyped according the genotyping protocols available on the Jackson Laboratories' homepage (www.jax.org).
For genotyping of *Foxp3gfp*.KI mice the following primers and PCR program were used:

Primer:

Primer	Sequence (5'-3')
Common forward	ACG CCC CAA CAA GTG CTC CAA T
Wild type reverse	GTG TGA GTC AGT AGG ACT GCA G
Mutant reverse	ACC CCT AGG AAT GCT CGT CAA G

PCR program:

Step	Temperature	Duration	Repeats
1	94 °C	2 min	
2	94 °C	30 s	
3	55 °C	30 s	
4	72 °C	40 s	Step 2-4 40x
5	4 °C	∞	

For genotyping of *Il23rgfp*.KI mice two PCRs with primers specific for the mutant allele and the wild type allele, respectively, were performed.

Primer:

Primer	Sequence (5'-3')
Wild type forward	GAT CAT CTT ATG GCT GGT CCT C
Wild type reverse	GAG TGA GAC AGT GTA GCC ACA GAT
Mutant forward	ACC CCT AGG AAT GCT CGT CAA G
Mutant reverse	TGG TTG CCT GCA CCA ATT TAA AAG

PCR program (both reactions)

Step	Temperature	Duration	Repeats
1	94 °C	2 min	
2	94 °C	30 s	
3	55 °C	30 s	
4	72 °C	30 s	Step 2-4: 40x
5	72 °C	10 min	
6	4 °C	∞	

4.2.1.4 Phenotyping

CD45.1 congenic and 2D2 mice were characterized by phenotyping. First, red blood cells were lysed (PharmLyse, BD) to enrich leukocytes in the sample. These were subsequently labeled with antibodies against CD4, CD45.1, and CD45.2 or CD4, Vα3.2, and Vβ11, respectively. Labeled cells were analyzed by flow cytometry.

4.2.2 Preparation of lymphocytes

Mice were sacrificed by isoflurane inhalation, immediately followed by cervical dislocation. In case of CNS preparation, mice were perfused using PBS. To obtain single cell suspensions from thymi, spleens, and lymph nodes, tissues were dissected and grated through a nylon mesh (cell strainer, BD) with 70 µm pores. In spleen cell suspensions red blood cell lysis was performed. Cells were then washed once with DMEM, resuspended in Clone medium and stored on ice until further processing. To determine the absolute cell numbers cells were mixed

with Trypan blue and counted using a Neubauer-improved haemocytometer. Dead cells were identified by Trypan blue exclusion.

If bone marrow cells were required, femurs and humeruses were cut at hip or shoulder joint, respectively, skin and muscle fibers were removed and bone marrow cells were flushed out the bone with 10 ml DMEM using a syringe and a 14-gauge needle. Red blood cells were lysed and cells were washed once with DMEM, resuspended in Clone medium, counted and stored on ice until their further processing.

For single cell suspensions of CNS cells, mice were perfused with 10 ml PBS. The brain was dissected. To obtain the spinal cord the vertebral canal was opened at the level of the sacral bone and the spinal cord was flushed out with PBS using a syringe and 20-gauge needle. The brain and the spinal cord were cut into small pieces with a scalpel and incubated in CNS digestion solution for 45 min at 37 °C. The tissue was then grated through a nylon mesh with 70 µm pores and centrifuged for 8 min at 1200 rpm. Pelleted cells were resuspended in 5 ml 70 % Percoll, which was afterwards covered by 5 ml 37 % Percoll. The tubes were centrifuged for 20 min at 1800 rpm without brake. The interphase was recovered and cells were washed once with DMEM, resuspended in Clone medium, counted and stored on ice until their further processing.

4.2.3 Flow cytometry

Flow cytometry or FACS® (fluorescence-activated cell sorting, brand name of BD) is a widely utilized, powerful method which uses the parameters cell size, cell granularity, and marker protein expression to distinguish different cell types within a single cell suspension. The operation mode is based on a flow of labeled cells that pass a focused laser-beam. Laser-light that strikes a particle or cell will be scattered in all angels which is detected by photomultipliers. Low-angle forward scatter can be used as a marker for cell size, whereas orthogonal, 90 ° side scatter gives information about the granularity or internal complexity.

Additionally, the use of fluorochrome-conjugated antibodies allows the detection of cell surface and intracellular protein markers. By passing the laser-beam, compounds of these fluorochromes absorb light which leads to a rising of electrons into an excited state. By returning to the ground state a quantum of light is emitted. Different fluorochromes emit light at different wavelength. By using detectors for different wavelengths, bandpass filters, and dichroic mirrors it is possible to detect simultaneously different fluorochromes on a single cell.

For flow cytometry experiments, up to $2x10^6$ cells were first incubated with Fc receptor blocking antibodies to prevent the unspecific binding of antibodies to Fc receptors and subsequently stained with 1:100 dilutions of the respective surface marker antibodies for 30 min at 4 °C. The staining panels always included dyes that specifically bind to dead cells, so that it was later possible to exclude them from analysis. The staining was performed in round-bottom 96-well plates in a total volume of 100 µl. After staining, cells were washed in FACS buffer, transferred to FACS-tubes, and analyzed using a CyAn ADP Analyzer (BeckmanCoulter).

In order to determine their cytokine secretion by flow cytometry, T cells were incubated for 2.5-4 h in restimulation medium, containing PMA (phorbol-12-myristate-13-acetate), ionomycin, and monensin (GolgiStop, BD Biosciences). The combination of PMA and ionomycin activates T cells while bypassing the requirements of antigen-induced signals. The phorbol ester PMA is structurally analogous to diacylglycerol (DAG), an endogenous signaling molecule. Just like DAG, PMA activates the protein kinase C (PKC). The calcium (Ca^{2+}) ionophore ionomycin enhances the intracellular Ca^{2+} concentration, partly by inducing the release of Ca^{2+} from internal stores (Yoshida and Plant, 1992, Muller et al., 2013). Increased intracellular Ca^{2+} levels lead to the hydrolysis of phosphoinositides into DAG which further activates PKC, and inositol trisphosphate (IP3). IP3 binds to Ca^{2+} channels found on the surface of the endoplasmic reticulum (ER) and triggers the release of Ca^{2+} into the cytoplasm. Additionally, free intracellular Ca^{2+} targets the Ca^{2+}-activated phosphatase calcineurin. Calcineurin together with PKC activates several transcription factors, such as nuclear factor of activated T cells (NFAT) (Jain et al., 1993) and nuclear factor κB (NF-κB) (Trushin et al., 1999), whose transcriptional activity leads to an upregulation of IL-2 receptors, proliferation, and cytokine production. The ionophore monensin effects a trans-membrane exchange of sodium ions for protons which results in a neutralization of acidic intracellular compartments such as the trans Golgi apparatus. Therefore, monensin blocks transport processes of the Golgi apparatus and thus, the secretion of cytokines (Tartakoff and Vassalli, 1977).

For intracellular cytokine staining, cells were fixed with 4 % paraformaldehyde (Cytofix®, BD Biosciences) for 20 min at 4 °C, permeabilized in a saponin containing permeabilization/wash buffer (Perm/Wash Buffer, BD Biosciences), and incubated overnight at 4 °C with monoclonal antibodies against cytokines. Antibodies were diluted 1:100 in permeabilization/wash buffer.

Afterwards, cells were washed once with permeabilization/wash buffer and analyzed. For intranuclear staining, the Foxp3 staining buffer set (eBioscience) was used.

4.2.4 Cell sorting

To purify a desired cell population out of a heterogeneous single cell suspension, fluorescence-activated or magnetic cell sorting was used.

During fluorescence-activated cell sorting the cell stream that passes the laser-beam is broken into uniform droplets. When a cell or a particle is detected whose scatter and fluorescence characteristics match the predefined sorting criteria, voltage is applied to the correspondent droplet. The stream which now contains charged and uncharged droplets enters a strong electric field generated by two deflecting plates. The charged droplets are attracted to the oppositional charged plate and hence deflected from the center of the stream, whereas the uncharged droplets are not affected by the electric field and thus remain in the center of the stream and are finally collected in the waste tank.

To perform fluorescence-activated cell sorting, cells were stained as described in chapter 4.2.3. Sorting was conducted at the Institute for Medical Microbiology, Immunology and Hygiene, TU Munich.

Magnetic cell sorting is based on the labeling of cells with antibodies that are conjugated to magnetic particles. Subsequently, these labeled cells are separated from unlabeled cells by passing the cell suspension through a column arranged in a magnetic field. After removing of the column from the magnet, labeled cells can be eluted.

Magnetic cell separation (MACS®, brand name of Miltenyi Biotec) was used to purify $CD4^+$ αβ T cells and γδ T cells and to deplete CD90.2 T cells out of bone marrow cell suspensions. Magnetic beads used during these purifications were purchased from Miltenyi Biotec. All purification procedures were performed following manufacturer's instructions.

4.2.5 T cell assays

4.2.5.1 T cell differentiation

For in vitro αβ T cell differentiation, spleens and peripheral lymph nodes were removed from naïve *Foxp3gfp*.KI mice and $CD4^+$ T cells were purified using magnetic cell sorting. $CD4^+$ T cells were further purified by fluorescence-activated cell sorting to obtain $CD4^+Foxp3^-CD44^-$

naïve or $CD4^+CD44^+$ memory T cells. After transferring cells into fresh Clone medium, $2x10^5$ T cells per well were seeded into a flat-bottom 96-well plate which had been coated with a monoclonal antibody to CD3 (4 µg/ml) for 24 h at 4 °C. For co-stimulation, an antibody against CD28 in a concentration of 2 µg/ml was added to the culture medium. By using antibodies to CD3 and CD28 T cells are activated polyclonally, which means in an antigen-independent manner. To induce differentiation, the following recombinant cytokines were used: human TGF-β1 (3 ng/ml), mouse IL-6 (30 ng/ml), mouse IL-21(100 ng/ml), mouse IL-12 (25 ng/ml), and mouse IL-23 (25 ng/ml, all R&D Systems). After 3 days of cultures, mRNA or protein expression was analyzed using Real-Time PCR or flow cytometry and ELISA, respectively.

For γδ T cell differentiation, spleens and peripheral lymph nodes were removed from naïve C57BL/6, *Foxp3gfp*.KI or *IL-23Rgfp*.KI mice. To prevent contaminations with αβ T cells, the single cell suspension was first depleted of $CD4^+$ T cells by magnetic cell sorting. Afterwards, a highly pure γδ T cell population was obtained by a two-step magnetic cell sorting process. γδ T cells were cultured in Clone medium in a round-bottom 96-well cell culture plate. Per well $1x10^5$ cells together with $6.67x10^4$ anti-CD3/anti-CD28 beads were seeded and recombinant mouse IL-23 (25 ng/ml) was added to the culture medium. After 4 days, mRNA or protein expression was analyzed using Real-Time PCR or flow cytometry and ELISA, respectively. Furthermore, an aliquot of the cell culture supernatants was stored for the application in conversion assays.

4.2.5.2 Conversion assay

For in vitro conversion assays, spleens and peripheral lymph nodes were removed from naïve *Foxp3gfp*.KI mice and $CD4^+$ T cells were purified using magnetic cell sorting. $CD4^+$ T cells were further purified by fluorescence-activated cell sorting to obtain $CD4^+Foxp3^-CD44^-$ naïve T cells. After transferring cells into fresh Clone medium, $2x10^5$ T cells per well were seeded into a flat-bottom 96-well plate which had been coated with anti-CD3 (4 µg/ml) for 24 h at 4 °C. For co-stimulation, an antibody against CD28 in a concentration of 2 µg/ml was added to the culture medium. To induce Foxp3 expressing regulatory T cells or as control conditions the following cytokine panels were used: human TGF-β1 (5 ng/ml) alone or in combination with mouse IL-6 (37.5 ng/ml), mouse IL-21 (100 ng/ml), or mouse IL-23 (25 ng/ml, all R&D Systems). To investigate the effect of γδ T cell supernatants on the conversion of naïve into

regulatory T cells half of the culture medium (which is 100 µl) was replaced by supernatants of γδ T cells that had been stimulated polyclonally in the presence or absence of IL-23. In addition to these supernatants, blocking antibodies to IL-6 or IL-21 or control goat IgG (all 12.5 µg/ml, R&D Systems) were used in some experiments.
After 3 days of culture, Foxp3 expression by means of GFP expression or cytokine expression was analyzed by flow cytometry.

4.2.5.3 Suppression assay

T cell suppression assays are used to evaluate the capacity of Treg cells to inhibit the proliferation of conventional T cells. There are two common assays to monitor the proliferation rate of T cells: First, proliferation can be measured by the incorporation of the radiolabelled DNA precursor ^{3}H-thymidine into the DNA replicated during cell division and second, by flow cytometric analysis of dye dilution. These dyes bind covalently to cytoplasmic or cell membrane compounds and are equally distributed during cell division between the two resulting daughter cells, so that the fluorescence of dividing cells decreases with every division cycle.
For suppression assays, spleens and peripheral lymph nodes were removed from naïve *Foxp3gfp*.KI mice and $CD4^+$ T cells were purified using magnetic cell sorting. $CD4^+$ T cells were further purified by fluorescence-activated cell sorting to obtain $CD4^+Foxp3^-$ responder T cells and $CD4^+Foxp3^+$ Treg cells. After transferring cells into fresh Clone medium, $2.5x10^4$ conventional T cells per well were cultured in a round-bottom 96-well plate together with $2.5x10^4$ Treg cells and $2x10^5$ irradiated (3.000 rad) syngeneic splenic γδ T cell-depleted APCs for 72 h. For TCR stimulation an antibody against CD3 (1 µg/ml) was added to the culture. To investigate the effect of γδ T cells on the suppressive capacity of regulatory T cells, $2.5x10^4$ γδ T cells were added to the culture. In analogous assays, responder T cells were co-cultured with Treg cells and irradiated (3.000 rad) syngeneic splenic γδ T cell-depleted APCs in the presence of supernatants of γδ T cells or IL-23 (25 ng/ml). 56 h after initiation of culture 1 µCi of ^{3}H-thymidine was added and 16 h later cells were harvested on glass fiber filters and ^{3}H-thymidine incorporation was measured in a β-counter.
To measure proliferation by dye dilution, responder T cells and Treg cells were labeled with PKH26 following manufacturer's instructions and analyzed using flow cytometry.

4.2.6 ELISA (enzyme-linked immunosorbent assay)

ELISA is an antibody-based technique that allows both the detection and the quantification of proteins in an aqueous solution. In immunology the most widely used type of ELISA is the so-called Sandwich ELISA. In a first step a 96-well reaction plate is coated with an antibody (capture antibody) which recognizes the antigen in the liquid sample. Consecutively, a second biotinylated antibody to the antigen, an enzyme-linked streptavidin-complex and the appropriate substrate is added. The enzyme, for example, Horse radish peroxidase, converts the substrate into a colored product, with the color intensity correlating to the amount of protein.

ELISA was used to determine secreted cytokines in cell culture supernatants that were collected after 72 h, in the case of αβ T cell cultures, or after 96 h, in the case of γδ T cell cultures and was performed according to manufacturer's instructions (R&D Systems).

4.2.7 Real-time PCR

Real-time PCR is a method to quantify mRNA. Therefore mRNA has to be isolated from cells or tissues and reverse transcribed into cDNA. To prevent the amplification of DNA originating from contaminating genomic DNA, primers are chosen that produce a PCR product that spans an exon-exon boundary. Using this strategy an amplification starting from genomic DNA can be excluded. Furthermore, to compare mRNA levels correctly, a stably expressed reference gene is used to normalize the cDNA concentration between different samples.

The 5'-nuclease probe assay (TaqMan® PCR assay) is one of the best established Real-time PCR techniques among the multitude of used techniques. This assay is based on the 5'-nuclease activity of the Taq DNA polymerase and the usage of single-stranded hybridization probes that are labeled with a fluorescent molecule at the 5'end and a quencher molecule at the 3'end of the probe. The fluorescent and the quencher molecule are in such a close proximity that they can interact via FRET (förster resonance energy transfer) which means that the fluorescent molecule transfers its excitation to the quencher molecule without photon emission which results in a highly decreased fluorescence signal. During the PCR, the probe binds together with specific primers to the target cDNA molecule. When the polymerase reaches the hybridized probe, it becomes cleaved, the fluorescent molecule and the quencher are physically separated, FRET cannot take place any longer, and the fluorescence signal increases. With every amplification cycle more probes hybridize and hence more probes can be cleaved, which results in a higher fluorescence signal. That means that the intensity of the fluorescence signal

correlates to the quantity of target cDNA molecules. To finally quantify and compare cDNA copy numbers, it is mandatory to define a fluorescence threshold that lies above the background fluorescence calculated by the Real-time instrument. For every sample the number of amplification cycles, that are necessary for the fluorescence signal to exceed the threshold, is determined. Accordingly, this value, called Ct value, is inversely proportional to the amount of cDNA present in the analyzed sample.

For Real-time PCR, cells were homogenized using QiaShredder®, RNA was isolated using the RNeasy® kit (both Qiagen) and transcribed into cDNA using the TaqMan Reverse Transcription reagents kit (Life Technologies) according manufacturer's instructions. Primers and probes were purchased from Life Technologies; the assays were performed on 96-well reaction plates (Life Technologies) and analyzed using a StepOne® system from Life Technologies. In all experiments β-actin was used as reference gene to normalize gene expression.

4.2.8 In vivo mouse experiments

4.2.8.1 EAE

EAE was induced by active immunization with MOG_{35-55} peptide emulsified in Complete Freund's Adjuvant (CFA) plus Pertussis toxin. An emulsion (1 part aqueous solution and 1 part CFA) was prepared containing 1 mg/ml MOG_{35-55} and 2.5 mg/ml Mycobacteria tuberculosis using two luer lock syringes connected by a three way stopcock. Emulsion was stored on ice and mixed frequently until a viscous liquid suspension was obtained. On day 0 mice received a subcutaneous injection of 200 µl emulsion at the base of tail. Additionally, on day 0 and day 2, 200 ng Pertussis toxin solved in 100 µl PBS were administered intravenously (i.v.). Mice were monitored daily until first signs of disease developed which was usually around day 10. Once to twice a day sick mice were weighted and scored as follows: 1-tail paralysis, 2-hind limb weakness, 3-hind limb paralysis, 4-front and hind limb paralysis, 5-moribund. Euthanasia was required when more than 20 % of body weight was lost or a score of 4 was reached.

In some experiments, Treg cells were depleted prior to EAE induction. For this purpose 500 µg of monoclonal antibody to CD25 (clone PC61) were injected intraperitoneally (i.p.) 5 days and 3 days before immunization.

Furthermore, EAE was explored in several adoptive transfer experiments. In a series of experiments, $5x10^6$ purified $CD3^+$ T cells or γδ T cell-depleted $CD3^+$ T cells from congenic

mice (CD90.1) were transferred i.v. into IL-23R deficient recipients 1 day prior to EAE induction. In some experiments, $3.5x10^6$ fluorescence-activated cell sorted naïve ($CD4^+CD44^-$ $Foxp3^-$) T cells were purified from 2D2x*Foxp3gfp*.KI mice and injected i.v. into Rag1 deficient recipients 2 days prior to EAE induction with or without co-transfer of $0.5x10^6$ wild type γδ T cells. Additionally, 500 µg of a monoclonal antibody to IL-6R were injected i.p. into the recipient mice on days 1, 3, 5, and 9 after cell transfer.

4.2.8.2 Bone marrow chimeras

For the generation of (mixed) bone marrow or fetal liver chimeras, *Rag1*$^{-/-}$ recipients were lethally irradiated. As a split-dose irradiation is known to reduce morbidity and mortality (Vavrova and Petyrek, 1988), the 11 Gy total dose was delivered as two 5.5 Gy doses 3 h apart. For irradiation, mice were separated into groups of up to 9 mice, placed into an antiseptically cleaned irradiation container, irradiated using an X-ray apparatus (Gulmay Medical D3-225) and put back into their cages. For bone marrow chimeras, $2x10^7$ bone marrow cells harvested from donor mice were suspended in 150 µl of sterile PBS and injected i.v. into the recipients the next day. As donor mice *Il23r*$^{+/-}$, *Il23r*$^{-/-}$, *Il1r1*$^{+/-}$ or *Il1r1*$^{-/-}$ congenic mice (CD45.1) were used. Irradiated mice were maintained on antibiotic containing water (Baytril, Bayer, 0.1 mg/ml) until reconstitution was ensured by analyzing a blood sample for chimerism by measuring T cells by flow cytometry. A sufficient reconstitution was usually achieved four weeks after cell transfer.

For mixed bone marrow chimeras, bone marrow cells derived from donors with different genotypes were mixed in a 50/50 ratio. A total of $2x10^7$ bone marrow cells were injected. For some experiments, thymi from neonatal mice were dissected and $1x10^7$ thymocytes were injected i.v. into the recipient on day 1 after irradiation. On day, 2 $1x10^7$ bone marrow cells, distinguishable from the thymocytes by the expression of a congenic marker, were injected.

For fetal liver cell transplantation experiments, fetal mice were prepared at day 13.5 of gestation. Subsequently, livers were isolated using a stereo microscope. $9x10^6$ liver cells were transferred either alone or in combination with $9x10^6$ congenic bone marrow cells into the recipient one day after irradiation.

In some experiments recipient mice were immunized with MOG_{35-55}/CFA to induce EAE 5 weeks after cell transfer.

4.2.9 Statistical analysis

Statistical evaluations were performed with the unpaired, two-tailed Student's t test when two groups were compared and a continuous probability distribution (normal distribution) of analyzed data was assumed such as cell frequency measurements or proliferation data. For the comparison of several groups, one-way ANOVA including the Tukey's Multiple Comparison Post-Hoc Test was performed. The Mann Whitney U test is a non-parametric test used to compare data that are not normally distributed or data that were measured on an ordinal scale. This test was applied to analyze clinical EAE scores. In general, p-values < 0.05 were considered significant. In this context, "*" means $p< 0.05$, "**" means $p< 0.01$, and "***" means $p< 0.001$.

5 Results

5.1 Characterization of IL-23R expressing γδ T cells

5.1.1 A subset of γδ T cells expresses the IL-23R constitutively

γδ T cells have been found to be a major source of IL-17 during a variety of infections (Lockhart et al., 2006, Shibata et al., 2007, Cho et al., 2010). However, compared to αβ T cells, the mechanisms of IL-17 induction in γδ T cells are poorly characterized. The robust production of IL-17 in αβ T cells depends on IL-23. The resistance of mice deficient for either IL-23 or its receptor to the induction of several autoimmune diseases, including EAE has been explained by the lack of pathogenic Th17 cells in these mice (Langrish et al., 2005, Awasthi et al., 2009). However, some studies show that IL-23 has an impact on inflammatory processes that is independent of αβ T cells (Uhlig et al., 2006, Buonocore et al., 2010). To address the question whether γδ T cells express the IL-23R and thus, are able to sense IL-23, an IL-23R reporter mouse was used. The IL-23R reporter mouse is a "knock-in" mouse in which an IRES-EGFP cassette was introduced in the endogenous *Il23r* locus (Awasthi et al., 2009). In heterozygous mice (*Il23r*$^{+/-}$) IL-23R expressing cells can be followed by their GFP expression, but remain fully responsive to IL-23 as they express one wild type allele. In contrast, cells of homozygous mice (*Il23r*$^{-/-}$) are unresponsive to IL-23 due to two mutated alleles. To determine the IL-23R expression of γδ T cells, cells from the thymus (THY), the spleen (SPL), and the lymph nodes (LN) of naïve *Il23r*$^{+/-}$ mice were isolated and examined for GFP expression using flow cytometry. IL-23R(GFP) was expressed on a subset of γδ T cells in all analyzed compartments (Figure 5.1). The highest numbers of IL-23R positive γδ T cells were found in the LN.

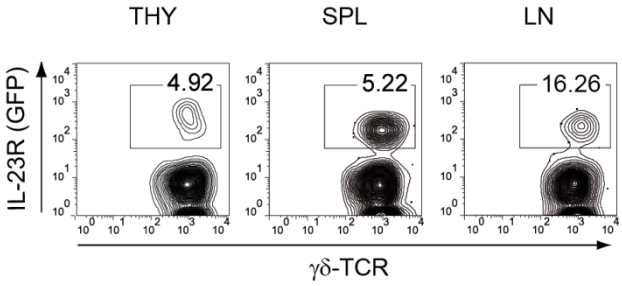


Figure 5.1: A subset of γδ T cells expresses the IL-23R constitutively
Thymocytes (THY), splenocytes (SPL), and lymph node (LN) cells were prepared from naïve *Il23r*$^{+/-}$ mice and analyzed by flow cytometry. Percentages indicate fractions of IL-23R(GFP)$^{+}$ γδ T cells. Representative dot plots out of six independent experiments are shown.

To analyze the impact of IL-23 on γδ T cells, γδ T cells were isolated from the spleen and lymph nodes of *Il23r*$^{+/-}$ mice, purified by magnetic cell sorting, and cultivated in vitro. Purified γδ T cells were stimulated in the presence of anti-CD3/anti-CD28 beads either without cytokines, with IL-12, or with IL-23. Stimulation with IL-12 served as control condition in several in vitro γδ T cell assays, because of the structural similarities between IL-12 and IL-23 and their receptors, respectively. Thus, specific IL-23-mediated effects could be distinguished from effects caused by both, IL-12 and IL-23. After three days of stimulation, the IL-23R expression was measured by flow cytometry. Stimulation with IL-23 resulted in an increase of the fraction of γδ T cells expressing the IL-23R compared to the stimulation without cytokines or with IL-12 (Figure 5.2A). To determine whether this increase was due to an expansion of IL-23R positive γδ T cells or to a conversion of IL-23R negative into IL-23R positive γδ T cells, dye dilution assays were performed. γδ T cells, purified from *Il23r*$^{+/-}$, were labeled with the lipophilic, fluorescent, membrane intercalating dye PKH26 and stimulated in the presence of anti-CD3/anti-CD28 beads either without cytokines, with IL-12, or with IL-23. While under neutral conditions or in the presence of IL-12, IL-23R negative γδ T cells were preferentially expanded, IL-23 promoted the proliferation of IL-23R positive γδ T cells (Figure 5.2B). This result indicates that IL-23 increased the IL-23R positive γδ T cell population by inducing the proliferation of IL-23R positive cells.

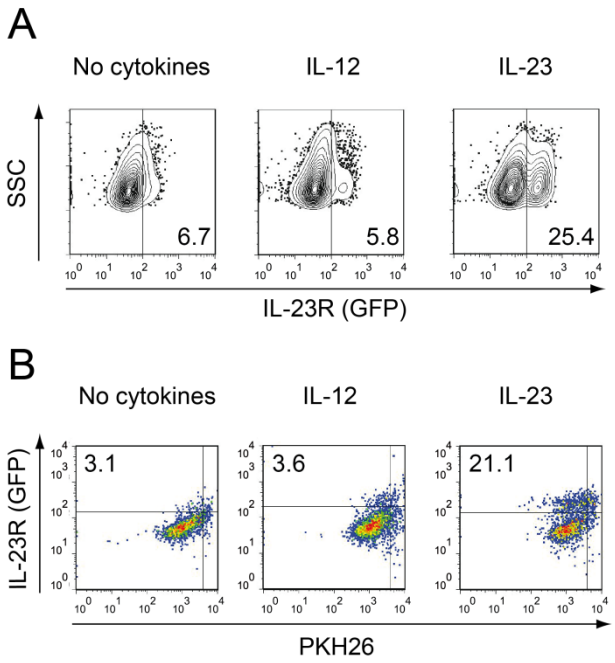


Figure 5.2: IL-23 augments IL-23R positive γδ T cell population
γδ T cells were isolated from spleens and lymph nodes of *Il23r*$^{+/-}$ mice and purified by magnetic cell sorting. (A) After in vitro stimulation with anti-CD3/anti-CD28-coated beads, γδ T cells were analyzed for IL-23R(GFP) expression. Numbers indicate percentages within the γδ-TCR gate. (B) *Il23r*$^{+/-}$ γδ T cells were labeled with PKH26 and stimulated with anti-CD3/anti-CD28 beads in the presence of the indicated cytokines. After 3 days, dilution of PKH26 was measured in the gate of blasting γδ T cells by flow cytometry. Numbers indicate percentages of proliferating IL-23R^{+} γδ T cells. Representative dot plots out of three independent experiments are shown.

Upon IL-23R inducing stimuli, cells from homozygous IL-23R reporter mice (*Il23r*$^{-/-}$), in which the inserted EGFP cassette destroys the encoded IL-23R gene, express an EGFP protein without expressing a functional IL-23R. These cells are henceforth referred to as "wannabe" cells. Flow cytometrical analysis of cells isolated from *Il23r*$^{-/-}$ mice showed that a lack of IL-23R resulted in reduced numbers of IL-23R positive γδ T cells in all analyzed compartments (Figure 5.3), which is in accordance with the finding that IL-23 itself increased transcription from the *Il23r* gene in γδ T cells.

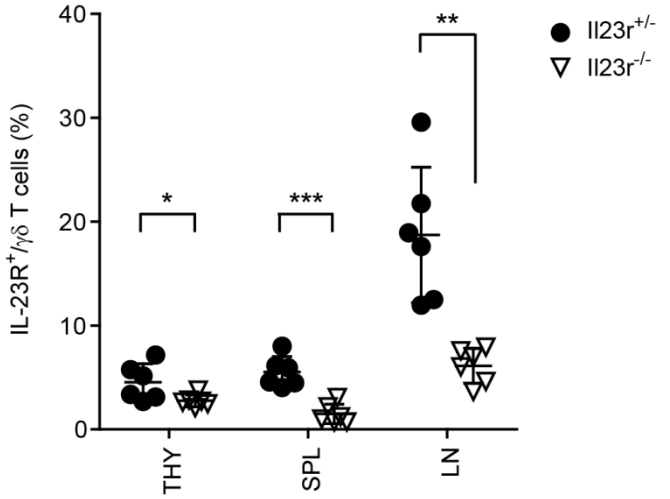


Figure 5.3*: Il23r*$^{-/-}$ mice harbor decreased numbers of IL-23R positive γδ T cells
γδ T cells were isolated from various tissues of naïve *Il23r*$^{+/-}$ or *Il23r*$^{-/-}$ mice. Frequencies of IL-23R(GFP)$^{+}$ cells within the γδ T cell compartment were analyzed by flow cytometry. Representative data out of three independent experiments.

5.1.2 γδ T cells produce high amounts of pro-inflammatory cytokines

γδ T cells were described as highly potent cytokine producers. In the next set of experiments, the role of IL-23 to induce the production of cytokines in γδ T cells was investigated. γδ T cells, purified from the spleen and lymph nodes of *Il23r*$^{+/-}$ and *Il23*$^{-/-}$ mice, were stimulated with anti-CD3/anti-CD28-coated beads in the presence of several cytokines or cytokine combinations. The cytokine production of γδ T cells was compared to those of CD4^{+} αβ T cells. CD4^{+} αβ T cells were purified by magnetic sorting from the spleen and lymph nodes of *Il23r*$^{+/-}$ and *Il23*$^{-/-}$ mice. Both, γδ T cells and αβ T cells stimulated in the presence of IL-12 produced high amounts of IFN-γ (Figure 5.4A and B). On a per cell basis, γδ T cells produced up to five fold more IFN-γ than αβ T cells. As expected, the IL-12-driven IFN-γ production was equal in cells isolated from *Il23r*$^{+/-}$ and *Il23r*$^{-/-}$ mice, i.e. was not affected by a lack of IL-23R. Furthermore, upon IL-23 stimulation IL-23R$^{+/-}$ γδ T cells produced large amounts of IL-17, nearly ten times more than αβ T cells (Figure 5.4C and D). In addition, the combination of TGF-β and IL-6, the cytokine combination that is used to differentiate conventional naïve T cells into Th17 cells, also induced IL-17 in γδ T cells. However, while αβ T cells isolated from *Il23r*$^{+/-}$ or *Il23r*$^{-/-}$ produced similar amounts of IL-17 when stimulated with TGF-β and IL-6 or TGF-β and IL-21, IL-23R$^{-/-}$ γδ T cells failed to produce IL-17 under any condition suggesting that the γδ T cell compartment of *Il23r*$^{-/-}$ mice did not contain γδ T cells that could be induced

to produce IL-17. Thus, all precusors of IL-17 producing γδ T cells (γδT17) cells are IL-23R positive.

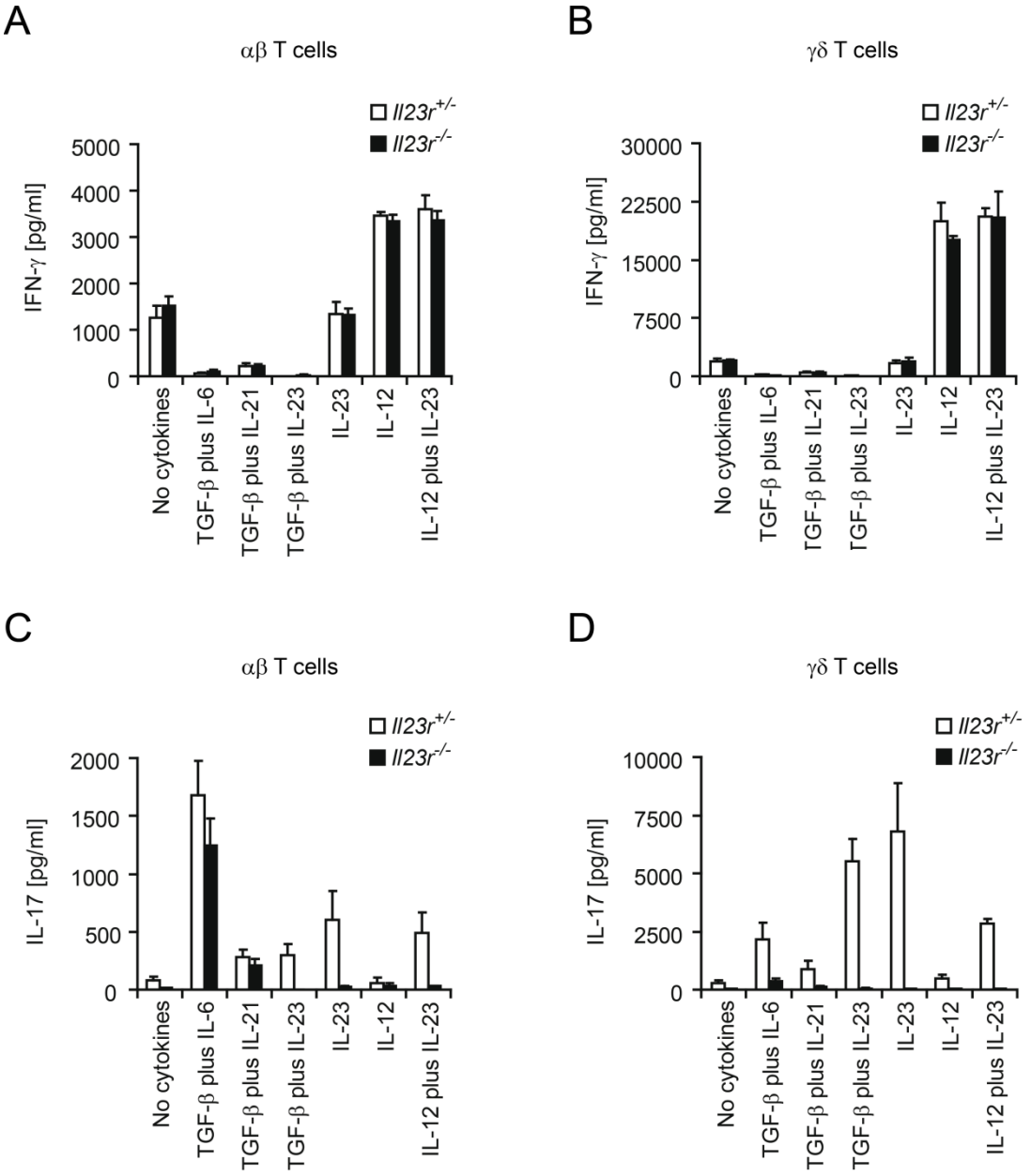


Figure 5.4: γδ T cells produce high amounts of IFN-γ and IL-17 in response to IL-12 and IL-23, respectively
$CD4^+$ αβ T cells and γδ T cells were isolated from *Il23r*$^{+/-}$ or *Il23*$^{-/-}$ mice, purified by magnetic cell sorting, and stimulated for 72 h with anti-CD3/anti-CD28-coated beads in the presence of the indicated cytokines. IFN-γ and IL-17 production were determined by ELISA. Amount of cytokines produced in triplicate wells, mean ± SD. Representative data out of three independent experiments.

In contrast to αβ T cells, γδ T cells leave the thymus as cells that already express activation/memory markers (Jensen et al., 2008, Narayan et al., 2012). However, to ensure a fair comparison between αβ T cells and γδ T cells the cytokine production capacity of γδ T

cells was then compared to those of antigen-experienced $CD4^+CD44^+$ memory αβ T cells. In response to IL-12 and IL-23, γδ T cells still produced significantly higher amounts of IFN-γ and IL-17 on a per cell basis, respectively (Figure 5.5).

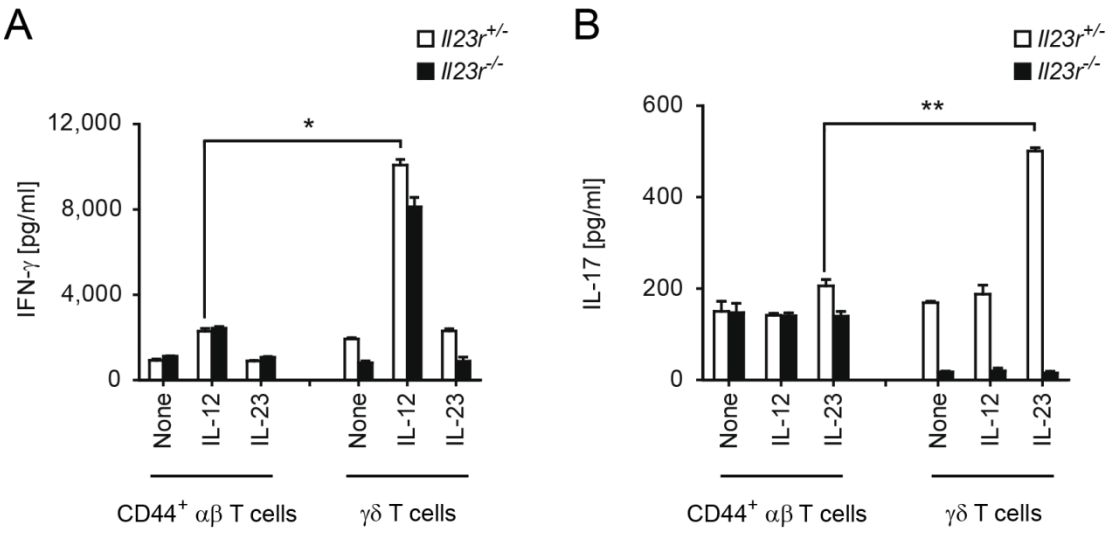


Figure 5.5: γδ T cells produce higher amounts of IFN-γ and IL-17 compared to memory αβ T cells
αβ T cells and γδ T cells were isolated from *Il23r*$^{+/-}$ or *Il23*$^{-/-}$ mice. $CD4^+CD44^+$ αβ T cells were purified by fluorescence-activated cell sorting, γδ T cells by magnetic cell sorting. Cells were stimulated for 24 h with plate-bound monoclonal antibodies to CD3 (0.5 mg/ml) and CD28 (0.25 mg/ml) in the presence of the indicated cytokines. IFN-γ and IL-17 production were determined by ELISA. Amount of cytokines produced in triplicate wells, mean ± SD. Representative data out of three independent experiments.

5.1.3 There are two subsets of γδ T cells with different surface marker expression and cytokine profiles

Upon stimulation with IL-12 and IL-23, γδ T cells produced high amounts of IFN-γ and IL-17, respectively. However, it was unclear, whether one given cell has the capacity to produce both, IFN-γ and IL-17, or whether there are functional subsets of γδ T cells that differ from each other concerning the cytokines they secrete. In order to address this question, the cytokine production of γδ T cells was analyzed on the single cell level. γδ T cells were purified from the spleens and lymph nodes of Il23r+/- mice, stimulated for 4 h with PMA, ionomycin, and monensin, and analyzed for IL-17 and IFN-γ production. When correlating the IL-23R expression with cytokine secretion, two γδ T cell subsets could be distinguished (Figure 5.6). IL-17 was produced by IL-23R expressing cells, while IFN-γ was exclusively secreted by IL-23R negative cells. Thus, two distinct γδ T cell subsets were identified that differ in their cytokine profile and in the expression of IL-23R. Besides IL-23R, additional surface markers

were found to characterize these two functional subsets, respectively. Those γδ T cells that were IL-23R positive and produced IL-17, expressed the chemokine receptor CCR6, which is also found on the surface of Th17 cells (Figure 5.6B). Accordingly, in CCR6 positive γδ T cells, purified from spleens and lymph nodes of wild type mice, highly increased amounts of Il23r mRNA were detected compared to CCR6 negative γδ T cells (Figure 5.6C). In contrast, IL-23R negative, IFN-γ producing γδ T cells were CCR6 negative, but expressed the natural killer cell-associated marker NK1.1 (Figure 5.6A). Thus, IL-23R expression in γδ T cells segregates with expression of CCR6 and lack of NK1.1 expression.

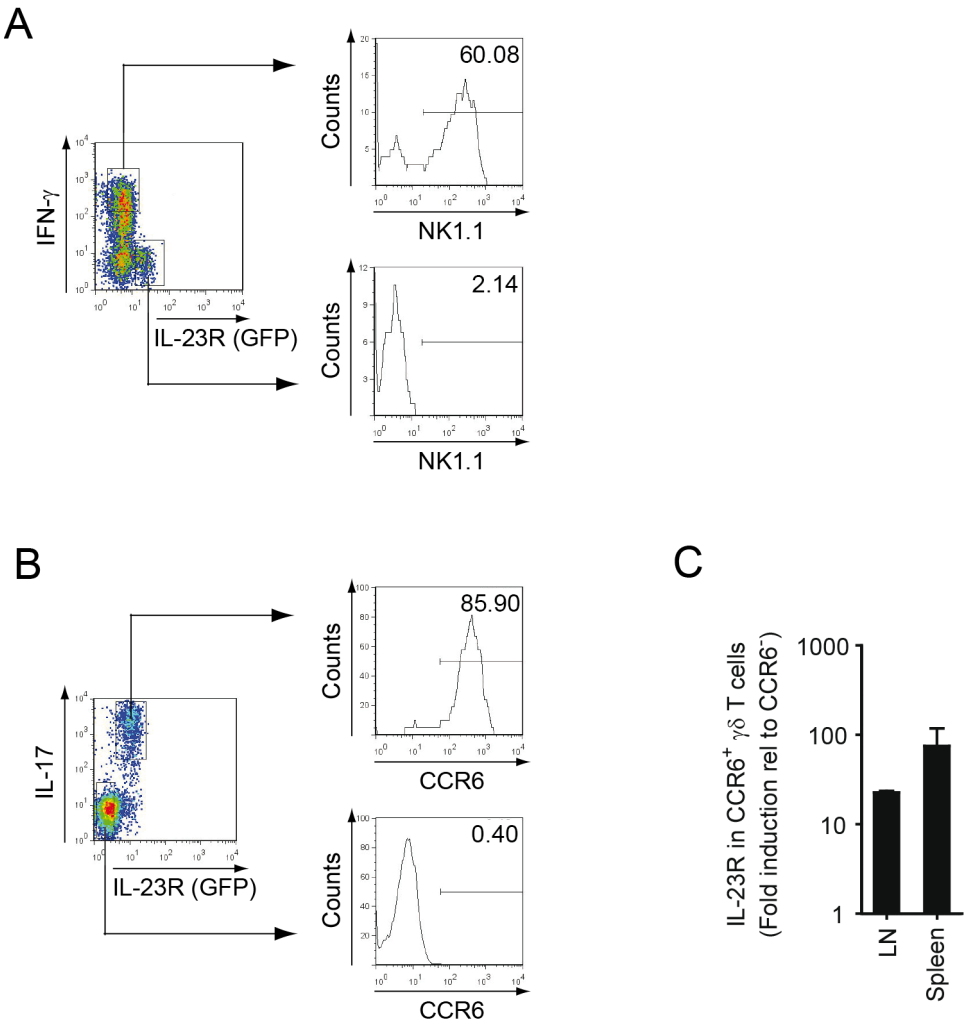


Figure 5.6: Correlation of IL-23R status of γδ T cells to their cytokine production
γδ T cells were isolated from lymph nodes and spleen of naïve *Il23r*$^{+/-}$ mice and ex vivo stimulated with PMA plus ionomycin. Cells were stained for NK1.1 and intracellular IFN-γ (A) or CCR6 and intracellular IL-17 (B). (C) CCR6^{+} γδ T cells were purified from lymph nodes and spleens of naïve wild type mice by fluorescence-activated cell sorting and analyzed for *Il23r* mRNA expression by Real-time PCR directly ex vivo. Fold induction of *Il23r* mRNA in CCR6^{+} γδ T cells is related to CCR6^{-} γδ T cells. All data are representative of at least two independent experiments.

5.1.4 The IL-23R$^+$ γδ T cell fraction is reduced in IL-6 deficient mice

TGF-β and IL-6 are required to induce Rorγt and IL-23R in αβ T cells during lineage commitment of Th17 cells and IL-6 deficient mice have been described to have a defect in the generation of Th17 cells (Korn et al., 2007a). Thus, the frequencies, surface marker expression patterns, and cytokine secreting profiles of γδ T cells purified from the spleens and lymph nodes of IL-6 deficient mice (*Il6*$^{-/-}$) were analyzed using flow cytometry. Although the overall numbers of γδ T cells were not reduced in *Il6*$^{-/-}$ mice compared to wild type mice, reduced fractions of CCR6$^+$ γδ T cells and higher fractions of NK1.1$^+$ γδ T cells were detected, both in the spleens and in the lymph nodes of IL-6 deficient animals (Figure 5.7).

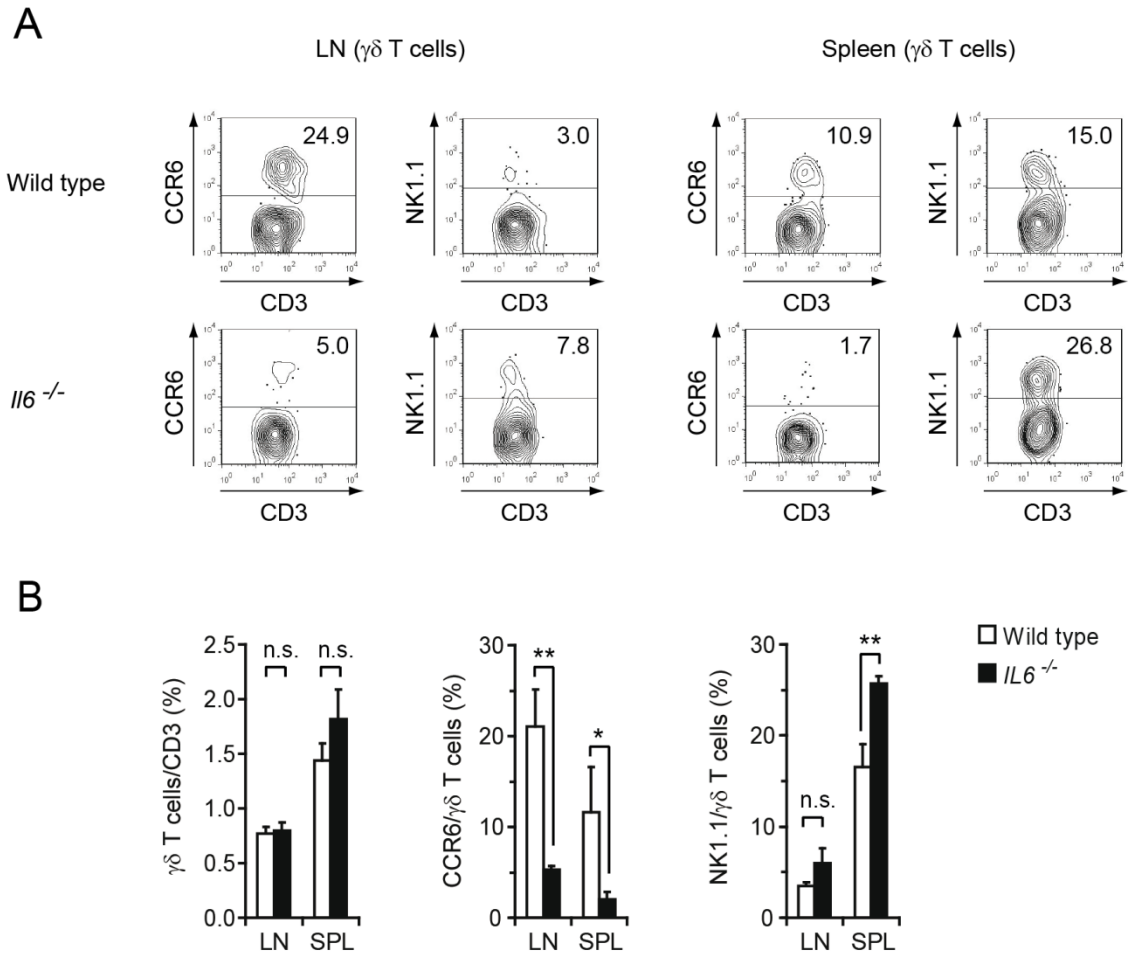


Figure 5.7: The frequency of CCR6$^+$ γδ T cells is reduced in the absence of IL-6
γδ T cells were isolated from lymph nodes (LN) and spleen (SPL) of naïve wild type and *Il6*$^{-/-}$ mice. The frequencies of γδ-TCR$^+$ cells within the CD3$^+$ gate (B) and the frequencies of CCR6$^+$ or NK1.1$^+$ cells within the γδ-TCR gate (A+B) were determined by flow cytometry. (A) Numbers indicate percentages within the γδ-TCR gate. (B) Mean frequencies ± SD. All data are representative of at least two independent experiments.

The reduced fraction of CCR6$^+$ γδ T cells correlated with a lower percentage of IL-17 producers in both analyzed compartments (Figure 5.8A and B). Next, the cytokine secretion of γδ T cells purified from *Il6*$^{-/-}$ x *IL23r*$^{+/-}$ mice was analyzed. IL-23R$^+$ γδ T cells and IL-23R$^+$, γδT17 cells were clearly reduced in *Il6*$^{-/-}$ x *Il23r*$^{+/-}$ compared to *Il23r*$^{+/-}$ control mice (Figure 5.8C). These experiments indicated that IL-6 is involved in the maintenance of γδT17 cells in the peripheral immune compartment.

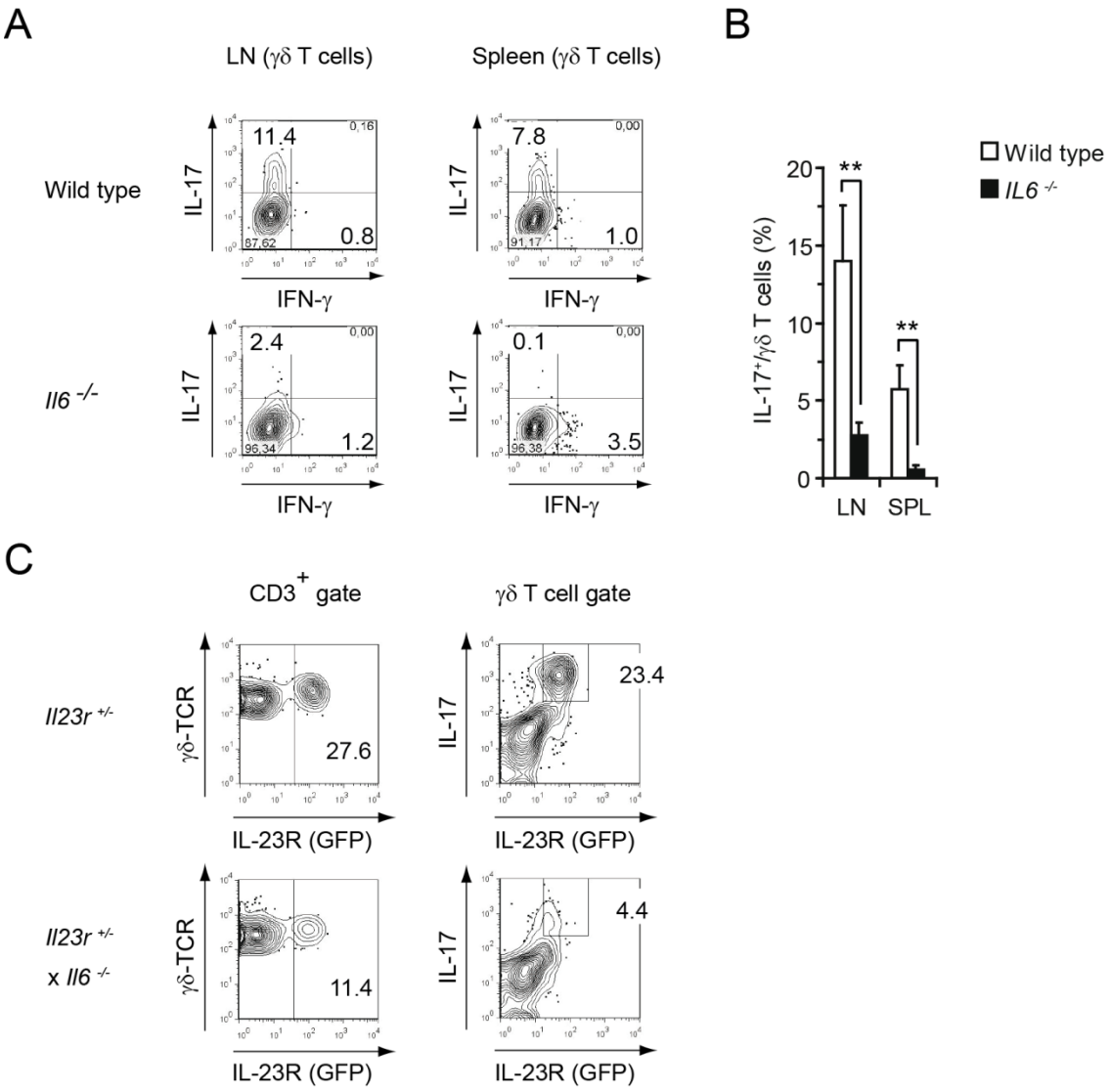


Figure 5.8: The fractions of γδT17 cells and of IL-23R$^+$ γδ T cells are reduced in the absence of IL-6
(A)+(B) γδ T cells were isolated from lymph nodes and spleen of naïve wild type or *Il6*$^{-/-}$ mice and ex vivo stimulated with PMA plus ionomycin. Cells were stained for intracellular IFN-γ and IL-17. (A) Numbers indicate percentages within the γδ-TCR gate. (B) Mean frequencies ± SD. (C) γδ T cells were isolated from lymph nodes (LN) and spleen (SPL) of naïve *Il23r*$^{+/-}$ or *Il-23r*$^{+/-}$ *x Il-6*$^{-/-}$ mice and ex vivo stimulated with PMA plus ionomycin prior to staining for intracellular IL-17. Numbers indicate fractions of IL-23R$^+$ γδ T cells (left panel) or fractions of IL-23R$^+$IL-17$^+$ γδ T cells (right panel). All data are representative of at least two independent experiments.

5.2 Development of IL-23R expressing γδ T cells

Previous experiments have shown that a subset of γδ T cells expresses the IL-23R constitutively. These cells immediately respond to IL-23 with enormous production of IL-17. In contrast to Th17 cells, which develop only upon antigen encounter in the peripheral immune compartment, γδ T cells seem to be pre-committed to the production of IL-17 when leaving the thymus. Similarly, another functionally distinct γδ T cell population (γδT1 cells) was identified to produce IFN-γ but not IL-17. γδT1 cells did not express IL-23R but produced IFN-γ after IL-12 stimulation. These observations led to the hypothesis that there are at least two distinct subsets of γδ T cells that develop separately in the thymus. Since IL-23 and IL-1 are cytokines known to promote the expansion and activation of γδT17 cells in the peripheral compartment (Sutton et al., 2009), a series of experiments was designed to test the role of IL-23 and IL-1 in thymic development of distinct γδ T cell subsets. In first experiments bone marrow cells from *Il23r*$^{+/-}$ mice were injected i.v. into lethally irradiated *Rag1*$^{-/-}$ recipients. Five to six weeks after cell transfer several lymphoid tissues were analyzed for the frequencies of IL-23R^{+} γδ T cells and compared to those of unmanipulated *Il23r*$^{+/-}$ mice. Surprisingly, the frequencies of IL-23R^{+} γδ T cells were significantly reduced in all analyzed tissues (Figure 5.9A). While the total fraction of γδ T cells in the thymus was also reduced, the total fractions of γδ T cells within the CD3 compartment were not altered in spleen and lymph nodes, suggesting that the defective generation of IL-23R^{+} γδ T cells was compensated by IL-23R negative γδ T cells filling the niche in the peripheral immune compartment (Figure 5.9B). Intracellular cytokine staining of lymph node cells either isolated from bone marrow-transplanted mice or from non-grafted *Il23r*$^{+/-}$ control mice revealed that those γδ T cells found in the periphery after bone marrow transplantation – although sufficient in IFN-γ production – did not produce IL-17, while CD4^{+} T cells were able to produce both, IFN-γ and IL-17 (Figure 5.9C and D). Therefore, it can be concluded that γδT17 cells selectively fail to develop after bone marrow transfer in adult recipients while there is no general defect in the generation of γδ T cells in the bone marrow recipients.

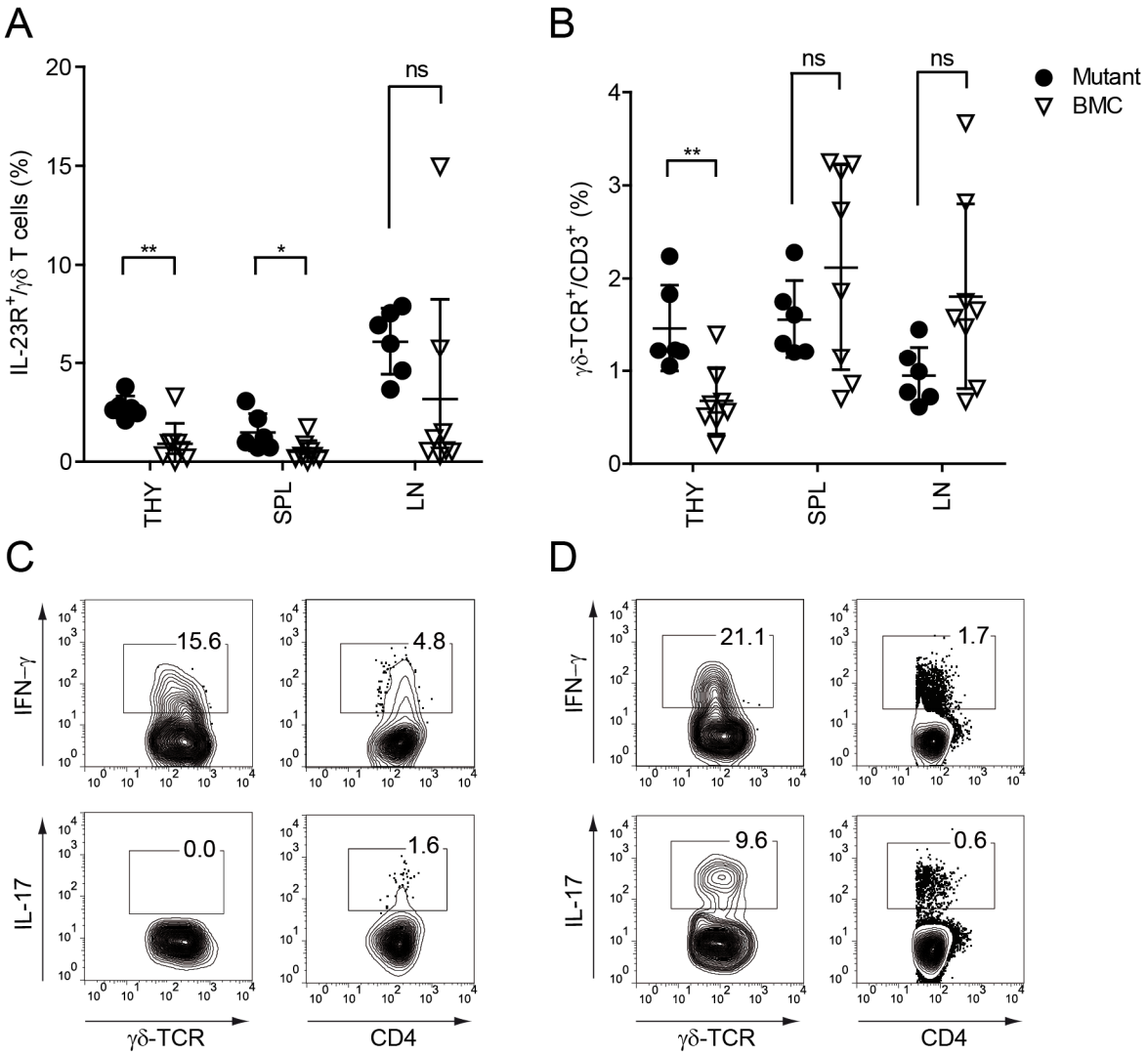


Figure 5.9: The development of IL-23R$^+$ γδ T cells is defective in adult mice
Lethally irradiated *Rag1*$^{-/-}$ mice were reconstituted with donor bone marrow from *Il23r*$^{+/-}$ mice. After 5-6 weeks, donor cells were re-isolated from various tissues and analyzed for frequencies of IL-23R(GFP)$^+$ γδ T cells (A) or for frequencies of γδ T cells within the CD3$^+$ gate (B). Cells isolated from bone marrow chimera (BMC) were compared to cells isolated from unmanipulated *Il23r*$^{+/-}$ mice (Mutant). (C) Donor cells re-isolated from lymph nodes (C) or lymph node cells isolated from naïve *Il23r*$^{+/-}$ mice (D) were ex vivo stimulated with PMA plus ionomycin and analyzed for frequencies of IFN-γ$^+$ cells and IL-17$^+$ cells within the γδ T cell gate or CD4$^+$ T cell gate. All data are representative of at least two independent experiments.

The adult thymus is not able to produce γδT17 cells after transfer of bone marrow cells. Thus, γδT17 cells must be generated in the fetal thymus and their population is likely maintained in the periphery by self-renewal. In order to analyze whether in the adult individual, the failure to generate γδT17 cells in the thymus was due to some intrinsic alterations of the thymus during ontogenesis after birth or due to altered properties of γδ T cell precursor cells from adult bone marrow fetal liver chimeras were generated. To this end fetal liver cells were isolated from *Cd45.1xIl23r*$^{+/-}$ mice on day 13.5 of gestation and transferred i.v. into lethally irradiated *Rag1*$^{-/-}$

recipients. Furthermore, the same number of bone marrow cells isolated from *Cd45.2xIl23r*$^{+/-}$ mice was co-transferred. Using this strategy, it was possible to analyze the development of γδ T cells from bone marrow precursors and fetal liver precursors side by side in the same mouse. 5 to 6 weeks after cell transfer donor cells were re-isolated from the thymus, the spleen, and lymph nodes and analyzed for the frequencies of IL-23R$^+$ cells by flow cytometry. Cells originating from the fetal liver graft or the bone marrow graft were distinguished with the help of the congenic marker (CD45.1 vs CD45.2). Again, the numbers of IL-23R$^+$ γδ T cells were highly reduced compared to non-grafted wild type controls (Figure 5.10) irrespective of whether the cells originated from fetal liver precursors or bone marrow precursors. Consistently, those γδ T cells found in the periphery were not able to produce IL-17 upon ex vivo stimulation with PMA/ionomycin (data not shown). To exclude the possibility that the presence of bone marrow cells hindered the development of IL-23R$^+$ γδ T cells from fetal liver precursors, fetal liver cells were also transferred without co-transfer of bone marrow cells. In this scenario, the numbers of IL-23R$^+$ γδ T cells were still strongly reduced (data not shown). Together, these data suggest that IL-23R$^+$ γδ T cells are not generated after precursor cell transplantation into adult mice because the adult thymus fails to provide an appropriate milieu for the generation of γδT17 cells.

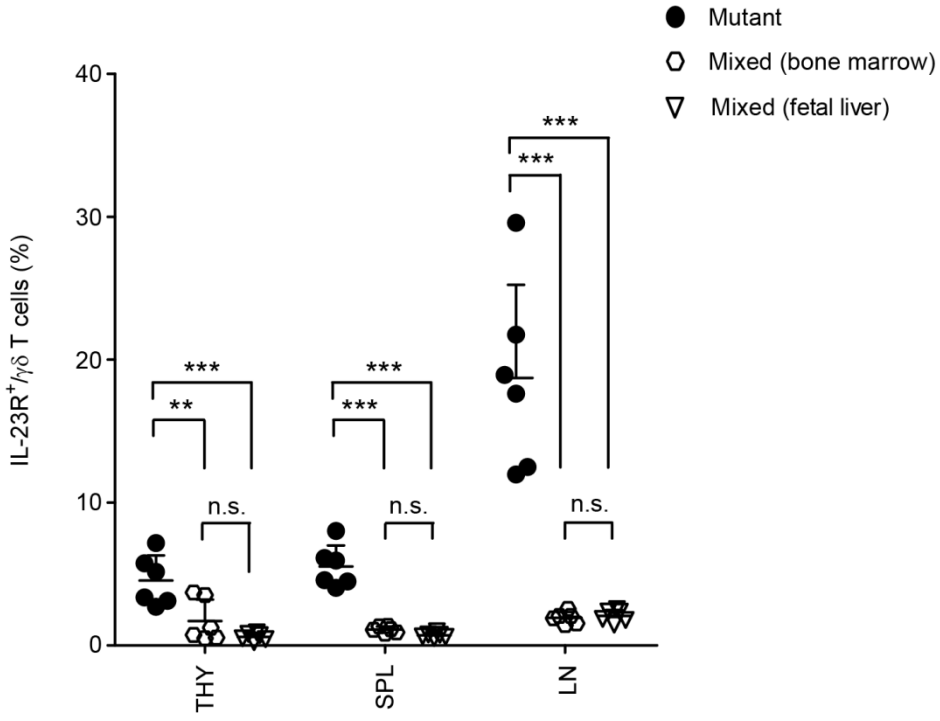


Figure 5.10: The development of IL-23R$^+$ γδ T cells is defective in adult mice after fetal liver cell transfer
Lethally irradiated *Rag1*$^{-/-}$ mice were reconstituted with a mixture of cells composed of fetal liver cells isolated from *Il23r*$^{+/-}$ mice on day 13.5 of gestation (CD45.1) and bone marrow cells isolated from adult *Il23r*$^{+/-}$ mice (CD45.2). After 5-6 weeks, donor cells were re-isolated from various tissues. Cells originating from fetal livers or bone marrow distinguishable by congenic markers were flow cytometrically analyzed for frequencies of IL-23R(GFP)$^+$ γδ T cells. Frequencies of IL-23R(GFP)$^+$ γδ T cells in chimeras were compared to those of unmanipulated *Il23r*$^{+/-}$ mice (Mutant). Representative data out of two independent experiments.

The intrathymic conditions that impact on the development of γδ T cells into different functional subsets are poorly characterized. Current hypotheses suggest that the strength of TCR ligation influences the γδ T cell commitment into IL-17 or IFN-γ producing cells. However, data concerning the intrathymic cytokine milieu during ontogenesis are limited. Further experiments addressed the question whether the development of γδT17 cells in the adult thymus is influenced by IL-23 and IL-1. It is conceivable that processes that occur after birth, for example the microbial colonization of the gut, increases the systemic levels of IL-23 and IL-1. IL-23 and IL-1 promote the maintenance and/or expansion of γδT17 cells but simutanously might act as a negative feedback loop that inhibits the generation of IL-23R$^+$ γδ T cells in the thymus. Therefore, the development of γδ T cells deficient for IL-23R or IL-1R was investigated. For this purpose, bone marrow chimeras were generated using bone marrow cells from *Il23r*$^{-/-}$ or *Il1r*$^{-/-}$ mice. After reconstitution the γδ T cell compartment was analyzed for the presence of γδ T cells with the potential to produce IL-17. As *Il1r*$^{-/-}$ mice did not express the IL-23R(GFP) construct other surface markers had to be established to characterize the IL-23R$^+$ γδ T cell population. Consistent with data provided by Haas et al, IL-23R$^+$ γδ T cells were found to be identified as CCR6$^+$ and CD27$^-$ cells within the γδ T cell compartment (Haas et al., 2009) (Figure 5.11B). Five to six weeks after cell transfer the γδ T cells were re-isolated from various lymhoid tissues and analyzed for the expression of CCR6 and CD27, and IL-23R(GFP), respectively. In *Il1r*$^{-/-}$ chimeras, CCR6$^+$CD27$^-$ γδ T cell fractions were signifcantly reduced in all analyzed tissues (Figure 5.11C). In the *Il23r*$^{-/-}$ chimeras, reduced numbers of GFP$^+$ γδ T cells (IL-23R "wannabe" cells) were found in all tissues, althought the difference in the lymph nodes did not reach significance (Figure 5.11A). Thus, these data suggest that IL-23 and IL-1 do not negatively influence the development of IL-23R$^+$ γδ T cells in the adult thymus.

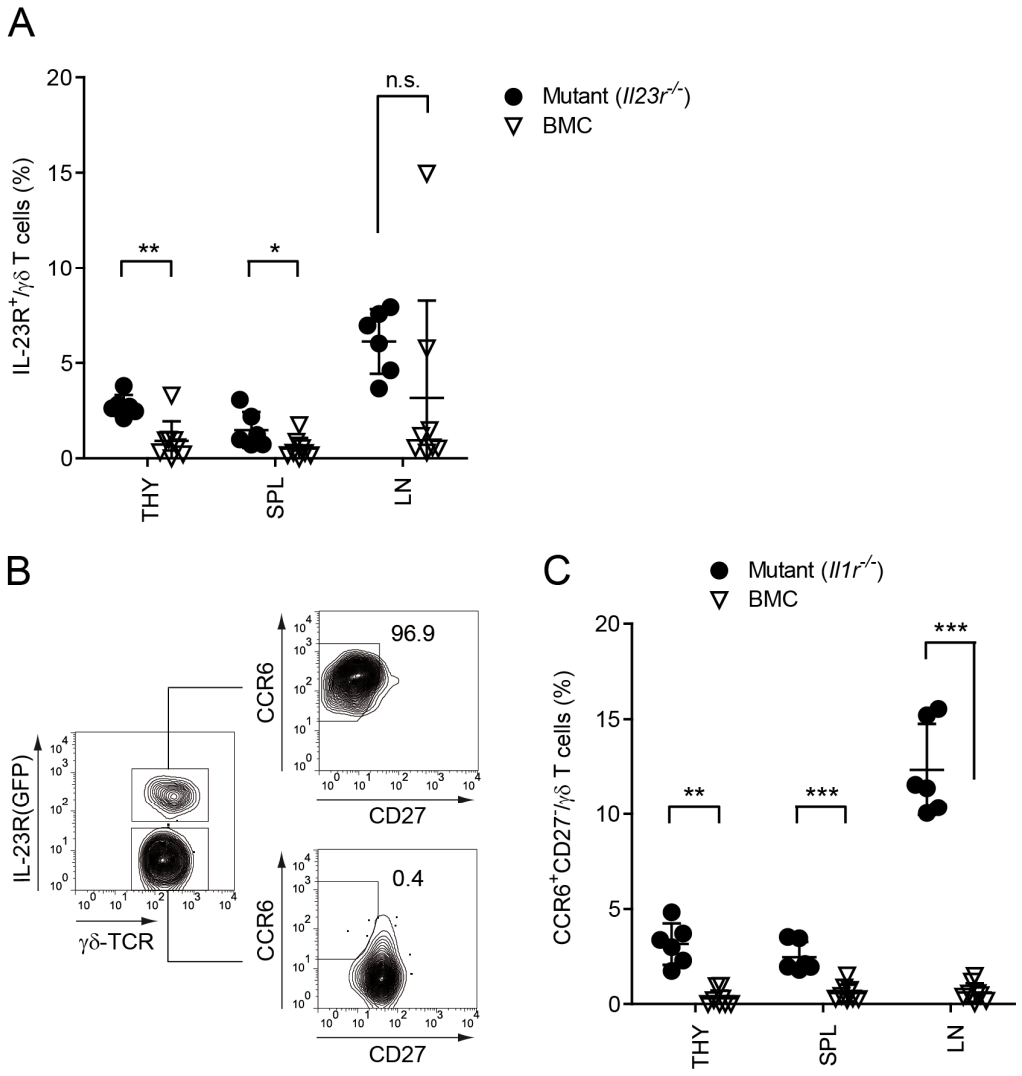


Figure 5.11: The development of IL-23R$^+$ γδ T cells and CCR6$^+$CD27$^-$ γδ T cells is defective in adult mice after transfer of bone marrow isolated from *Il23r*$^{-/-}$ or *Il1r*$^{-/-}$ mice, respectively
(A) Lethally irradiated *Rag1*$^{-/-}$ mice were reconstituted with donor bone marrow from *Il23r*$^{-/-}$ mice. After 5-6 weeks, donor cells were re-isolated from various tissues and analyzed for frequencies of IL-23R(GFP)$^+$ γδ T cells. Cells isolated from bone marrow chimeras (BMC) were compared to cells isolated from unmanipulated *Il23r*$^{-/-}$ mice (Mutant). (B) γδ T cells were isolated from lymph nodes of naïve *Il23r*$^{+/-}$ mice. Expression of IL-23R(GFP) within the γδ-TCR gate was determined by flow cytometry. IL-23R(GFP)$^+$ and IL-23R(GFP)$^-$ γδ T cells were analyzed for the expression of CCR6 and CD27. (C) Lethally irradiated *Rag1*$^{-/-}$ mice were reconstituted with donor bone marrow from *Il1r*$^{-/-}$ mice. After 5-6 weeks, donor cells were re-isolated from various tissues and analyzed for frequencies of CCR6$^+$CD27$^-$ γδ T cells. Cells isolated from bone marrow chimeras (BMC) were compared to cells isolated from unmanipulated *Il1r*$^{-/-}$ mice *(*Mutant). All data are representative of at least two independent experiments.

5.3 Role of IL-23R$^+$ γδ T cells during autoimmunity

IL-17 producing CD4$^+$ αβ T cells, called Th17 cells and IL-23, which serves as an indispensable maturation factor for Th17 cells, have emerged as major players during chronic inflammation and autoimmune diseases, including EAE. Further characterization of IL-17 has led to the finding that γδ T cells are prominent producers of IL-17 during various infection models. Previous experiments have shown that γδ T cell-derived IL-17 is exclusively produced by IL-23R expressing γδ T cells and that these cells represent an ontogenetically distinct subset of γδ T cells.

Next, the behavior of IL-23R$^+$ γδ T cells during an autoimmune response in vivo was analyzed. *Foxp3gfp*.KI and *Il23r*$^{+/-}$ mice were immunized with MOG$_{35-55}$/CFA plus Pertussis toxin. Wild type mice usually show first signs of disease 10 to 14 days after immunization and reach the peak of disease two to three days after the onset. Mice recover completely or partially within 7 to 10 days after the peak of disease. As expected, *Foxp3gfp*.KI and *Il23r*$^{+/-}$ mice developed EAE in a manner similar to wild type mice. A representative EAE course is shown in Figure 5.12.

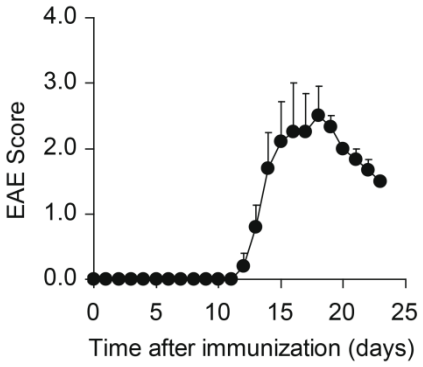
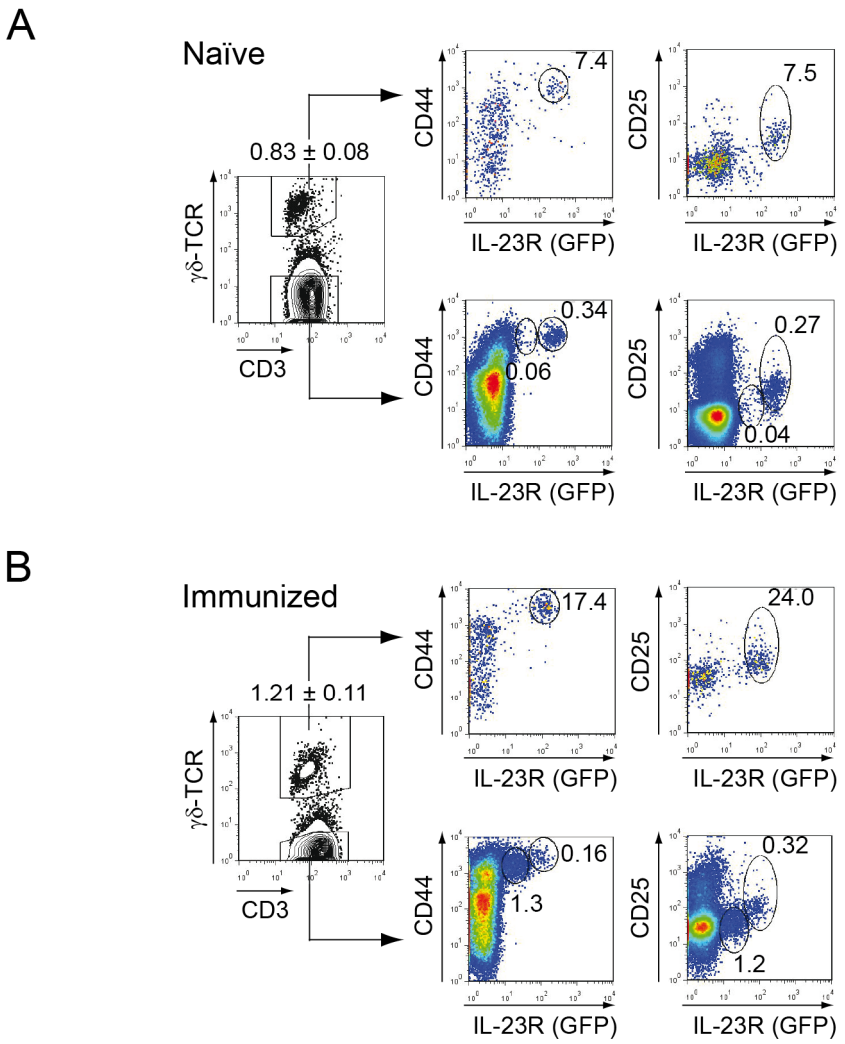


Figure 5.12: Representative EAE course
Foxp3gfp.KI and *Il23r*$^{+/-}$ mice were immunized with MOG$_{35-55}$/CFA plus Pertussis toxin. Both strains develop EAE in a manner similar to wild type mice (mean clinical score ± SEM, n=5). Representative data out of at least five independent experiments.

In first experiments, EAE was induced in *Il23r*$^{+/-}$ mice. Hence, IL-23R expressing cells could be followed by their GFP expression. Before immunization two IL-23R$^+$ T cell populations were determined within the CD3$^+$ compartment: γδ T cells and CD4$^-$CD8$^-$ (DN) cells (data not shown). DN αβ T cells probably represent a heterogeneous population consisting of mucosal-associated invariant T cells and Natural Killer T cells (Figure 5.13A and C). Both IL-23R$^+$

populations were characterized by the expression of the effector/memory T cell markers CD44 and CD25. Immunization did not result in a change of DN T cell numbers, but a new IL-23R^{low} CD44^{+}CD25^{dim} population emerged within the αβ T cell compartment (Figure 5.13B and C). These cells expressed CD4 (data not shown), the effector/memory cell marker CD44 and IL-23R and therefore it is likely that these cells were Th17 cells that were primed in an antigen-specific manner. In addition, the fraction of γδ T cells in the CD3^{+} compartment in draining lymph nodes expanded (data not shown). Notably, while in lymph nodes of naïve mice only 7 % of γδ T cells were positive for IL-23R, now one-fifth or more of the γδ T cells expressed IL-23R (Figure 5.13D). Nearly all of these IL-23R^{+} γδ T cells were CCR6^{+} and NK1.1^{-} and expressed the TCR gamma-chain Vγ4 (Figure 5.13D).



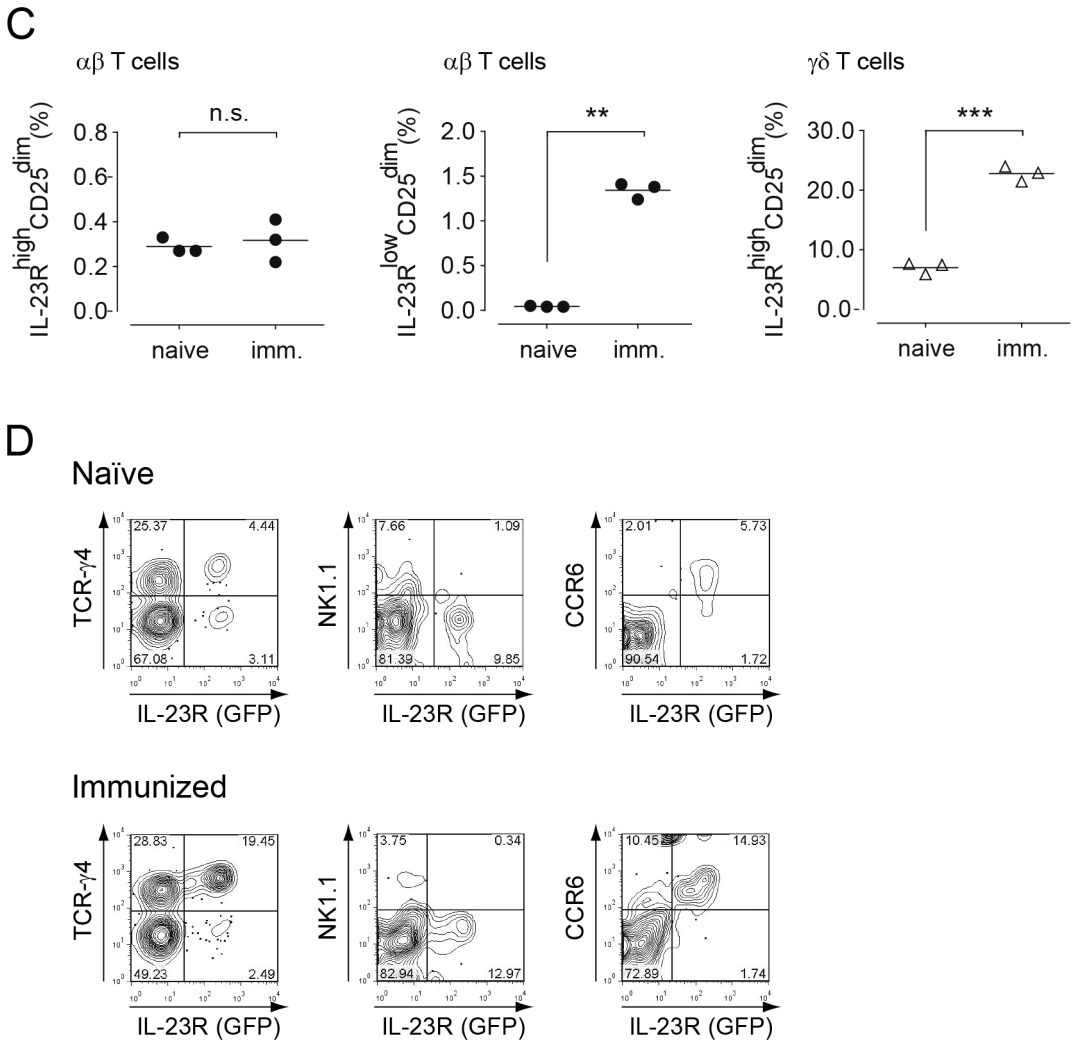


Figure 5.13: Upon senzitation with MOG_{35-55}/CFA IL-23R$^+$ γδ T cells expand in the lymph nodes
Lymph node cells were isolated from naïve *Il23r*$^{+/-}$ mice (A) or from *Il23r*$^{+/-}$ mice that were immunized with MOG_{35-55}/CFA plus Pertussis toxin (B) and analyzed for expression of IL-23(GFP), CD44, and CD25 within the γδ T cell compartment (CD3$^+$γδ-TCR$^+$) and the αβ T cell compartment (CD3$^+$γδ-TCR$^-$) by flow cytometry. Numbers indicate percentages. (C) Percentages of IL-23R^{high} (CD4$^-$CD8$^-$) and IL-23R^{low} (CD4$^+$) cells within the αβ T cell compartment (filled circles) and of IL-23R$^+$ cells within the γδ T cell compartment (open triangles) of individual mice before and after immunization. (D) Lymph node cells were isolated from naïve *Il23r*$^{+/-}$ mice (upper panel) or from *Il23r*$^{+/-}$ mice that were immunized with MOG_{35-55}/CFA (lower panel) and analyzed for expression of IL-23(GFP), CCR6, NK1.1, and TCR-Vγ4 within the γδ T cell compartment (CD3$^+$γδ-TCR$^+$). Numbers in the quadrants indicate percentages within the CD3$^+$γδ-TCR$^+$ gate. All data are representative of at least two independent experiments.

In order to compare the population dynamics of IL-23R$^+$ γδ T cells during EAE to those of IL-23R$^-$ γδ T cells, Th17 cells, and Treg cells, *Foxp3gfp*.KI and *Il23r*$^{+/-}$ mice were immunized with MOG_{35-55}/CFA plus Pertussis toxin. Upon EAE induction, Th17 cells (IL-23R$^+$γδ-TCR$^-$ CD3$^+$ T cells) expanded in draining lymph nodes and accumulated in the spleen prior to clinical signs of disease (Figure 5.14 and Figure 5.16). When the animals became sick, the Th17 numbers declined in the spleen but increased in the CNS, suggesting that the splenic pool

of Th17 cells drained into the target tissue. γδ T cells expanded in the peripheral lymphoid organs (SPL and LN) until the peak of disease and accumulated in the CNS at the peak of disease (Figure 5.14 and Figure 5.16). During the recovery of EAE the γδ T cell numbers again declined both in the peripheral immune compartment and in the CNS. In the peripheral lymphoid organs IL-23R$^+$ γδ T cells never outnumbered IL-23R$^-$ γδ T cells. In contrast, most of the accumulating γδ T cells in the CNS at the peak of disease expressed the IL-23R. Here, the fraction of IL-23R$^+$ γδ T cells within the CD3$^+$ T cell population increased ~8-fold until the peak of disease, and the absolute numbers of IL-23R$^+$ γδ T cells increased even 20-fold. Thus, the population dynamics of IL-23R$^+$ γδ T cells in the CNS clearly reflected the clinical course of EAE and the state of inflammation in the target tissue.

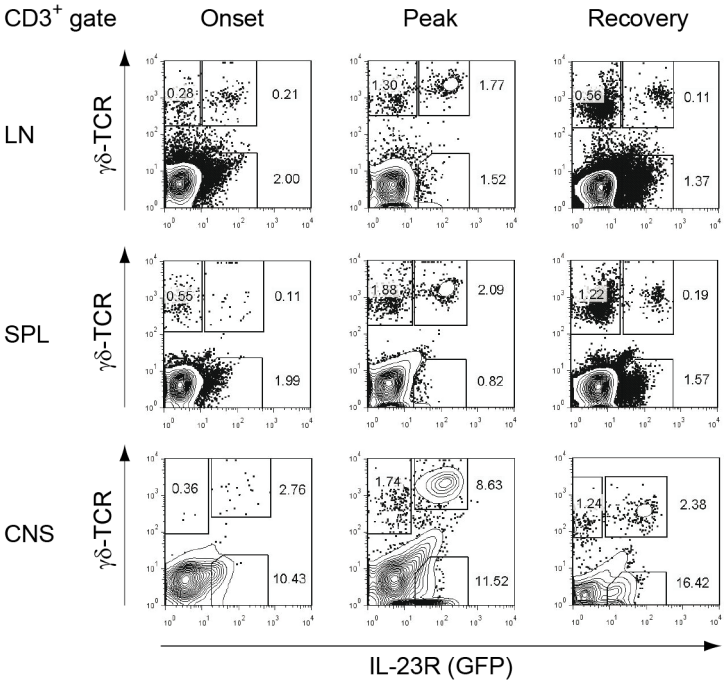


Figure 5.14: IL-23R$^+$ γδ T cells accumulate in the CNS at the peak of EAE and rapidly contract during recovery
Il23r$^{+/-}$ mice were immunized with MOG_{35-55}/CFA plus Pertussis toxin. At the onset and peak of disease and during the recovery phase splenocytes (SPL), cells from the draining lymph nodes (LN), and mononuclear cells from the CNS were isolated and analyzed for IL-23R(GFP) expression by flow cytometry. Numbers indicate percentages within the CD3$^+$ T cell gate. Representative dot plots out of three independent experiments are shown.

Previous experiments have shown that IL-23R$^+$ γδ T cells produce IL-17 while IL-23R$^-$ γδ T cells produce IFN-γ. Consistent with this finding, γδ T cells contributed substantially to the IL-17 production, but not the IFN-γ production in the CNS during EAE (Figure 5.15).

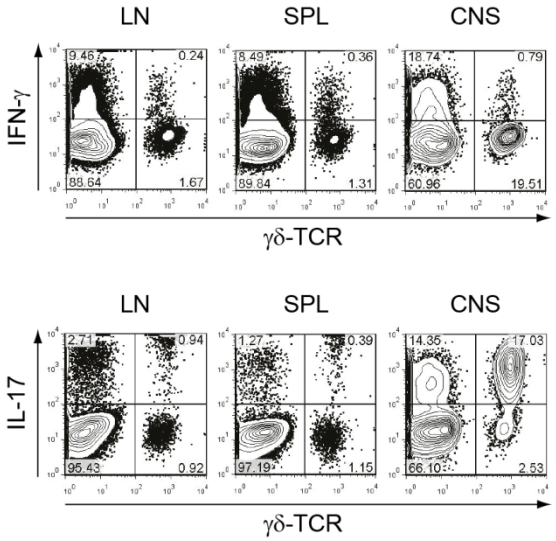


Figure 5.15: γδ T cells contribute substantially to the production of IL-17 during EAE
Il23r$^{+/-}$ mice were immunized with MOG_{35-55}/CFA plus Pertussis toxin. At peak of disease splenocytes (SPL), cells from the draining lymph nodes (LN), and mononuclear cells from the CNS were isolated and stimulated with PMA/ionomycin. Cells were analyzed for production of IFN-γ and IL-17 by intracellular cytokine staining and subsequent flow cytometry. Numbers in the quadrants indicate percentages within the $CD3^+$γδ-TCR^+ gate. Representative dot plots out of three independent experiments are shown.

Interestingly, when analyzing Treg cell numbers during EAE in *Foxp3gfp*.KI mice $Foxp3^+$ Treg cells showed inverse population dynamics compared to IL-$23R^+$ γδ T cells, reaching their highest numbers during the recovery phase after the attrition of IL-$23R^+$ γδ T cells (Figure 5.16). Recovery from EAE requires an efficient suppression of effector functions by Treg cells. However, it seems that $Foxp3^+$ Treg cells are able to control effector functions of αβ T cells only in the absence of γδ T cells. These data suggest, that IL$23R^+$ γδ T cells might be able to influence $Foxp3^+$ Treg cell population dynamics in the inflammed tissue during autoimmunity.

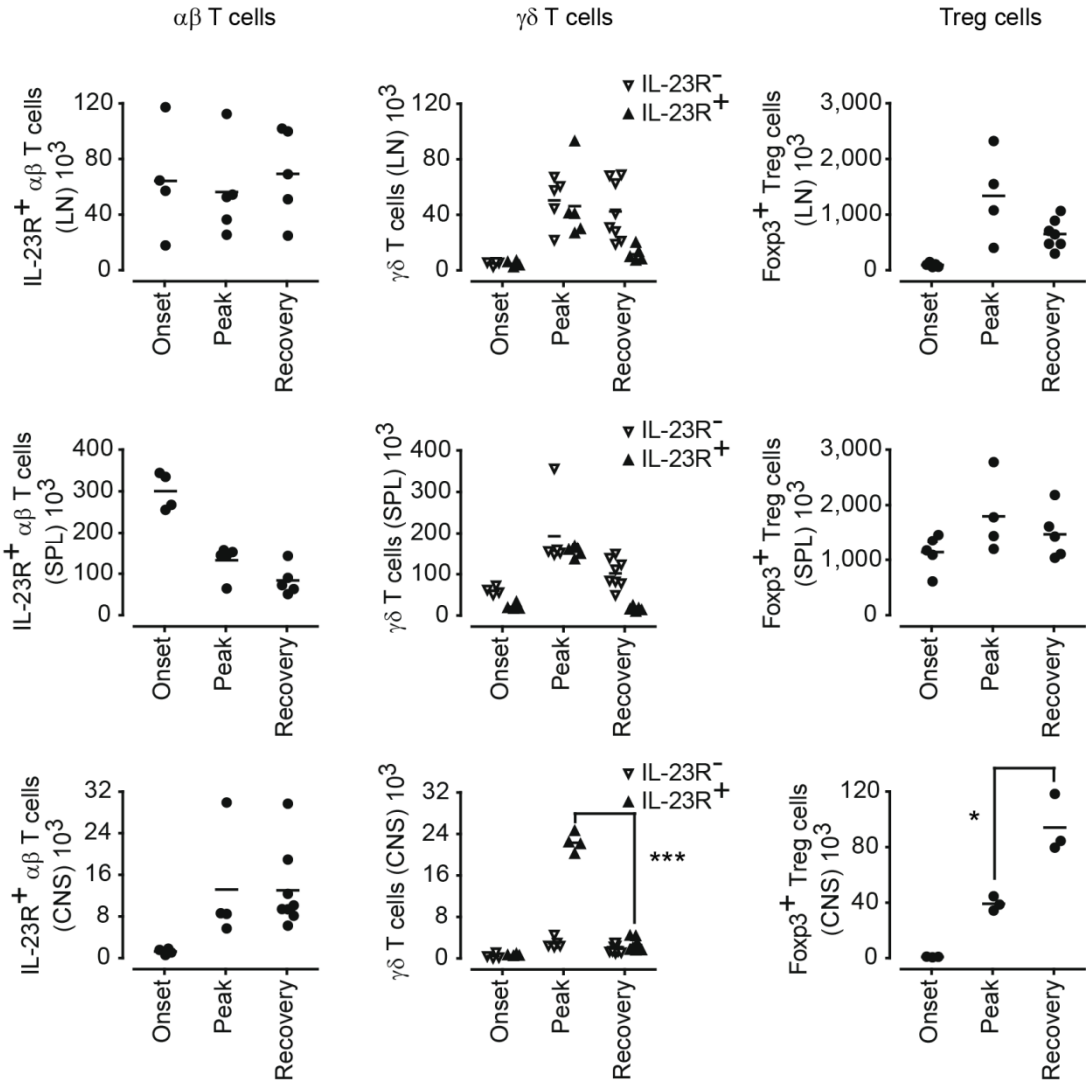


Figure 5.16: Population dynamics of Th17 cells, γδ T cells, and Treg cells during the course of EAE
Foxp3gfp.KI and *Il23r*$^{+/-}$ mice were immunized with MOG_{35-55}/CFA plus Pertussis toxin. Lymph nodes (LN) cells, splenocytes (SPL), and mononuclear cells from the CNS were prepared at different stages of EAE and analyzed for IL-23R(GFP) expression within the $CD3^+$γδ-TCR^+ gate (middle column) or for expression of IL-23R(GFP) (left column) and Foxp3(GFP) (right column) within the $CD3^+$γδ-TCR^- gate. Absolute numbers were calculated on the basis of percentages obtained in the flow cytometric analysis and assessment of total live mononuclear cells in a Trypan blue exclusion analysis. IL-$23R^+CD3^+$γδ-TCR^- cells in the CNS correspond to Th17 cells. Representative data out of three independent experiments.

In the course of EAE, effector T cells migrate to the CNS and create an inflammatory milieu, particularly by producing pro-inflammatory cytokines. Th17 cells and the IL-17 that they produce have been found to play a major role in the pathogenesis of EAE and MS. IL-$23R^+$ γδ T cells that accumulated in the CNS at the peak of disease contributed substantially to the total IL-17 production in the CNS. However, at the peak of disease IL-17 producing Th17 cells have already invaded the CNS in great numbers. Thus, although their IL-17 production promotes tissue inflammation during EAE, additional functions of IL-$23R^+$ γδ T cells were likely. The

inverse population dynamics of IL-23R$^+$ γδ T cells and Treg cells raised the question whether γδ T cells influenced the induction or function of Treg cells in an IL-23-dependent manner.

Cells communicate with each other by direct cell-cell contacts or by the secretion of effector molecules. To investigate, whether γδ T cells secrete substances that act on Treg cells, an initial in vitro screening approach was performed. γδ T cells, isolated from lymph nodes and spleen of wild type mice, were stimulated polyclonally in the presence or absence of IL-23. After four days of culture, the cell-free culture supernatants were collected and tested for their ability to modulate the conversion of conventional T cells into Foxp3$^+$ Treg cells. For conversion assays naïve CD4$^+$CD44$^-$Foxp3$^-$ T cells were purified from lymph nodes and spleen of *Foxp3gfp*.KI mice by fluorescence-activated cell sorting. Naïve T cells were then stimulated in the presence of TGF-β to induce the expression of Foxp3. The TGF-β-driven Foxp3 expression in conventional T cells was then tested in the presence of γδ T cell supernatants or a series of recombinant cytokines as control conditions. After three days of culture the induction of Foxp3$^+$ Treg cells was analyzed on the basis of GFP expression by flow cytometry. As already demonstrated in other studies, the generation of Foxp3$^+$ Treg cells was induced by TGF-β whereas the cytokine combinations TGF-β/IL-6 and TGF-β/IL-21 inhibited the conversion of naïve T cells into Treg cells but promoted Th17 cell development (Figure 5.17A, Th17 induction not shown). Interestingly, addition of supernatants of γδ T cells that were activated with IL-23 also suppressed the development of Foxp3$^+$ Treg cells while the supernatants that were generated without IL-23 did not mediate suppression (Figure 5.17B). The missing suppression of the combination of TGF-β plus IL-23 demonstrated that the observed effect is not caused by IL-23 alone but by a factor that is secreted by γδ T cells in an IL-23-dependent fashion. However, in contrast to TGF-β/IL-6 and TGF-β/IL-21 the combination of TGF-β plus the IL-23-supernatant did not induce the differentiation of Th17 cells (data not shown). To further characterize the nature of the factor that provoked this observed suppression of Treg cell conversion, supernatants were boiled and again tested in conversion assays. This time, IL-23-activated γδ T cells supernatants failed to inhibit the TGF-β-driven conversion of naïve T cells into Foxp3$^+$ Treg cells (Figure 5.17C). Thus, a protein identity of this unknown γδ T cell-derived factor must be assumed.

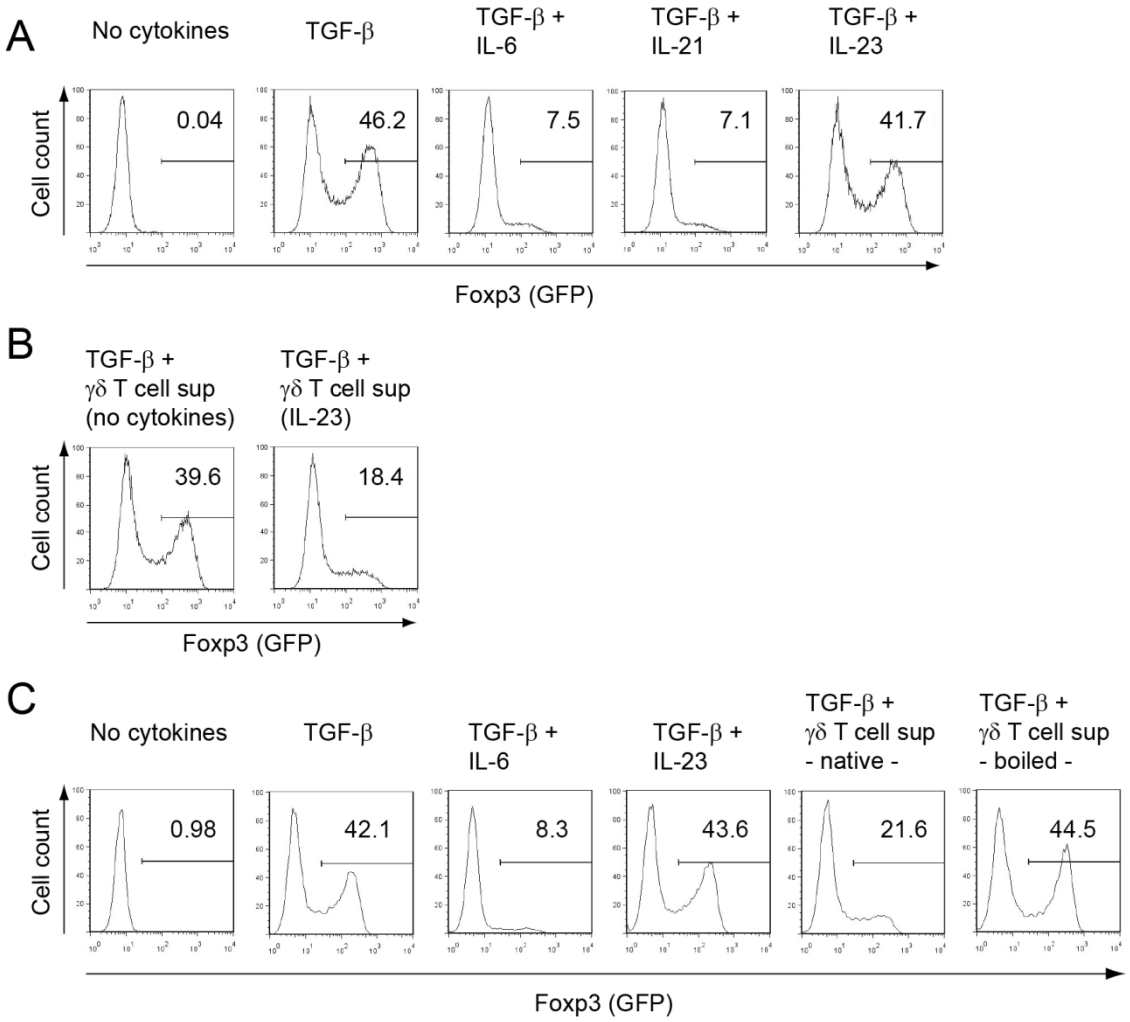


Figure 5.17: Cell-free supernatant from IL-23-activated γδ T cells abrogates the TGF-β-driven conversion of conventional T cells into Foxp3$^+$ Treg cells
Naïve CD4$^+$Foxp3$^-$CD44$^-$ T cells were isolated from *Foxp3gfp*.KI mice by fluorescence-activated cell sorting and stimulated with plate-bound anti-CD3 and soluble anti-CD28 in the presence of different cytokines, cytokine combinations (A), or γδ T cell culture supernatants (B). After three days, Foxp3(GFP) expression was analyzed by flow cytometry. γδ T cell culture supernatants were collected from γδ T cells that were isolated from wild type mice by magnetic cell sorting and stimulated four days with anti-CD3/anti-CD28 beads in the presence or absence of IL-23. (C) γδ T cells were stimulated with anti-CD3/anti-CD28 beads in the presence of IL-23. After four days, culture supernatants were collected from culture and boiled for 5 min at 95 °C. (A-C) Numbers indicate percentages. All data are representative of at least three independent experiments.

By performing quantitative Real-time PCR it was possible to analyze the cytokine profile of γδ T cells that were activated with IL-23. These analyses included the genes that code for the cytokines IL-4, IL-6, IL-21, and IL-27 that have been described to block the generation of Foxp3$^+$ Treg cells. Compared to γδ T cells that were cultured in the absence of IL-23, IL-23-activated γδ T cells expressed higher levels of IL-17 (IL-17A), IL-17F, IL-21, and IL-22, but no IL-4, IL-6, and IL-27 (Figure 5.18). However, analogous conversion assays showed that IL-17A, IL-17F, and IL-22 did not suppress the TGF-β-driven conversion of conventional T cells

into Treg cells (data not shown). Thus, except IL-21, no known cytokine was produced by IL-23-activated γδ T cells that could account for the suppression of Treg cell conversion.

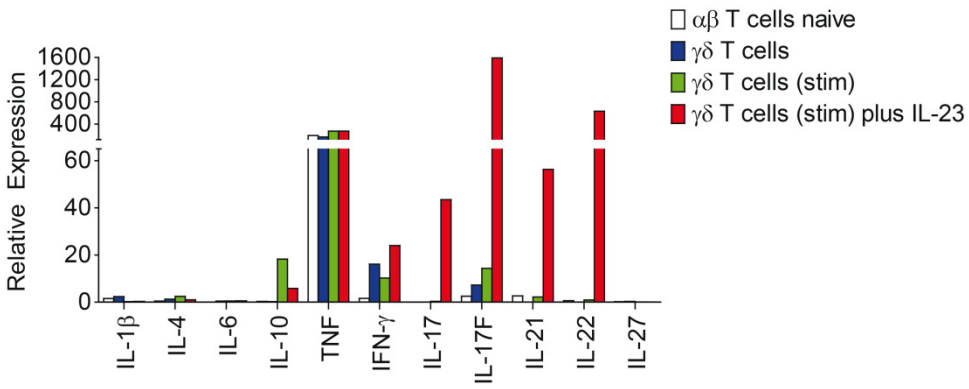


Figure 5.18: IL-23-activated γδ T cells produce IL-17A, IL-17F, IL-21, and IL-22
Quantitative Real-time PCR analysis was performed in γδ T cells that had been isolated from wild type mice by magnetic cell sorting and stimulated four days with anti-CD3/anti-CD28 beads in the presence or absence of IL-23. Naïve $CD4^+Foxp3^-CD44^-$ T cells, isolated from *Foxp3gfp*.KI mice by fluorescence-activated cell sorting, and un-stimulated γδ T cells served as controls. Representative data out of two independent experiments.

To analyze whether IL-21 could be the unknown factor and to exclude definitely that it was IL-6, which is the most prominent cytokine described to inhibit Treg cell differentiation, conversion assays with neutralizing antibodies against IL-6 and IL-21 were performed. As expected, IL-6 and IL-21 induced the production of IL-17 (Figure 5.19B). The addition of neutralizing antibodies against IL-6 and IL-21 abolished the IL-6- and IL-21-induced IL-17 production, respectively which proved the functionality of the neutralizing antibodies (Figure 5.19B). However, neither anti-IL-6 nor anti-IL-21 blocked the bioactivity of the supernatant derived from IL-23-stimulated γδ T cells to suppress the conversion of conventional T cells into $Foxp3^+$ Treg cells (Figure 5.19A). Thus, it could be concluded that the unknown factor comprised in this supernatant is neither IL-6 nor IL-21.

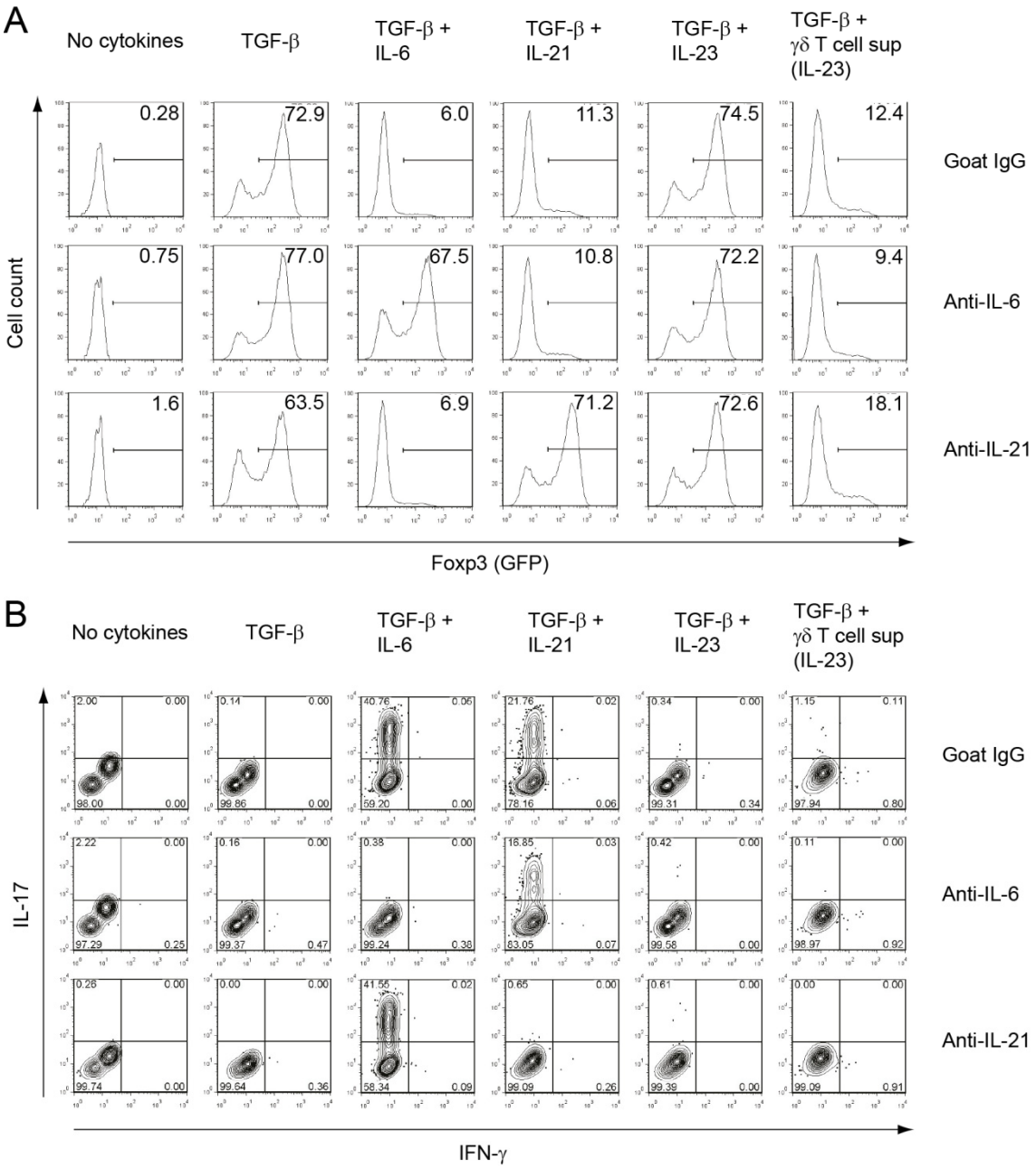


Figure 5.19: Suppression of the TGF-β-driven conversion of conventional T cells into Foxp3$^+$ Treg cells by cell-free supernatant from IL-23-activated γδ T cells is IL-6 and IL-21 independent
Naïve CD4$^+$Foxp3$^-$CD44$^-$ T cells were isolated from *Foxp3gfp*.KI mice by fluorescence-activated cell sorting and stimulated with plate-bound anti-CD3 and soluble anti-CD28 and the indicated cytokines, cytokine combinations, or γδ T cell culture supernatants in the presence of isotype (goat IgG) or blocking antibodies to IL-6 or IL-21. γδ T cell culture supernatants were collected from γδ T cells that were isolated from wild type mice by magnetic cell sorting and stimulated four days with anti-CD3/anti-CD28 beads in the presence of IL-23. (A) After three days, Foxp3(GFP) expression was analyzed by flow cytometry. Numbers indicate percentages. (B) After three days, cells were stimulated with PMA plus ionomycin prior to staining for intracellular IL-17 and IFN-γ. Numbers in the quadrants indicate percentages. All data are representative of at least two independent experiments.

Further hints for the identity of the unknown soluble mediator were expected from analysis of the transcriptome of the αβ T cells that served as responder cells during conversion assays. The

genes that were induced in those responder cells that were cultivated in the presence of TGF-β and IL-23-activated γδ T cell supernatant included T-bet, the IL-12 receptor subunit β2, and IL-22, while the gene for IL-17 was down-regulated (Figure 5.20). These data suggest that γδ T cells that were stimulated in the presence of IL-23 create a cytokine milieu that influences the intrinsic αβ T cell developmental program in a way that promotes a Th1-like phenotype but inhibits the generation of induced Treg cells.

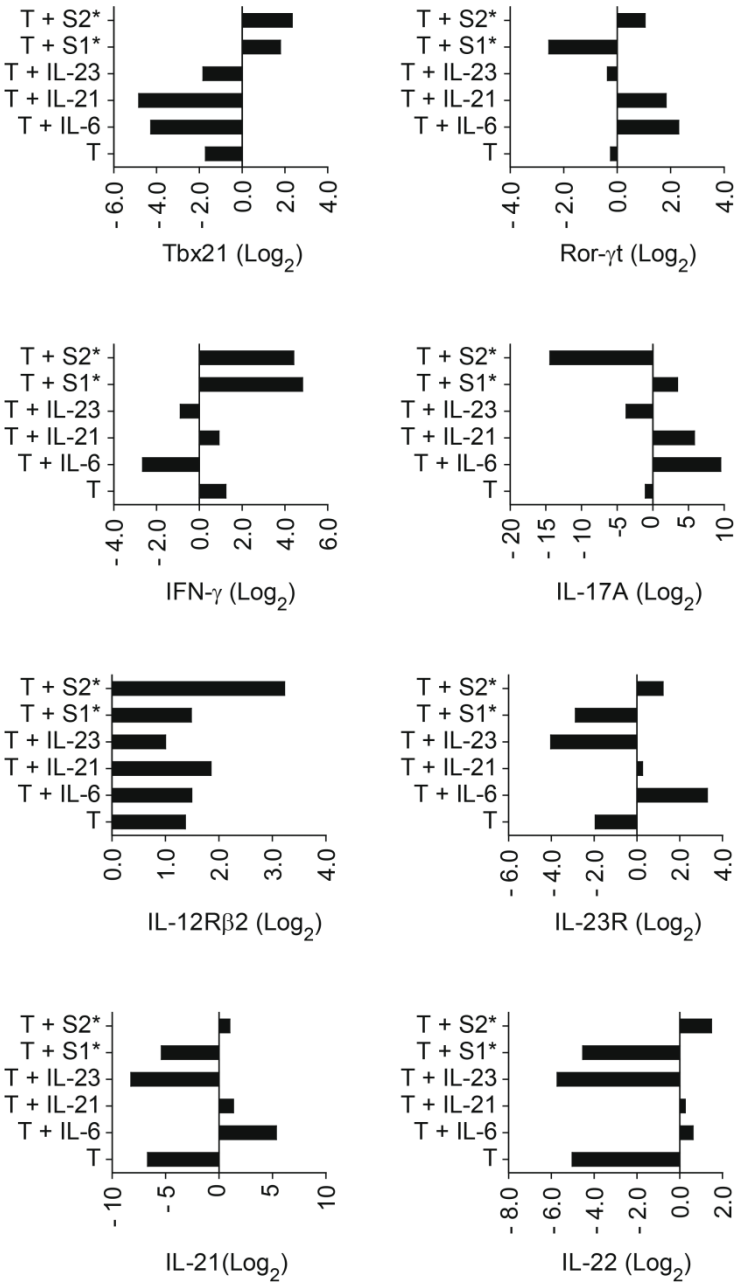


Figure 5.20: Expression profile of responder αβ T cells upon exposure with supernatant from IL-23-activated γδ T cells
Quantitative Real-time PCR analysis was performed in naïve $CD4^+Foxp3^-CD44^-$ T cells that had been isolated from *Foxp3gfp*.KI mice by fluorescence-activated cell sorting and stimulated for three days under the indicated cytokine conditions. (T=TGF-β, S1*=supernatant from γδ T cells, S2*=supernatant from IL-23-activated γδ T cells). Relative expression of the indicated genes in αβ responder T cells in relation to the expression level in αβ responder T cells stimulated under neutral conditions (no cytokine condition), which was arbitrarily set to 1.0. Representative data out of two independent experiments.

To investigate whether γδ T cells have an impact on Treg cells in vivo, two experiments were performed that examined how the absence or presence of γδ T cells correlate with Treg cell expansion or induction in vivo, respectively. In a first set of experiments, $CD3^+$ T cells were purified from lymph nodes and spleen from naïve wild type CD90.1 expressing mice and adoptively transferred into *Il23r*$^{-/-}$ mice (CD90.2). CD90 is a pan T cell surface marker that exists in two alleles in mice, which code for CD90.1 and CD90.2, and differ by one amino acid (Rege and Hagood, 2006). By using CD90.1 donor mice and CD90.2 recipient mice transferred cells could be distinguished from host cells during flow cytometry. Before transfer, donor $CD3^+$ cells were either left untreated or depleted of γδ T cells by magnetic cell sorting. One day after cell transfer, recipient mice were immunized with MOG_{35-55} emulsified in CFA. Two weeks after immunization, donor cells were re-isolated from draining lymph nodes and analyzed for the expression of γδ-TCR and $Foxp3^+$. As the recipient mice did not express IL-23R the priming of αβ T cells occurred either in the presence or absence of donor-derived IL-23-responsive γδ T cells. In the presence of IL-$23R^+$ γδ T cells, reduced fractions of $Foxp3^+$ Treg cells within the donor-derived $CD4^+$ T cell compartment were detected (Figure 5.21). This finding led to the conclusion that IL-$23R^+$ γδ T cells restrain the expansion of $Foxp3^+$ Treg cells during inflammatory responses in vivo.

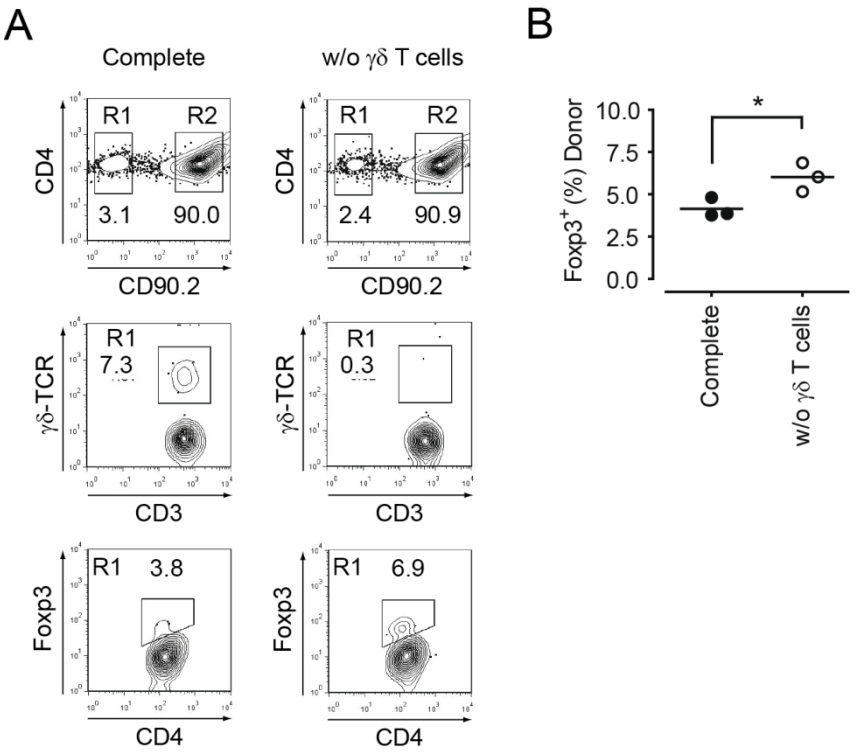


Figure 5.21: γδ T cells inhibit Treg cell expansion in vivo
$CD3^+$ T cells were isolated from congenic wild type mice (*Cd90.1*) and left untreated or depleted of γδ T cells by magnetic cell sorting. Subsequently, cells ($5x10^6$) were transferred intravenously into *Il23r*$^{-/-}$ recipients that were immunized with MOG_{35-55}/CFA one day later. Two weeks after immunization donor cells were re-isolated from draining lymph nodes and analyzed for the expression of γδ-TCR and intranuclear Foxp3 by flow cytometry (R1 = donor cells, R2 = host cells). (A) Numbers indicate percentages within the total $CD4^+$ gate (upper panel), within the $CD90.1^+CD3^+$ gate (middle panel), or within the $CD90.1^+CD4^+$ gate (lower panel). (B) Fractions of $Foxp3^+$ Treg cells within the $CD90.1^+CD4^+$ gate. Representative data out of two independent experiments.

In a second set of experiments it was investigated whether IL-$23R^+$ γδ T cells have the capability to reduce the conversion of naïve T cells into $Foxp3^+$ Treg cells in vivo. Here, $CD4^+Foxp3^-$ T cells were isolated from lymph nodes and spleen of 2D2x*Foxp3gfp*.KI mice and purified by fluorescence-activated cell sorting. The 2D2 mouse is a TCR transgenic mouse whose T cells are all specific for MOG_{35-55}. Additionally, γδ T cells were isolated from lymph nodes and spleen of wild type mice and purified by magnetic cell sorting. Isolated MOG_{35-55}-specific $CD4^+Foxp3^-$ cells were transferred with or without co-transfer of wild type γδ T cells into recipient mice that lack T and B cells (*Rag1*$^{-/-}$). One day after cell transfer recipient mice were immunized with MOG_{35-55}/CFA and additionally treated with a monoclonal antibody against IL-6 because CFA is known to induce massive amounts of IL-6. As IL-6 inhibits the conversion of conventional T cells into Treg cells, high levels of IL-6 would disguise any effects mediated by IL-$23R^+$ γδ T cells. On day 15 after immunization donor cells were re-isolated from draining lymph nodes, spleen, and CNS and the frequency of $Foxp3(GFP)^+$ Treg

cells within the CD4$^+$ T cell gate was analyzed by flow cytometry. Without co-transfer of γδ T cells sensitization with MOG$_{35-55}$/CFA resulted in a measurable conversion of Foxp3$^-$ cells into Foxp3$^+$ cells (Figure 5.22). However, when γδ T cells were co-transferred this conversion was abolished in all three analyzed compartments. These results suggested that γδ T cells restrain the conversion of conventional Foxp3$^-$ T cells into Foxp3$^+$ Treg cells during inflammation in vivo.

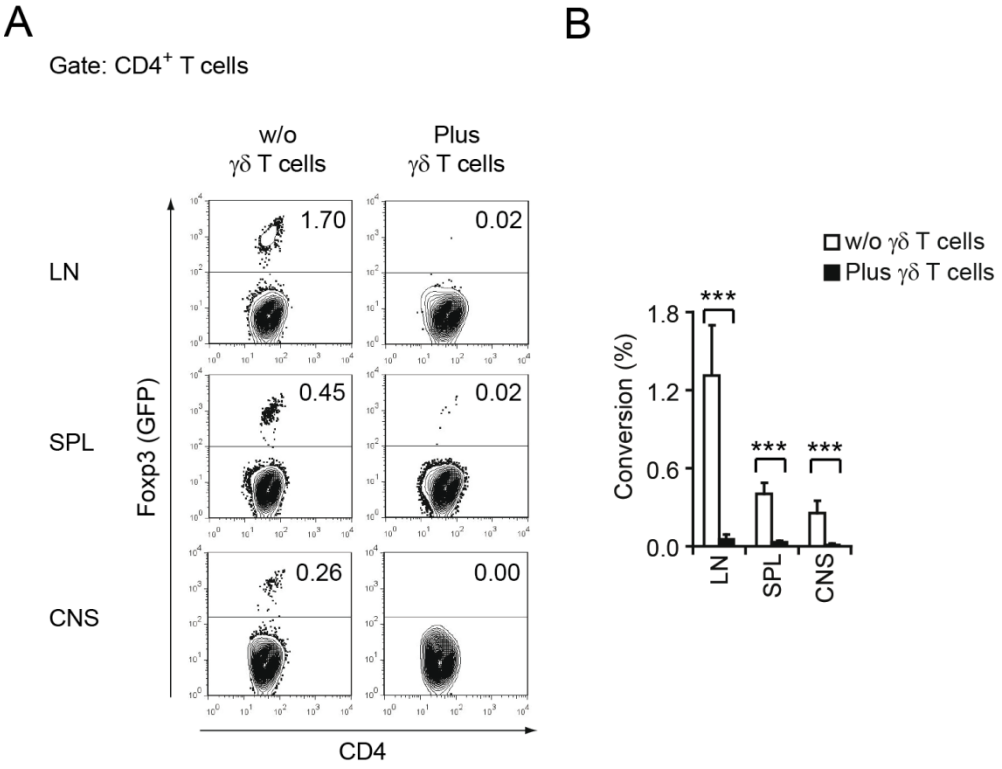


Figure 5.22: γδ T cells inhibit Treg cell induction in vivo
CD4$^+$Foxp3$^-$ T cells were isolated from 2D2x*Foxp3gfp*.KI mice by fluorescence-activated cell sorting followed by intravenous transfer into *Rag1*$^{-/-}$ recipients without or with co-transfer of γδ T cells that had been isolated from wild type mice by magnetic cell sorting. Recipient mice were immunized with MOG$_{35-55}$/CFA and treated with an antibody to IL-6 i.p. 15 days after immunization donor cells were re-isolated from lymph nodes, spleen, and CNS and analyzed for the expression of Foxp3(GFP) by flow cytometry. (A) Numbers indicate percentages within the CD4$^+$ gate. (B) Fractions of 2D2 T cells converted into Foxp3$^+$ Treg cells within the CD4$^+$ gate; mean ± SD. Representative data out of two independent experiments.

Previous experiments have shown that γδ T cells restrain the induction and conversion of Treg cells in vivo during the course of inflammation. To further investigate whether γδ T cells also repress Treg cell functions, additional in vitro assays were performed. An established assay for the analysis of Treg cell function is the suppression assay. In this in vitro assay, polyclonally stimulated CD4$^+$ effector T cells are co-cultured with Treg cells. In the absence of Treg cells,

stimulation of effector T cells via CD3 and CD28 results in a massive proliferation that can be measured by the incorporation of radioactive thymidine into newly synthesized DNA or by dye dilution assays. However, when Treg cells are added to the culture, the proliferation of effector T cells is suppressed. In a first set of suppression assays, polyclonally stimulated effector T cells were co-cultured with Treg cells and γδ T cells in the presence or absence of IL-23. All cell populations were isolated from lymph nodes and spleen of *Foxp3gfp*.KI mice. Effector T cells ($CD4^+Foxp3^-$) and Treg cells ($CD4^+Foxp3^+$) were purified by fluorescence-activated cell sorting, γδ T cells by magnetic cell sorting. After 56 hours of culture, tritium-labeled thymidine was added. 16 hours later, the proliferation was measured by means of tritium incorporation. Polyclonally stimulated effector T cells showed massive proliferation when cultivated without Treg cells and γδ T cells irrespective of whether or not IL-23 was present in the culture (Figure 5.23A). As expected, the addition of Treg cells resulted in a suppression of proliferation. When γδ T cells and IL-23 were added to the culture the Treg cell-mediated suppression of effector T cell proliferation was diminished. As this was not observed in the absence of IL-23, it was concluded that IL-23-activated γδ T cells released substances that were responsible for the enhanced proliferation of responder cells even in the presence of Treg cells. In a second set of experiments, effector T cells were stimulated polyclonally in the presence of Treg cells and γδ T cell supernatants that were generated with or without IL-23. An enhanced proliferation of effector T cells was measured only upon addition of IL-23-activated γδ T cell supernatant to the culture, whereas IL-23 alone had no suppressive effect on the proliferation (Figure 5.23B).

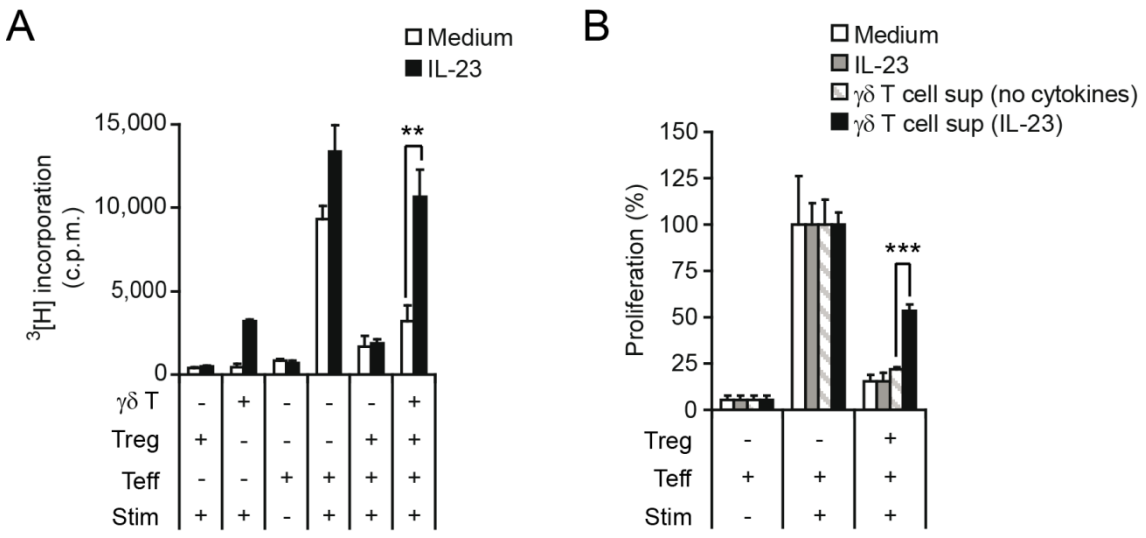


Figure 5.23: Products from IL-23-activated γδ T cells reverse Treg cell-mediated suppression of αβ T cells
(A) $CD4^+Foxp3^-$ T cells were isolated from *Foxp3gfp*.KI mice by fluorescence-activated cell sorting and stimulated with an antibody to CD3 and syngeneic irradiated and γδ T cell-depleted splenocytes as APCs in the presence of wild type Treg cells and wild type γδ T cells (Ratio T responder cells:Treg cells:γδ T cells: 1:1). IL-23 was added at a concentration of 25 ng/ml, as indicated. Cell proliferation was measured by incorporation of ^{3}H-thymidine into DNA. (B) In an autologous assay, $CD4^+Foxp3^-$ T cells were co-cultured with Treg cells in the presence of an antibody to CD3 and APCs. IL-23, γδ T cell supernatants, or IL-23-activated γδ T cell supernatants were added, as indicated. Cell proliferation was measured by incorporation of ^{3}H-thymidine into DNA and normalized to the unsuppressed condition (absence of Treg cells) within each condition. Data are representative of at least two independent experiments.

Treg cells did not to express the IL-23R (Figure 5.24A). Thus, it is unlikely that the observed change in thymidine incorporation is due to a direct effect of IL-23 on Treg cells, e.g. an altered proliferation behavior of Treg cells in the absence or presence of IL-23. However, in order to accurately evaluate the contribution to the proliferative response of individual cell populations within mixed cultures, dye dilution experiments were performed. Effector T cells were stimulated polyclonally in the presence of Treg cells and γδ T cell supernatants. Polyclonally stimulated effector T cells showed a strong proliferation which was not diminished by the γδ T cell supernatants (Figure 5.24). Addition of Treg cells suppressed the proliferation of effector T cells. However, Treg cells failed to suppress the proliferative response of effector T cells in the presence of IL-23-stimulated γδ T cell supernatant. Yet, IL-23-stimulated γδ T cell supernatant did not induce Treg cell proliferation.
These results suggest that IL-23-activated γδ T cells create a cytokine milieu that not only suppresses induction and expansion of Treg cells, but also their functionality.

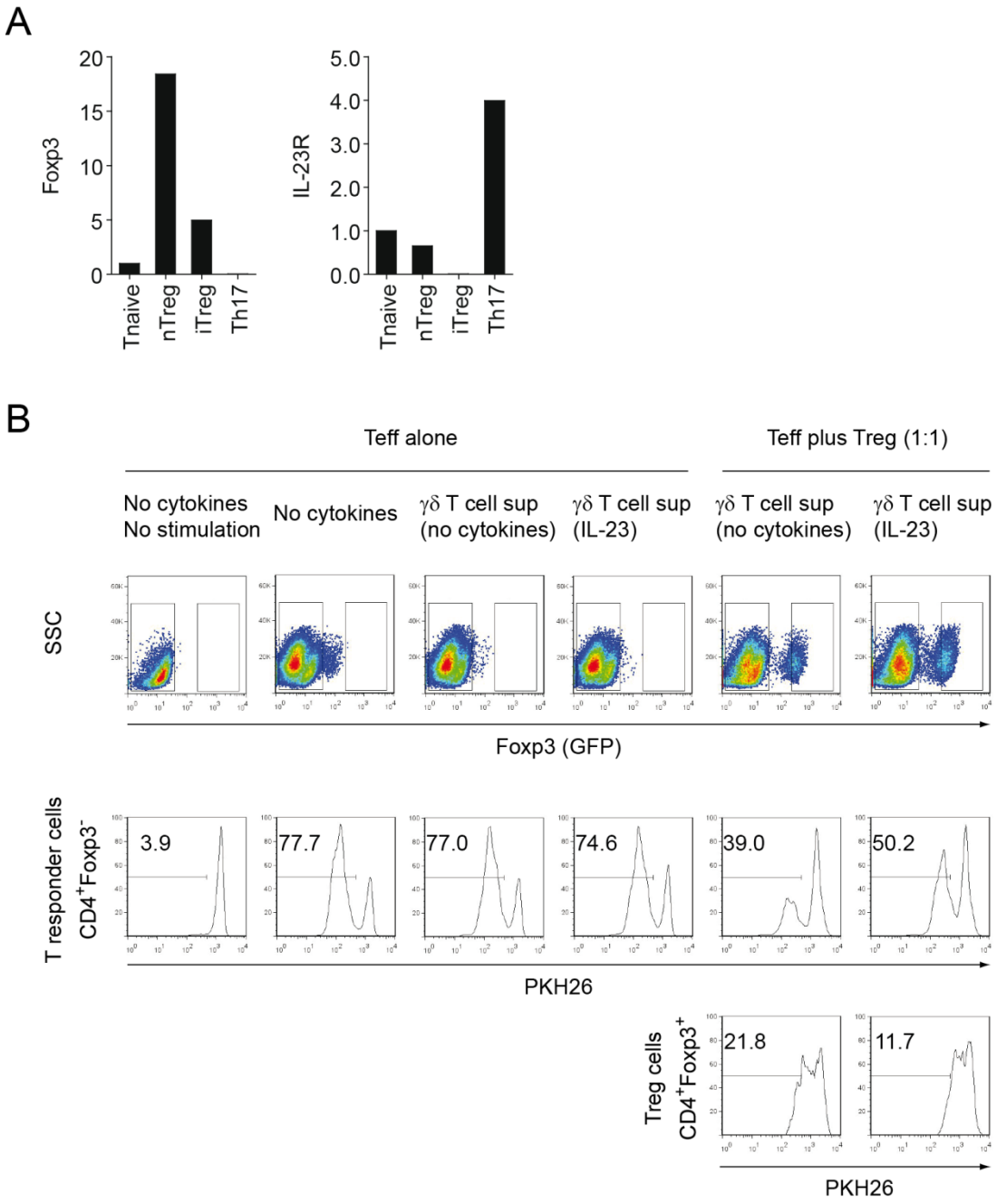


Figure 5.24: Reduced Treg cell-mediated suppression of αβ T cells caused by supernatants of IL-23-activated γδ T cells is not due to an enhanced Treg cell proliferation
(A) Naïve $CD4^+Foxp3^-CD44^-$ T cells or $CD4^+Foxp3^-$ Treg cells (nTreg cells) were isolated from *Foxp3gfp*.KI mice by fluorescence-activated cell sorting. Naïve T cells were differentiated into iTreg cells or Th17 cells in the presence of TGF-β (5 ng/ml) or TGF-β (2 ng/ml) plus IL-6 (50 ng/ml), respectively. Total RNA was isolated from indicated cell populations and tested for relative abundance of *Foxp3* mRNA and *Il23r* mRNA. (B) $CD4^+Foxp3^-$ T cells were isolated from Foxp3gfp.KI mice by fluorescence-activated cell sorting and labeled with the red dye PKH26. $CD4^+Foxp3^-$ T cells were co-cultured with Treg cells in the presence of anti-CD3/anti-CD28 beads and γδ T cell supernatants or IL-23-activated γδ T cell supernatants, as indicated. After three days, the percentages of $PKH26^{dim}$ cells were determined separately within the $Foxp3(GFP)^-$ and $Foxp3(GFP)^+$ T cell gate. Representative histograms out of three independent experiments are shown.

γδ T cells restrained induction, expansion, and function of Treg cells. These observations raised the question how the Treg compartment is constituted in mice that lack γδ T cells. Lymph nodes and spleen of unmanipulated γδ T cell deficient mice (*Tcrd*$^{-/-}$) were isolated and analyzed for Foxp3^{+} Treg cell fractions within the CD4^{+} T cell compartment by flow cytometry. Compared to age-matched wild type mice, *Tcrd*$^{-/-}$ mice bred in the same facility harbored higher fractions of Foxp3^{+} Treg cells both in lymph nodes and spleen (Figure 5.25).

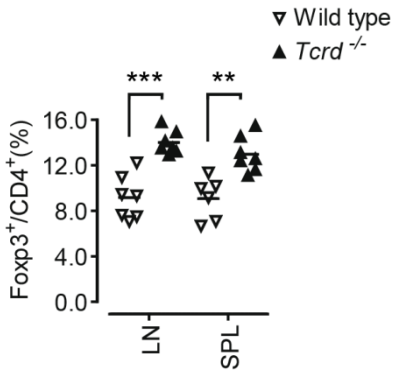


Figure 5.25: γδ T cell deficient mice harbor higher numbers of Foxp3^{+} Treg cells
Single cell suspensions were prepared from lymph nodes (LN) and spleen (SPL) of unmanipulated *Tcrd*$^{-/-}$ and wild type mice and stained for intranuclear Foxp3. The percentage of Foxp3^{+} Treg cells within the CD4^{+} T cell compartment was determined by flow cytometry. Data are representative of at least two independent experiments.

To analyze the population dynamics of effector T cells and Foxp3^{+} Treg cells during the priming phase of EAE, *Tcrd*$^{-/-}$ mice and aged-matched controls were immunized with MOG_{35-55}/CFA. At day 6 after immunization lymph node and spleen cells were isolated and analyzed for the percentages of activated (CD103^{+}) Foxp3^{+} Treg cells and effector cytokine producing Foxp3^{-} T cells within the CD4^{+} T cell compartment by flow cytometry. During the priming phase of EAE, *Tcrd*$^{-/-}$ mice had significantly increased fractions of activated Treg cells both in lymph nodes and spleen, but reduced fractions of IL-17 producing T cells and IFN-γ producing T cells in the spleen, respectively (Figure 5.26). These findings pointed to enhanced antigen-driven Treg cell responses in an in vivo setting where γδ T cells were lacking.

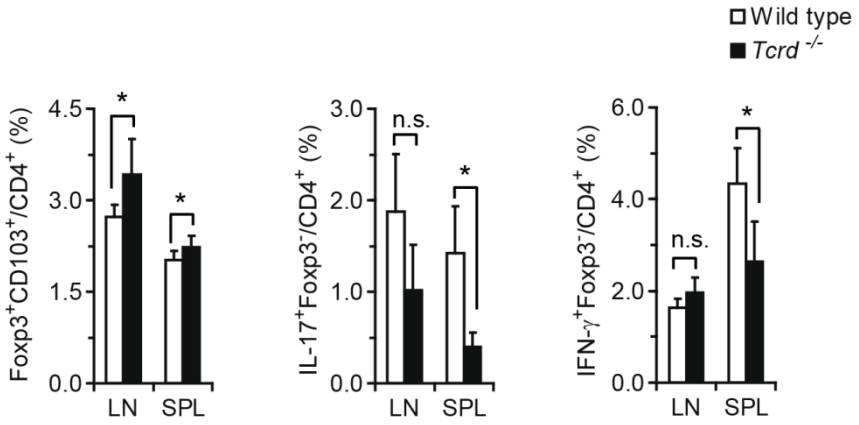
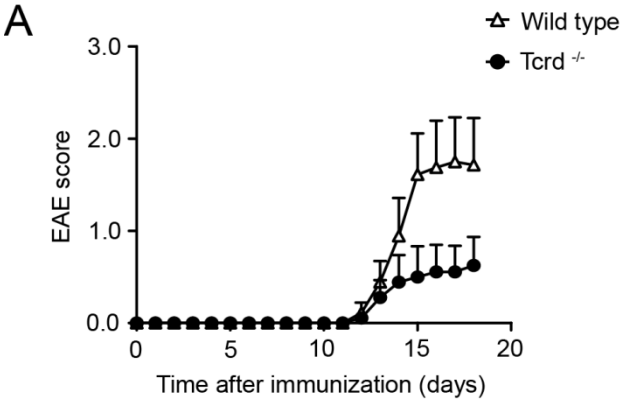


Figure 5.26: Antigen-driven Treg cell responses are enhanced in the absence of γδ T cells
Tcrd$^{-/-}$ mice and wild type mice were immunized with MOG_{35-55}/CFA. 6 days after sensitization, lymph node (LN) cells and splenocytes (SPL) were isolated and stained for intranuclear Foxp3 or intracellular cytokines. The percentages of Foxp3$^+$CD103$^+$ Treg cells, IL-17$^+$Foxp3$^-$, and IFN-γ$^+$Foxp3$^-$ CD4$^+$ effector T cells were determined by flow cytometry; mean ± SD. Representative data out of two independent experiments.

Consistent with these results, *Tcrd*$^{-/-}$ mice experienced a milder EAE compared to wild type mice suggesting that the inflammation is better controlled in *Tcrd*$^{-/-}$ mice by a strengthened Treg cell compartment (Figure 5.27A). In order to analyze whether effector responses were intrinsically fully functional in *Tcrd*$^{-/-}$, Treg cells were depleted in *Tcrd*$^{-/-}$ mice using an anti-CD25 antibody prior to EAE induction. Compared to *Tcrd*$^{-/-}$ mice that were treated with a control antibody of the same isotype (rat IgG), CD25-depleted mice regain full susceptibility to EAE (Figure 5.27B and Table 5.1)



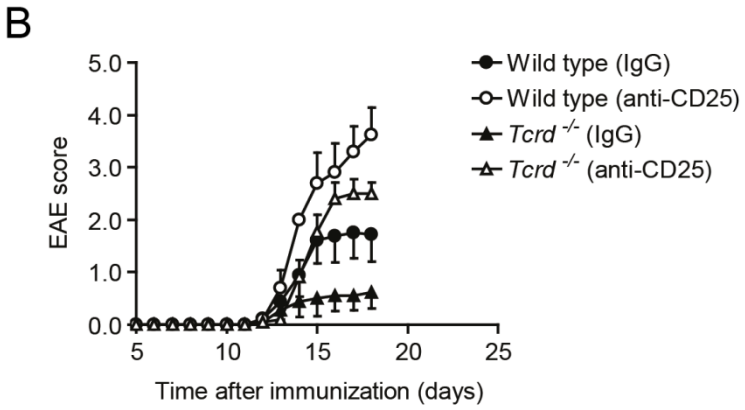


Figure 5.27: Treg cell depletion in *Tcrd*$^{-/-}$ mice restores full susceptibility to EAE
(A) *Tcrd*$^{-/-}$ and wild type mice were immunized with MOG_{35-55}/CFA. (B) *Tcrd*$^{-/-}$ and wild type mice were treated with a Treg depleting antibody to CD25 five days and three days before immunization with MOG_{35-55}/CFA; means of clinical score ± SEM. Representative data out of two independent experiments.

At the peak of disease, cells were recovered from the CNS parenchyma and analyzed for Foxp3 expression and effector cytokine production. CD25-depleted *Tcrd*$^{-/-}$ mice had higher percentages of Foxp3^{+} Treg cells in the CNS compared to CD25-depleted wild type mice which indicated that Treg cells showed faster recovery dynamics in γδ T cell free environments (Figure 5.28). Accordingly, Treg cell-depleted wild type mice developed a fulminant disease with marked mortality. Treg depletion in *Tcrd*$^{-/-}$ mice fully restored fractions and absolute numbers of IFN-γ producers and IL-17 producers in the CNS compared to IgG-treated wild type controls. The percentages of IL-17 producers and IFN-γ producers within the CD4^{+}Foxp3^{-} compartment were not different between Treg cell-depleted wild type and IgG-treated *Tcrd*$^{-/-}$ mice. However, the latter had significantly reduced absolute numbers of cytokine producers in the CNS. Taken together, these results demonstrate that the priming of effector T cells is not impaired in a cell-intrinsic manner in *Tcrd*$^{-/-}$ mice but that the absence of γδ T cells enhances Treg cell responses which results in efficiently suppressed antigen-specific effector T cell responses and thus, in a relative protection from EAE.

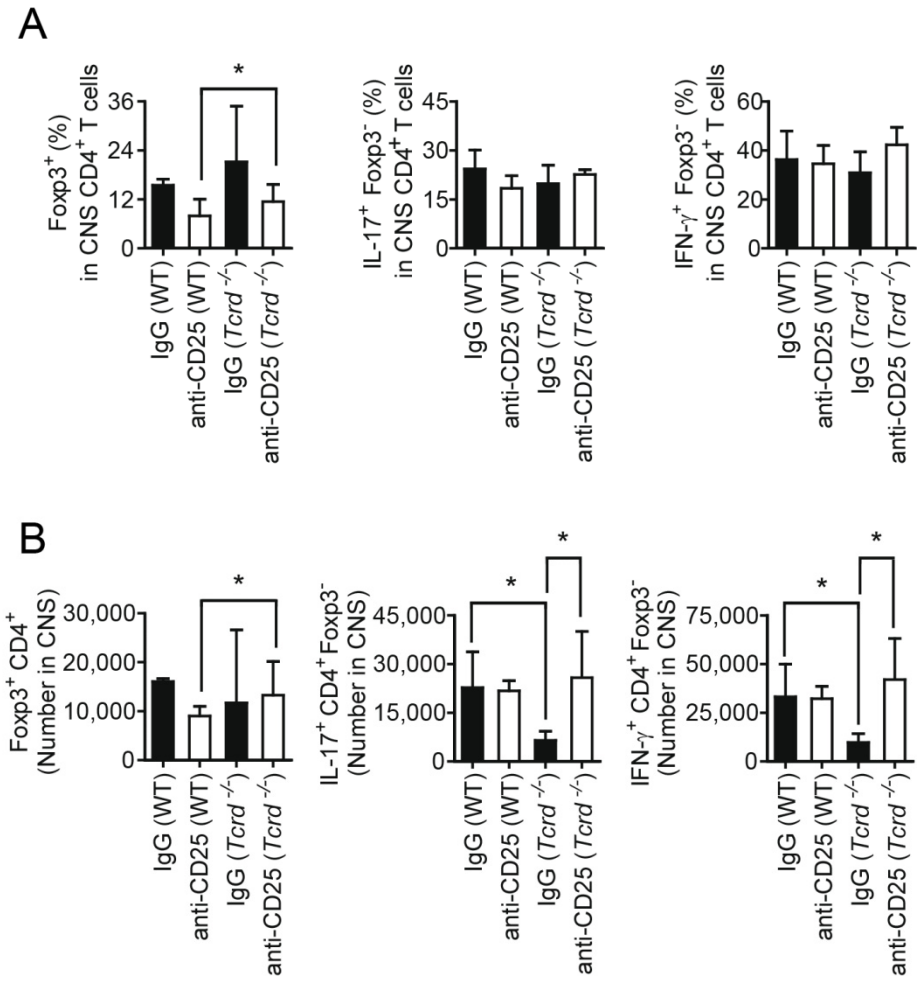


Figure 5.28: Treg cell depletion restores absolute numbers of cytokine producers in the CNS in *Tcrd*$^{-/-}$ mice
Tcrd$^{-/-}$ and wild type mice were treated with a Treg depleting antibody to CD25 five days and three days before immunization with MOG_{35-55}/CFA. At the peak of disease (d18), mononuclear cells from the CNS were isolated and analyzed for the expression of intranuclear Foxp3 and intracellular cytokines within the $CD4^+$ T cell compartment by flow cytometry. Fractions of $Foxp3^+$ cells and $cytokine^+$ cells within the CNS in the $CD4^+$ T cell gate (A) and absolute numbers (B) of $Foxp3^+$ cells, IL-17^+Foxp3^-, and IFN-γ^+Foxp3^- $CD4^+$ effector T cells are depicted; mean ± SD. Representative data out of two independent experiments.

Table 5.1: EAE in *Tcrd*$^{-/-}$ mice

	Wild type (IgG)	Wild type (anti-CD25)	*Tcrd*$^{-/-}$ (IgG)	*Tcrd*$^{-/-}$ (anti-CD25)
Incidence	7/9 = 78 %	5/5 = 100 %	3/9 = 33 %	11/11 = 100 %
Mortality	0/9 = 0 %	1/5 = 20 %	0/9 = 0 %	0/11 = 0 %
Day of onset	14.3 ± 0.70	13.8 ± 0.20	14.0 ± 1.0	14.9 ± 0.4
Maximum EAE score	2.2 ± 0.46	3.6 ± 0.40	0.7 ± 0.34[a]	2.7 ± 0.22[b]

Table 5.1 shows cumulative results of two independent experiments. [a] Compared to wild type (IgG) mice: $p < 0.03$; calculated by Mann-Whitney U rank sum test. [b] Compared to *Tcrd*$^{-/-}$ (IgG) mice: $p < 0.0009$; calculated by Mann-Whitney U rank sum test.

6 Discussion

6.1 Identification of a pro-inflammatory subset of γδ T cells

IL-17 is a pro-inflammatory cytokine that plays a key role in host defense against various pathogens particularly by inducing mobilization of neutrophils (Ye et al., 2001). Furthermore, IL-17 is also implicated in the establishment and maintenance of autoimmunity and chronic inflammation. As major source of IL-17, a distinct subset of effector T helper cells, so-called Th17 cells, was identified as an independent T helper cell lineage besides Th1 and Th2 cells. Most studies on IL-17 focused on $CD4^+$ αβ T cells even though γδ T cells have been described to produce high amounts of IL-17, and in some conditions, are even the dominant source of IL-17. However, the knowledge on the role of γδ T cells in infections and conditions of chronic inflammation and autoimmunity has been limited.

Hence, this thesis aimed at characterizing the requirements of IL-17 production in γδ T cells and at investigating the potential role of γδT17 cells in autoimmune inflammation.

The full differentiation of Th17 cells requires the combined actions of TGF-β, IL-6, and IL-23 with IL-23 being a maturation factor rather than a differentiation factor. IL-23 has no effect on naïve T cells as they do not express IL-23R. Only when a T cell senses TGF-β and IL-6, IL-23R becomes up-regulated.

Interestingly, using an IL-23R(GFP) reporter mouse strain it has been shown here that a subset of naïve γδ T cells expressed the IL-23R constitutively. Upon stimulation with IL-23, γδ T cells produced high amounts of IL-17, IL-21, and IL-22, while stimulation with the structurally similar cytokine IL-12 resulted in the production of IFN-γ. A more detailed analysis of the cytokine producing cells revealed that the γδ T cell compartment in secondary lymphoid tissues consisted of functionally distinct subsets of γδ T cells that were discriminated by the expression of IL-23R. While all IL-23R expressing γδ T cells produce IL-17, IFN-γ is exclusively produced by IL-23R negative γδ T cells. Bone marrow chimera experiments suggested that IL-17 producing and IFN-γ producing γδ T cells develop separately in the thymus and leave the thymus as pre-committed T cells, ready to immediately deploy effector functions in peripheral tissues upon adequate stimulation. While γδT1 cells are continuously reproduced in the adult thymus, the population of $IL\text{-}23R^+$ γδT17 cells is exclusively generated in the fetal thymus and maintained in the periphery throughout the life span of an individual by

self-renewal. Furthermore, this population of IL-23R$^+$ γδ T cells was found to promote autoimmune inflammation by suppressing early Treg cell responses. γδ T cells activated by IL-23 selectively accumulated in the CNS during EAE and restrained both the generation and function of Foxp3$^+$ Treg cells. Thus, this thesis identified an IL-23-dependent mechanism by which γδ T cells shift the balance between suppressive Treg cell responses and conventional effector T helper cell responses in favor of effector responses during inflammation.

6.2 γδ T cells bridge innate and adaptive immune responses

γδ T cells use a rearranged CD3-associated γδ T cell receptor and thus, belong, strictly speaking, to the adaptive immune system. However, their phenotype and functions differ in several aspects from those of αβ T cells which let them rather resemble innate immune cells. First, γδ T cells populate distinct anatomical sites, mostly at the interface of the organism with its environment, such as, for example, epithelial surfaces. Second, γδ T cells recognize qualitatively distinct TCR-antigens and can even be activated in a TCR-independent manner, and third, immune responses exerted by γδ T cells are characterized by much faster kinetics.

By preventing the entry of exogenous pathogens epithelial surfaces perform important functions in host defense. Importantly, epithelia do not simply serve as physical, but also as immunological barriers. While representing only a small fraction of T cells in the lymphoid organs and blood, γδ T cells are found in high abundance on mucosal and epithelial surfaces. γδ T cells have been found to respond very early to states of infection by the secretion of cytokines and by cytotoxic activity (Ferrick et al., 1995). Consistently, γδ T cells are equipped with pattern recognition receptors (Martin et al., 2009), which enable them to sense common microbial molecules without the need of time consuming MHC-associated antigen presentation and TCR ligation.

The finding that a subset of γδ T cells constitutively expressed the IL-23R and produced IL-17 upon IL-23 stimulation fits well in the concept of γδ T cells as part of a “first line of defense” machinery against invading pathogens. Innate immune cells, such as macrophages and dendritic cells, respond to infections by the production of IL-23. IL-23 then stimulates resident IL-23R expressing γδ T cells to secrete large amounts of IL-17 irrespective of further γδ-TCR ligation. Remarkably, upon IL-23 stimulation γδ T cells, isolated from naïve mice, produced more than 10 times higher amounts of IL-17 than CD4$^+$ αβ T cells. This is partly due to the fact

that the bulk $CD4^+$ T cell population included naïve αβ T cells that do not express IL-23R but need to be activated through their TCR and have to sense TGF-β and IL-6 to up-regulate IL-23R (Parham et al., 2002). IL-23R expression on their surface enables them to sense IL-23 and respond to it by IL-17 secretion. In contrast, memory Th17 cells that have been generated during previous infections rapidly respond to IL-23 (Haines et al., 2013) and concomitant TCR ligation. Thus, γδT17 cells seem to be pre-activated effector cells that show similar response kinetics as activated or memory Th17 cells but are even less dependent on TCR ligation. When directly comparing $CD44^+$ conventional T cells with γδ T cells, IL-23 induced higher amounts of IL-17 in γδ T cells. Again similar to memory Th17 cells, γδ T cells produced IL-17 independently of IL-6. Nevertheless, the fractions of $CCR6^+$ and $IL\text{-}23R^+$ γδ T cells were significantly reduced in *Il6*$^{-/-}$ mice, suggesting a role of IL-6 in the maintenance of this population but not in their functionality. In line with this observation, Lochner et al. demonstrated that IL-6 is not necessary to induce IL-17 in Rorγt expressing γδ T cells, although it was not tested in this study whether IL-6 has a role in the maintenance of Rorγt expressing γδ T cells (Lochner et al., 2008).

Besides IL-17, γδ T cells also produced the Th17-associated cytokines IL-21 and IL-22 upon IL-23 stimulation which further supported the idea of γδ T cells being innate-like correlates to Th17 cells.

In addition to IL-23, γδ T cells have been shown to produce IL-17 in response to IL-1β which has also been described to be implicated in the development of Th17 cells (Chung et al., 2009). IL-1β alone induces similar amounts of IL-17 in γδ T cells as IL-23, while the combination of IL-23 and IL-1β cooperatively even further increases the amount of IL-17 that is produced by γδ T cells (Sutton et al., 2009, Raifer et al., 2012). This redundancy in cytokine activity suggests that IL-17 production by γδ T cell is highly important for host immunity.

While γδ T cells produced IL-17 in remarkable amounts, $IL\text{-}23R^-$ γδ T cells did not produce IL-17 but secreted large amounts of IFN-γ upon γδ-TCR ligation. This IFN-γ production was promoted by IL-12 which was also able to induce IFN-γ in $IL\text{-}23R^-$ γδ T cells in the absence of TCR stimulation (data not shown). Again the quantity of IFN-γ released by γδ T cells was much higher as compared to both bulk $CD4^+$ αβ T cells and memory αβ T cells. Because of their structural similarities, IL-12 and IL-23 belong to the same cytokine family. Both cytokines are produced by dendritic cells and phagocytes in response to pathogens during infection and both play a role in linking the innate immune response to adaptive responses. But

while IL-23 is implicated in the development of Th17 cells, IL-12 promotes the generation of IFN-γ producing Th1 cells. This thesis shows that a similar classification in IL-23-responsive IL-17 producing cells and IL-12-responsive IFN-γ producing cells also exists in the γδ T cell compartment. These data are in accordance with results published by Shibata et al. identifying IL-17 and IFN-γ producing γδ T cells in the peritoneal cavity as distinct functional subsets during Escherichia coli infection (Shibata et al., 2007).

As a whole, these findings lead to the conclusion that γδ T cells are pre-activated effector cells that immediately produce high amounts of pro-inflammatory cytokines in the first hours of infection when adaptive immune responses are not yet available. Interestingly, analogous to the classification of T helper cells, there are distinct subsets of γδ T cells that differ from each other by the cytokines they produce. However, in contrast to αβ T helper cells, the γδ T cell "lineage" commitment seems to proceed already in the thymus. Therefore γδ T cells leave the thymus as committed T cells and rapidly start producing cytokines upon infection. In this process, the cytokine milieu in the periphery does not further differentiate γδ T cells into distinct cytokine profiles but rather determines which "pre-committed" subset of γδ T cells is activated to secrete its signature cytokines.

6.3 γδ T cells are functionally programmed in the thymus

γδ T cells leave the thymus as cells that resemble antigen-experienced conventional T cells able to exert immediate effector functions in the periphery. The phenomenon of exportation of cells with an activated and/or memory T cell-like phenotype from the thymus has also been described for other innate-like cells that develop in the thymus from $CD4^+CD8^+$ progenitors including NKT cells and non-conventional $CD8^+$ T cells (Kronenberg and Gapin, 2002, Berg, 2007).

Similarly to memory T cells, γδ T cells preserve their phenotype once they have been functionally programmed. However, in contrast to αβ T cells the functional programming of γδ T cells occurs in the thymus (see Figure 6.1). By performing global gene expression analysis, Narayan et al. recently identified several molecularly distinct immature and mature γδ T cell subtypes in the thymus (Narayan et al., 2012). These subsets include canonical (niche-restricted) and non-canonical γδ T cells and are distinguished by a unique expression pattern of TCR γ and δ chains, cytokine receptors, chemokine receptors, and transcription factors. In this

study the adult intrathymic γδ T cell population is categorized into two distinct subsets based on TCR repertoire and gene expression profiles. In good accordance with the data presented here, one γδ T cell subset expresses Vγ4, IL-23R, and CCR6 and produces IL-17 upon activation in the peripheral immune compartment, while the second subset is characterized by the expression of Vγ1 and CXCR3 and the production of IFN-γ.

There are several studies dealing with the question how functional differentiation of γδ T cells in the thymus is controlled. Jensen et al. postulated that γδ T cells that had encountered their cognate antigen in the thymus develop towards an IFN-γ producing fate while ligand-naïve γδ T cells produce IL-17 (Jensen et al., 2008). However, if γδT17 cells develop in the absence of TCR ligation, the need of a strongly restricted TCR-γ repertoire is difficult to understand. Furthermore, in another study CD27 has been identified not only as a marker of γδT1 cells, which is consistent with the data presented in this thesis, but also as a positive regulator of the differentiation of these cells (Ribot et al., 2009).

Another pathway reported to be implicated in the development of γδT17 cell is the Notch/Hes1 pathway. In the absence of Hes1, IL-17$^+$ γδ T cell numbers are decreased while other γδ T cell subsets develop normally (Shibata et al., 2011). Similarly to T helper subsets, the commitment of distinct subsets of γδ T cells in the thymus relies on the action of distinct transcription factors. Rorγt and Sox13 drive the development of γδT17 cells (Gray et al., 2013, Malhotra et al., 2013) while NF-κB, NFAT, Egr2, and T-bet have been shown to be implicated in the development of γδT1 cells (Turchinovich and Hayday, 2011). Turchinovich and Hayday have shown that Skint-1, a surface molecule specifically expressed on thymic epithelial cells and keratinocytes, promotes the development of γδT1 cells by activating NF-κB, NFAT, and Egr2 and by simultaneously suppressing Rorγt and Sox13 (Turchinovich and Hayday, 2011). Furthermore, the B lymphoid kinase (Blk) (Laird et al., 2010) and the NF-κB family members RelA and RelB (Powolny-Budnicka et al., 2011) have been demonstrated to play a role in the development of γδT17 cells.

Because of the functional similarities between γδT17 cells and Th17 cells, the instructive signals required for Th17 development have been analyzed for their relevance during γδ T cell development. STAT3, which is an essential part of the IL-6 signaling pathway and thus, crucially involved in Th17 development, has been found to be dispensable for the development of γδT17 cells (Shibata et al., 2011). These data seem to be contradictory to results obtained in this thesis showing reduced numbers of IL-23R$^+$ γδT17 cells in *Il6*$^{-/-}$ mice. However, one can

speculate that IL-6 does not interfere with γδ T cell development but does stabilize the γδT17 cell population in the periphery. In contrast, Do et al. have demonstrated that TGF-β, which is besides IL-6 required for Th17 development, also plays a key role in the generation of γδT17 cells (Do et al., 2010). Accordingly, γδ T cells deficient for TGF-β or SMAD3, which is involved in TGF-β signaling, express lower levels of IL-17. Taken together, cytokines essential for Th17 commitment also influence γδT17 cells. TGF-β seems to play a role during development, IL-6 in the maintenance of the γδT17 population in the periphery, and IL-23, as shown in this thesis, as an activator of effector functions of γδT17 cells.

IL-23R$^+$ γδ T cells could not be detected in secondary lymphoid organs and peripheral tissues after bone marrow cell or fetal liver cell transplantation into adult recipients while IL-23R$^-$ γδ T cells were readily generated and populated the peripheral lymphoid tissues. Therefore, it has to be concluded that the embryonic thymus offers favorable conditions for the development of IL-23R$^+$ γδ T cells but that the intrathymic conditions are then modified after birth in a way that prevents the generation and exportation of IL-23R$^+$ γδ T cells from the thymus in adulthood. Furthermore, these data suggest that those IL-23R$^+$ γδ T cells found in the periphery are long-lived cells with the ability of self-renewal. This hypothesis is supported by Haas et al. who demonstrated a steady-state proliferation of CCR6$^+$CD27$^-$ γδ T cells in the periphery (Haas et al., 2012). However, the mechanisms that account for the defective development of IL-23R$^+$ γδ T cells in the adult thymus are currently poorly understood. Narayan et al. have shown that Vγ4$^+$ γδ T cells up-regulate IL-23R and IL-1R during maturation in the thymus (Narayan et al., 2012). And IL-1R$^+$ γδ T cells have been found to expand during microbial colonization (Duan et al., 2010). Together these studies propose that maturing γδ T cells might become responsive to cytokines that are generated during microbial colonization shortly after birth. However, transfer of bone marrow cells isolated from *Il23r*$^{-/-}$ or *Il1r*$^{-/-}$ did not restore the γδT17 cell population in the peripheral immune compartment.

This thesis is consistent with the report of Haas et al., who have shown that CCR6$^+$CD27$^-$ γδ T cells develop exclusively in the embryonic thymus (Haas et al., 2012). They propose that IL-17 producing αβ T cells and innate-like thymocytes negatively regulate the development of γδT17 cells in the adult thymus, as the mixed transfer of *Tcra*$^{-/-}$ and *Il17a/f* $^{-/-}$ bone marrow into *Il17a/f* $^{-/-}$ recipients partly restore the numbers of γδT17 cells in the periphery. However, the mechanism how IL-17 disrupts the development of γδT17 cells was not analyzed on the molecular level. In another study, it was proposed that the transfer of neonatal thymocytes in

an adult recipient could restore γδT17 cells in the periphery suggesting that adult progenitor cells lose the capability to develop into γδT17 cells (Gray et al., 2011). However, it is likely that the transferred thymocyte suspension had already contained mature γδT17 cells that then populated their niches in the secondary lymphoid organs and peripheral tissues to assemble a detectable population by homeostatic proliferation. In fact, in this thesis, it was shown that transplantation of embryonic hematopoietic precursor cells (fetal liver cells) were unable to develop into γδT17 cells in the recipient adult thymus.

In summary, IL-23R$^+$ γδT17 cells develop in the embryonic and maybe in the neonatal thymus. Then the intrathymic conditions change and the development of γδT17 cells ceases. The physiological relevance of this phenomenon is unclear. One can speculate that it is either just not necessary to produce more of these cells as they are stable and long-lived or it might even be dangerous to continuously produce γδT17 cells as they are pre-activated, highly potent producers of pro-inflammatory cytokines and crucially involved in the development of autoimmunity (discussed below).

Further approaches are needed to better characterize the alteration of the intrathymic cytokine and chemokine milieu after birth and its consequences for the development of γδT17 cells.

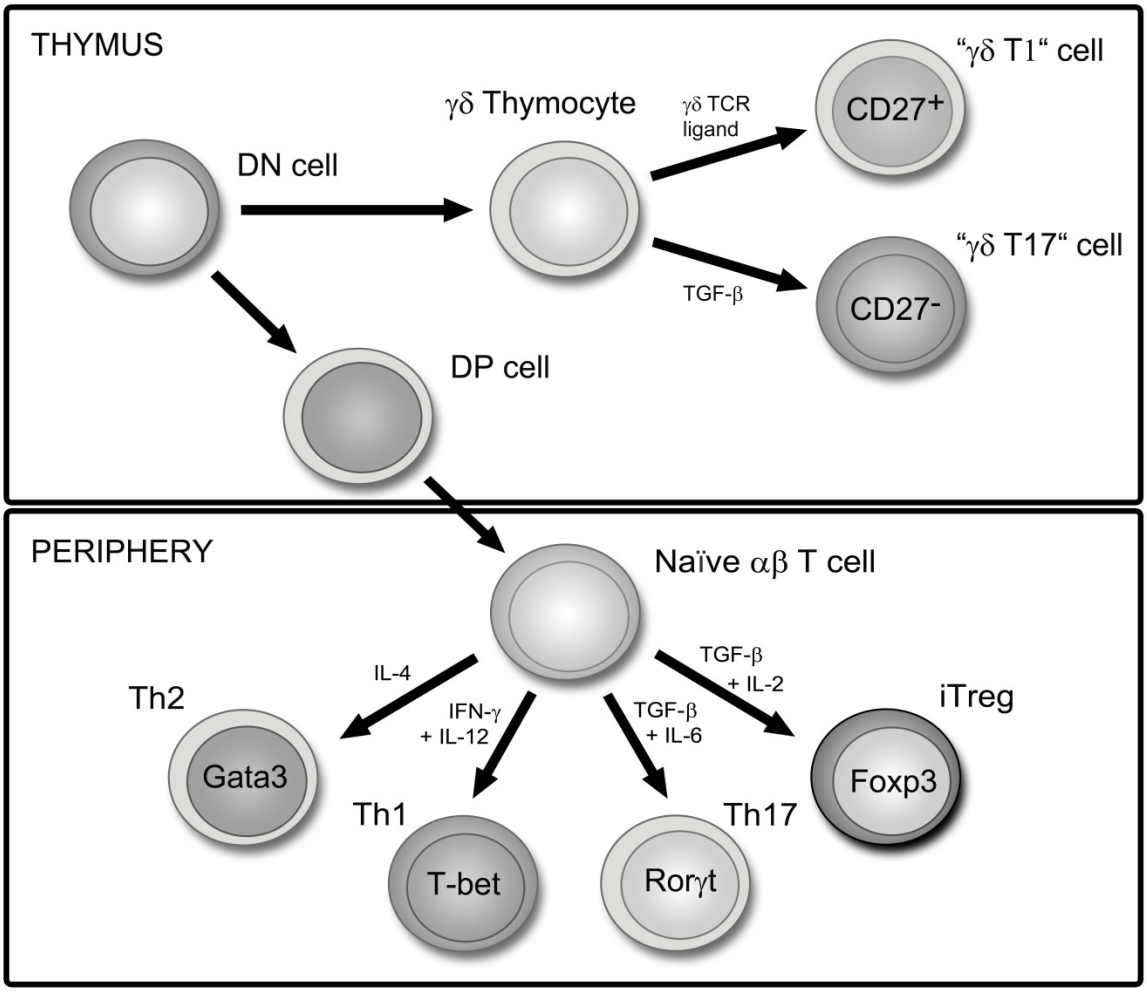


Figure 6.1: Acquisition of effector phenotypes within the αβ and γδ T cell lineage
αβ T cells and γδ T cells are generated in the thymus from a common double negative ($CD4^-CD8^-$, DN) precursor. γδ T cells develop in the thymus into $CD27^+$ IFN-γ producing cells ("γδT1") or into $CD27^-$ IL-17 producing cells ("γδT17"). In contrast, αβ T cell lineage committed precursors progress through a double positive (DP) stage to a single positive (SP) stage. αβ T cells leave the thymus as $CD4^+$ or $CD8^+$ SP, naïve cells. In the periphery naïve $CD4^+$ T cells can differentiate into Th1, Th2, Th17, and Treg cells, depending on the cytokine environment at the time of their encounter of cognate antigen in secondary lymphoid tissues. The transcription factors Gata3, T-bet, Rorγt, and Foxp3 are, amongst others, required for the induction of Th2, Th1, Th17, and iTreg cells. (From Korn and Petermann, 2012)

6.4 IL-23R$^+$ γδ T cells promote autoimmunity

6.4.1 Impact of γδ T cells on EAE severity

Consistent with reports from Spahn et al. (Spahn et al., 1999) *Tcrd*$^{-/-}$ mice were found to experience a less severe EAE after immunization with MOG_{35-55}/CFA. Also other studies identified a disease promoting effect of γδ T cells (Rajan et al., 1996, Rajan et al., 1998, Rajan

et al., 2000, Odyniec et al., 2004). Several mechanisms of increasing disease severity by γδ T cells were proposed including: i) regulation of the influx of inflammatory cells into the spinal cord, ii) enhancement of the expression of pro-inflammatory cytokines by the inflammatory infiltrates (Rajan et al., 1998), and iii) production of chemokines (Rajan et al., 2000). In contrast, Kobayashi et al. demonstrated an aggravated EAE in the absence of γδ T cells, suggesting a protective role of γδ T cells in EAE (Kobayashi et al., 1997). Many studies that investigated the role of γδ T cells in inflammation did not use *Tcrd*$^{-/-}$ mice but instead depleted γδ T cells in wild type mice with monoclonal antibodies (mAB) directed against γδ TCR. However, using Tcrd-H2b-eGFP.KI reporter mice, in which γδ T cells can be followed by their GFP expression, Koenecke et al. have recently shown that these mAB actually fail to physically deplete γδ T cells but instead functionally deplete or even activate γδ T cells (Koenecke et al., 2009). Thus, many of the published reports using γδ T cell depletion approaches are difficult to interpret. Furthermore, in previous studies on γδ T cells the functional dichotomy of γδT1 cells vs γδT17 cells has been neglected.

This thesis shows that those γδ T cells that accumulate in the CNS parenchyma during EAE represent a unique functional subset of γδ T cells as they express IL-23R and produce IL-17. Although IL-23 has long been implicated in the generation of Th17 cell, its role in γδ T cell biology during inflammatory responses in vivo has not been well understood.

6.4.2 The IL-23/IL-17 axis during inflammation and autoimmunity

IL-23 is now well established to act as a major pathogenic factor in a number of murine models of autoimmunity and inflammation including EAE, CIA, and intestinal inflammation (Cua et al., 2003, Murphy et al., 2003, Hue et al., 2006, Kullberg et al., 2006, Uhlig et al., 2006, Yen et al., 2006). Pathogenic mechanisms of IL-23 have primarily been analyzed in the context of Th17-mediated inflammation. IL-23 was found to promote full differentiation of activated T cells into Th17 effector cells, to maintain the Th17 effector phenotype, and to expand Th17 memory cells (Aggarwal et al., 2003, Cua et al., 2003, Gyulveszi et al., 2009, McGeachy et al., 2009). Mice deficient for either IL-23 or its receptor are protected from several autoimmune diseases, including EAE, which has been attributed to a failure of normal Th17 cell responses in these mice (Langrish et al., 2005, Awasthi et al., 2009). IL-23 has been shown to increase the capacity of Th17 cells to secrete IL-17. IL-17, which has been considered as the key determinant of Th17 pathogenicity, acts on several cell types to induce the expression of pro-

inflammatory cytokines (IL-6, IL-1β, TNF, GM-CSF) and chemokines, in particular CCL20, which can attract CCR6 expressing Th17 cells in a positive feed forward loop, and ELR^+ (glutamic acid (E), leucine (L), arginine (R)) members of the CXC family of chemokines (CXCL1 and CXCL2) that are strong attractants for neutrophils (Carlson et al., 2008). Accordingly, mice deficient for the IL-17A and IL-17F receptor IL-17RA have severe deficiencies in host defense against Toxoplasma gondii (Kelly et al., 2005) and Trypanosoma cruzi (Tosello Boari et al., 2012) due to defective neutrophil recruitment to the site of infection. On the other hand, IL-17RA deficient mice are less susceptible to autoimmunity and chronic inflammation (Koenders et al., 2005, Zhang et al., 2006, Hu et al., 2010, Toh et al., 2010).

A pathogenic role of IL-17 during autoimmune inflammation is widely accepted. However, while IL-23 deficient mice are resistant to EAE, IL-17 deficient mice are not resistant to EAE although they experience a milder disease compared with wild type littermates. Therefore, it has been suggested that IL-23 promotes autoimmunity beyond the induction of IL-17. IL-23 might play a role in the late plasticity of committed Th17 cells inducing a shift in function towards an IL-17 and IFN-γ double producing fate. However, the molecular mechanisms that contribute to the pathogenicity of IL-23 are still incompletely understood.

6.4.3 γδ T cell population dynamics during CNS autoimmunity

$IL\text{-}23R^+$ γδ T cells were identified as potent producers of IL-17, IL-21, and IL-22. During EAE these cells expanded in the peripheral lymphoid organs and accumulated in the CNS. Using a bioluminescence approach, Wohler et al. demonstrated that γδ T cells rapidly migrate to the site of MOG peptide injection and undergo expansion in the cervical lymph nodes and spleen starting as early as three days after EAE induction (Wohler et al., 2009). As shown in this thesis, sensitization with $MOG_{35\text{-}55}$/CFA resulted in an expansion of both $IL\text{-}23R^+$ γδ T cells and $IL\text{-}23R^-$ γδ T cells, which suggests that the inflammation induced by subcutaneous immunization activated both subsets of γδ T cells alike. In line with the preferential Vγ4 usage of $IL\text{-}23R^+$ γδ T cell precursors, the majority of expanded $IL\text{-}23R^+$ γδ T cells were also Vγ4 positive. It is unknown whether γδ T cells respond to a specific antigen in the immunization inoculum (e.g. a peptide or lipid antigen in the mycobacterium extract of the adjuvant preparation). However, $IL\text{-}23R^+$ γδ T cells may not even need to be activated via their TCR but can produce IL-17 in response to IL-1β and IL-23 without further TCR engagement (Sutton et al., 2009). Moreover, Ribot et al. demonstrated that the survival and proliferation of γδT17

cells depends on TLR/MyD88-mediated signals during malaria infection, while γδT1 cells depend on TCR signals (Ribot et al., 2010).

Notably, almost all γδ T cells that reached the CNS expressed the IL-23R and produced IL-17 while IFN-γ producing γδ T cells were almost absent in the inflamed CNS. It has been suggested that the expression of IL-23R is (indirectly) required for the homing of MOG-specific T cells to the CNS during EAE (Gyulveszi et al., 2009). Thus, the ability to sense IL-23 might decide whether a cell can become equipped to enter the CNS. This could apply to the population of γδ T cells in an analogous manner although the molecular mechanisms remain unclear.

Like Th17 cells, IL-23R$^+$ γδ T cells were found to express the chemokine receptor CCR6. The paper of Reboldi et al. shows that CCR6 expression is critical for entry of Th17 cells into the CNS at early, preclinical phases of EAE. Accordingly, CCR6 deficient mice were shown to be resistant to the induction of EAE but became susceptible after the transfer of CCR6-sufficient T cells (Reboldi et al., 2009). Therefore, it is conceivable that the expression of CCR6 licenses IL-23R$^+$ γδ T cell to invade the CNS parenchyma as pioneering cells in early (preclinical) phases of EAE. Smith et al. further proposed that γδ T cells traffic to the CNS in a β2-integrin-dependent manner (Smith and Barnum, 2008). Unfortunately, this study did not analyze potential differences in the β2-integrin expression of distinct γδ T cells subsets.

In summary, γδ T cells reached their highest accumulation shortly before the peak of disease and rapidly declined after the peak of clinical symptoms. In summary, these findings led to the conclusion that IL-23R$^+$ γδ T cells are crucially involved in the development of EAE by setting the stage for adaptive immune responses.

6.4.4 Function of IL-23R$^+$ γδ T cells during CNS autoimmunity

Tcrd$^{-/-}$ mice developed an EAE with attenuated clinical symptoms. After Treg cell depletion the disease phenotype in *Tcrd*$^{-/-}$ mice was similar to wild type mice suggesting that the antigen-specific priming of αβ T effector cells was not impaired in a cell-intrinsic manner when γδ T cells were missing. However, *Tcrd*$^{-/-}$ mice had increased numbers of Treg cells in the secondary lymphoid organs under naïve conditions, harbored increased percentages of activated (CD103$^+$) Treg cells in the lymph nodes and spleen upon immunization with MOG_{35-55}/CFA, and tended to have higher fractions of Treg cells in the inflamed CNS compared to

wild type mice. Therefore, I hypothesized that the relative protection of *Tcrd*$^{-/-}$ mice from EAE might be due to an enhanced Treg cell response.

In wild type mice Treg cells are crucially involved in the recovery from EAE by suppressing myelin-specific effector T cell responses. However, in this thesis it was noticed that the Treg cell response only controlled inflammation in the CNS once the number of IL-23R$^+$ γδ T cells in the CNS declined suggesting that IL-23R$^+$ γδ T cells contribute to CNS inflammation during EAE not only by producing IL-17 but also by restraining Treg cell responses. In fact, in further experiments during this project, γδ T cells were identified to inhibit Treg cell induction and function in an IL-23-dependent manner. It remains unclear whether γδ T cells directly influenced the development and function of Treg cells or whether they strengthened the ability of αβ T cells to resist Treg cell-mediated suppression. Several studies demonstrated that Treg cell numbers are not reduced during autoimmunity but that the suppressive capacities of Treg cells to inhibit proliferation and cytokine production of effector T cells are impaired at the site of inflammation (Korn et al., 2007b, Wehrens et al., 2011). Korn et al. demonstrated that CNS-derived CD4$^+$ effector T cells are resistant to Treg cell-mediated suppression when they were isolated at the onset or peak of EAE whereas cells isolated during recovery were at least partially susceptible to suppression (Korn et al., 2007b).

Therefore, one can speculate that IL-23-activated γδ T cells present at the site of inflammation at early phases inhibit Treg cell responses in a locally constrained manner. In vitro assays showed that IL-23-activated γδ T cells secreted heat-sensitive mediators that prevented the development of induced Treg cells and inhibited Treg cell-mediated suppression of responder T cell proliferation. Several cytokines have been described to inhibit the TGF-β-driven conversion of conventional T cells into Foxp3$^+$ Treg cells (Awasthi et al., 2007, Korn et al., 2007a, Dardalhon et al., 2008). The most prominent ones are IL-6 and IL-21. In contrast to IL-6, IL-21 is produced by γδ T cells. However, neutralization of IL-21 did not reverse the suppression of Treg cell induction by supernatants from IL-23-activated γδ T cells. Other cytokines that have been identified to interfere with Treg cell induction, such as IL-4 and IL-27, are not produced by γδ T cells upon IL-23 stimulation. The identity of the IL-23-induced, γδ T cell-derived mediator(s) responsible for these effects remains to be determined. However, one way to narrow down these factor(s) was the analysis of the transcription profile of the responder T cells exposed to IL-23-activated γδ T cell supernatant. Unlike IL-6 or IL-21, which also inhibit the TGF-β-driven induction of Foxp3, IL-23-activated γδ T cell supernatant

(together with TGF-β) did not induce a Th17 profile in responder T cells but led to the upregulation of Th1-associated molecules. In a study by Sutton et al. supernatants of γδ T cells that were stimulated with IL-1β and IL-23 did enhance the IL-17 production of $CD4^+$ αβ T cells (Sutton et al., 2009). However, direct effects of exogenous IL-1 (still present in the γδ T cells supernatant) on the responding αβ T cells in the "secondary" culture were not excluded.
In summary, by inducing a soluble factor or a panel of mediators, IL-23 enables γδ T cells to inhibit the TGF-β-driven induction of Treg cells and to suppress Treg cell function in vitro and in vivo. As a result αβ T cell responses are disinhibited and thus, enhanced (see Figure 6.2).

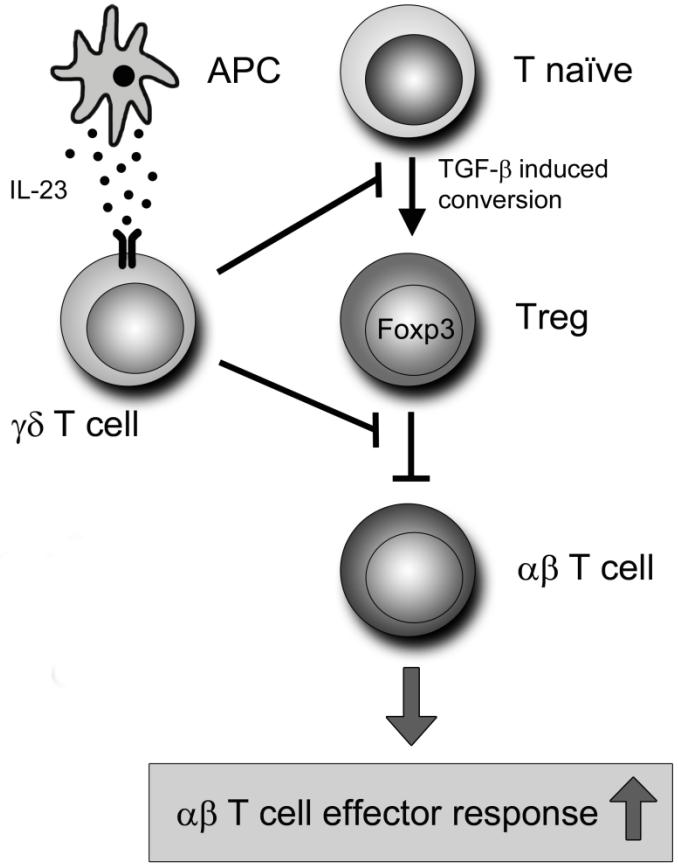


Figure 6.2: γδ T cells restrain regulatory T cell responses.
γδ T cells sense IL-23 produced by antigen-presenting cells (APC) via the constitutively expressed IL-23 receptor. In response to IL-23 γδ T cells inhibit the TGF-β-driven conversion of naïve T cells into Foxp3 expressing Treg cells and antagonize Treg cell-mediated suppression of αβ T cells. This enhances the adaptive immune response by effector αβ T cells. (From Petermann and Korn, 2011).

6.4.5 Role of γδ T cells in other models of autoimmune diseases

A few years ago γδ T cells were identified as potent IL-17 producers during infection. Because of the established role of IL-17 during autoimmunity, the role of γδ T cells has been tested also in other models of induced autoimmunity besides EAE.

CIA is a murine model of the human disease rheumatoid arthritis. Several studies demonstrated a disease promoting effect of γδ T cells in CIA. Already in 1993, Peterman et al. reported that the depletion of γδ T cells leads to a delayed onset and reduced severity in CIA (Peterman et al., 1993). Roark et al. found that the Vγ4$^+$ γδ T cell population is the only activated γδ T cell subset during CIA and the vast majority of these cells produce IL-17 both in the draining lymph node and in the joints (Roark et al., 2007). Moreover, a selective depletion of Vγ4$^+$ γδ T cells results in a less severe disease course. These findings were further supported by Ito et al. who demonstrated that γδ T cells are the major producers of IL-17 during CIA. In this study it was shown that IL-17 in γδ T cells is induced by IL-1β and IL-23 independently of T cell receptor ligation (Ito et al., 2009).

Topical treatment with imiquimod, a ligand for TLR7 and TLR8, has been reported as a novel mouse model for psoriasis-like skin inflammation (van der Fits et al., 2009). CCR6$^+$Rorγt$^+$ γδ T cells found in the dermis of naïve mice were demonstrated to be pre-committed to produce IL-17 and to fail to reconstitute after irradiation and bone marrow transplantation (Gray et al., 2011). Recently, Pantelyushin et al. identified skin invading γδ T cells and Rorγt$^+$ innate lymphocytes as potent producers of IL-17 and IL-22 during skin inflammation after imiquimod application and thus, as key players during psoriatic plaque formation (Pantelyushin et al., 2012). While most studies concentrated on the IL-17 production of γδ T cells during autoimmune inflammation, Van Belle et al. further elucidated the role of IL-22 during psoriasis (Van Belle et al., 2012). They found that IL-22 which is produced in large amounts by γδ T cells and cells of the innate immune system is essential for imiquimod-induced skin inflammation.

Finally, γδ T cells have been implicated to play a key role in the pathogenesis of colitis. A population of Vγ4$^+$ γδT17 cells has been found to be highly increased in a model of spontaneous colitis while the absence of γδ T cells abolishes colitis (Park et al., 2010). Similarly, Do et al. demonstrated that IL-17$^+$ γδ T cells but not IL-17$^-$ γδ T cells promote colitis by the generation of colitogenic Th17 cells (Do et al., 2011).

Taken together, γδ T cells, particularly IL-23R$^+$ γδT17 cells, have been shown to play a crucial role in the development of various autoimmune diseases. However, it seems that γδ T cells promote autoimmunity not only by the production of massive amounts of pro-inflammatory cytokines but also by enhancing adaptive effector T cell responses while restraining suppressive influences of Treg cells.

Although the majority of studies demonstrated a disease aggravating effect, there are some experimental data that suggest a protective role of γδ T cells during autoimmune inflammation (Chen et al., 2002). The mechanistic explanation for these functional differences is still missing. However, these findings might point to functions of γδ T cells that are more finely tuned than currently understood.

6.5 Development and function of human γδ T cells

The TCR V region (variable amino-terminal region) repertoire of human γδ T cells is even less variable compared with mice. Moreover, like in mice, the diversity of human γδ T cells in the peripheral immune compartment and peripheral tissues is even more restricted in the sense that subsets of γδ T cells that are characterized by the use of the same Vγ and Vδ chains localize to distinct tissues. The majority of human γδ T cells are either Vδ1$^+$ or Vδ2$^+$ with most of the circulating γδ T cells being Vγ9Vδ2 (Thedrez et al., 2007). During the embryonic development the thymus produces waves of γδ T cells expressing oligoclonal Vδ2 segments. However, after birth, the production switches to Vδ1 expressing γδ T cells (Ness-Schwickerath and Morita, 2011). Neonatal Vγ9Vδ2 γδ T cells have been found to produce IL-17 but no IFN-γ upon stimulation with IL-23 and the aminobisphosphate zoledronate, while cells stimulated with zoledronate alone produce exclusively IFN-γ (Moens et al., 2011). Interestingly, the induction of an IL-17$^+$IFN-γ$^-$ population is observed only in neonatal γδ T cells, but not in γδ T cells isolated from adult individuals. Yet, it has been shown that the IL-17 production in Vγ9Vδ2 γδ T cells is induced in vitro upon isopentenyl pyrophosphate (IPP) stimulation in the presence of a cytokine cocktail comprising TGF-β, IL-1β, IL-6, and IL-23 (Caccamo et al., 2011). However, it is currently not clear, whether the commitment of functional γδ T cell subsets in humans takes place already in the thymus, as described in this thesis for mice, or whether human γδ T cells can adopt distinct effector phenotypes in the periphery depending on environmental signals. The description of IFN-γ/IL-17 double producing γδ T cells in HIV

patients suggests that a clear discrimination of IL-17 and IFN-γ producing γδ T cells may not exist in humans (Fenoglio et al., 2009).

In addition to HIV, γδ T cells have been shown to participate in several inflammatory disorders. Peng et al. demonstrated that the fractions of γδT17 cells in peripheral blood are markedly increased in patients suffering from tuberculosis compared to healthy controls (Peng et al., 2008). Increased frequencies of Vγ9Vδ2 γδ T cells compared to healthy controls are found in children with bacterial meningitis (Caccamo et al., 2011). Here, up to 50 % of peripheral blood Vγ9Vδ2 γδ T cells and more than 70 % of Vγ9Vδ2 γδ T cells of the cerebrospinal fluid produce IL-17.

In vitro, human Vγ2Vδ2 γδ T cells were found to process and present antigens to αβ T cells with an efficiency close to that of DCs (Brandes et al., 2005). Consistently, Hu et al. reported that γδ T cells isolated from patients with rheumatoid arthritis up-regulate APC-specific molecules in vitro upon IPP stimulation (Hu et al., 2012). However, affirmative evidence for a relevant APC function of γδ T cells in vivo is missing.

Although γδ T cells have been shown to play important roles during EAE, their contribution to MS pathology is still not clear. Several studies identified increased numbers of γδ T cells in the cerebrospinal fluid (CSF) (Mix et al., 1990, Shimonkevitz et al., 1993) or peripheral blood of MS patients (Stinissen et al., 1995). Furthermore, γδ T cells were found to accumulate in acute MS plaques (Wucherpfennig et al., 1992). Recently, Schirmer et al. demonstrated that a population of γδT17 cells that is characterized by the surface expression of CD161 and CCR6 is enriched in the CSF and blood of MS patients (Schirmer et al., 2013). These findings point to a contribution of γδ T cells to acute inflammatory processes in MS, although molecular mechanisms still need to be further elucidated.

6.6 Conclusion and Outlook

This thesis shed light both on the development of IL-23R$^+$ γδ T cells and on their function during CNS autoimmunity. IL-23R$^+$ γδ T cells are generated exclusively in the embryonic thymus and leave the thymus as already pre-committed effector cells. Although IL-23R$^+$ γδ T cells are stable and long-lived, they seem to be of particular importance for host defense in the first stages of life when adaptive mechanisms have not yet evolved. Moreover, IL-23R$^+$ γδ T cells have been identified to be crucially involved in the induction of CNS autoimmunity. IL-

23 that is exclusively expressed during inflammation arms γδ T cells to restrain Treg cell-mediated suppression of αβ T cell proliferation and function. γδ T cells, once activated by IL-23, secrete a soluble factor that inhibits the induction and function of Treg cells. As a result αβ T cell responses are allowed to unfold in an unrestricted manner during autoimmunity.

This mechanism of IL-23-dependent promotion of inflammation by γδ T cells might be important not only for autoimmunity but also for host defense at epithelial surfaces where γδ T cells are a major source of IL-17. Because of their population dynamics it is likely that γδ T cells restrain Treg cell-mediated suppression of αβ T cell responses particularly in early phases of inflammation in order to allow for proper adaptive immune responses to build up.

Several points that are beyond the scope of this thesis remain to be further elucidated: First, IL-23R$^+$ γδ T cells acquire the capability to secrete IL-17 prior to exiting the thymus and interestingly, do not develop in the adult thymus. Future approaches will be needed to understand the signal pathways that regulate the commitment of IL-23R$^+$ γδ T cells both in the embryonic and adult thymus.

Second, the specific contributions of IL-23R$^+$ γδ T cells in inflammatory disorders are currently difficult to analyze as appropriate mouse models are missing. For a long time, the γδ TCR was the only marker specific for γδ T cells. However, recently, Kisielow et al. identified a novel surface marker, termed Scart2, that is exclusively expressed on γδT17 cells (Kisielow et al., 2008).Thus, it will be possible to create mouse models in which γδT17 cells are specifically tagged and can be deleted. Generation of Scart2-controlled Cre-recombinase expression may even allow abrogating the expression of genes of interest conditionally in γδT17 cells.

Third, γδ T cells restrain Treg cell responses in an IL-23-dependend manner. The mechanism appears to rely on a yet unknown secreted factor. However, complex protein biochemical approaches will help to clarify its identity.

And fourth, IL-23 activates γδ T cells to produce large amounts of cytokines, including IL-17 and IL-22. However, while the role of IL-17 has been analyzed in various models of infection and autoimmunity, the biological role of IL-22 remains poorly characterized. IL-22 has been found to be dispensable during EAE (Kreymborg et al., 2007), but to be required in γδ T cell-dominated disorders such as psoriasis (Van Belle et al., 2012). Thus, analyzing the whole spectrum of γδ T cell functions might help to understand the pathogenesis not only of EAE but also of other inflammatory disorders.

In summary, this thesis offers an explanation why Treg cells fail to control αβ T cell responses during highly active stages of inflammatory disorders. While this mechanism helps the host to clear pathogens more efficiently by promoting early adaptive immune responses, it also opens the door for deleterious autoimmune tissue inflammation. Understanding Treg cell restraining processes in early stages of autoimmunity might lead to the development of new therapeutic strategies for autoimmunity and chronic inflammatory disorders.

7 Bibliography

AARVAK, T., CHABAUD, M., MIOSSEC, P. & NATVIG, J. B. 1999. IL-17 is produced by some proinflammatory Th1/Th0 cells but not by Th2 cells. *J Immunol,* 162**,** 1246-51.

AFKARIAN, M., SEDY, J. R., YANG, J., JACOBSON, N. G., CEREB, N., YANG, S. Y., MURPHY, T. L. & MURPHY, K. M. 2002. T-bet is a STAT1-induced regulator of IL-12R expression in naive CD4+ T cells. *Nat Immunol,* 3**,** 549-57.

AGGARWAL, S., GHILARDI, N., XIE, M. H., DE SAUVAGE, F. J. & GURNEY, A. L. 2003. Interleukin-23 promotes a distinct CD4 T cell activation state characterized by the production of interleukin-17. *J Biol Chem,* 278**,** 1910-4.

AMIEL, S. A. 1976. The effects of Bordetella pertussis vaccine on cerebral vascular permeability. *Br J Exp Pathol,* 57**,** 653-62.

AMOR, S., GROOME, N., LININGTON, C., MORRIS, M. M., DORNMAIR, K., GARDINIER, M. V., MATTHIEU, J. M. & BAKER, D. 1994. Identification of epitopes of myelin oligodendrocyte glycoprotein for the induction of experimental allergic encephalomyelitis in SJL and Biozzi AB/H mice. *J Immunol,* 153**,** 4349-56.

AWASTHI, A., CARRIER, Y., PERON, J. P., BETTELLI, E., KAMANAKA, M., FLAVELL, R. A., KUCHROO, V. K., OUKKA, M. & WEINER, H. L. 2007. A dominant function for interleukin 27 in generating interleukin 10-producing anti-inflammatory T cells. *Nat Immunol,* 8**,** 1380-9.

AWASTHI, A., RIOL-BLANCO, L., JAGER, A., KORN, T., POT, C., GALILEOS, G., BETTELLI, E., KUCHROO, V. K. & OUKKA, M. 2009. Cutting edge: IL-23 receptor gfp reporter mice reveal distinct populations of IL-17-producing cells. *J Immunol,* 182**,** 5904-8.

BALANDINA, A., LECART, S., DARTEVELLE, P., SAOUDI, A. & BERRIH-AKNIN, S. 2005. Functional defect of regulatory CD4(+)CD25+ T cells in the thymus of patients with autoimmune myasthenia gravis. *Blood,* 105**,** 735-41.

BARTLETT, P. F. 1982. Cloned T cell progenitors from mouse bone marrow give rise to lymphocytes with multiple anti-H-2 reactivities. *Adv Exp Med Biol,* 149**,** 281-7.

BAUQUET, A. T., JIN, H., PATERSON, A. M., MITSDOERFFER, M., HO, I. C., SHARPE, A. H. & KUCHROO, V. K. 2009. The costimulatory molecule ICOS regulates the expression of c-Maf and IL-21 in the development of follicular T helper cells and TH-17 cells. *Nat Immunol,* 10**,** 167-75.

BECHER, B., DURELL, B. G. & NOELLE, R. J. 2002. Experimental autoimmune encephalitis and inflammation in the absence of interleukin-12. *J Clin Invest,* 110**,** 493-7.

BEN-NUN, A., MENDEL, I. & KERLERO DE ROSBO, N. 1997. Immunomodulation of murine experimental autoimmune encephalomyelitis by pertussis toxin: the protective activity, but not the disease-enhancing activity, can be attributed to the nontoxic B-oligomer. *Proc Assoc Am Physicians,* 109**,** 120-5.

BEN-NUN, A., WEKERLE, H. & COHEN, I. R. 1981. Vaccination against autoimmune encephalomyelitis with T-lymphocyte line cells reactive against myelin basic protein. *Nature,* 292**,** 60-1.

BENNETT, C. L., CHRISTIE, J., RAMSDELL, F., BRUNKOW, M. E., FERGUSON, P. J., WHITESELL, L., KELLY, T. E., SAULSBURY, F. T., CHANCE, P. F. & OCHS, H. D. 2001. The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. *Nat Genet,* 27**,** 20-1.

BERG, L. J. 2007. Signalling through TEC kinases regulates conventional versus innate CD8(+) T-cell development. *Nat Rev Immunol,* 7**,** 479-85.

BETTELLI, E., CARRIER, Y., GAO, W., KORN, T., STROM, T. B., OUKKA, M., WEINER, H. L. & KUCHROO, V. K. 2006. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature,* 441**,** 235-8.

BETTELLI, E., DAS, M. P., HOWARD, E. D., WEINER, H. L., SOBEL, R. A. & KUCHROO, V. K. 1998. IL-10 is critical in the regulation of autoimmune encephalomyelitis as demonstrated by studies of IL-10- and IL-4-deficient and transgenic mice. *J Immunol,* 161**,** 3299-306.

BETTELLI, E., PAGANY, M., WEINER, H. L., LININGTON, C., SOBEL, R. A. & KUCHROO, V. K. 2003. Myelin oligodendrocyte glycoprotein-specific T cell receptor transgenic mice develop spontaneous autoimmune optic neuritis. *J Exp Med,* 197**,** 1073-81.

BETTELLI, E., SULLIVAN, B., SZABO, S. J., SOBEL, R. A., GLIMCHER, L. H. & KUCHROO, V. K. 2004. Loss of T-bet, but not STAT1, prevents the development of experimental autoimmune encephalomyelitis. *J Exp Med,* 200**,** 79-87.

BIGBY, M., MARKOWITZ, J. S., BLEICHER, P. A., GRUSBY, M. J., SIMHA, S., SIEBRECHT, M., WAGNER, M., NAGLER-ANDERSON, C. & GLIMCHER, L. H. 1993. Most gamma delta T cells develop normally in the absence of MHC class II molecules. *J Immunol,* 151**,** 4465-75.

BOISMENU, R., FENG, L., XIA, Y. Y., CHANG, J. C. & HAVRAN, W. L. 1996. Chemokine expression by intraepithelial gamma delta T cells. Implications for the recruitment of inflammatory cells to damaged epithelia. *J Immunol,* 157**,** 985-92.

BONNEVILLE, M., ITO, K., KRECKO, E. G., ITOHARA, S., KAPPES, D., ISHIDA, I., KANAGAWA, O., JANEWAY, C. A., MURPHY, D. B. & TONEGAWA, S. 1989. Recognition of a self major histocompatibility complex TL region product by gamma delta T-cell receptors. *Proc Natl Acad Sci U S A,* 86**,** 5928-32.

BRANDES, M., WILLIMANN, K. & MOSER, B. 2005. Professional antigen-presentation function by human gammadelta T Cells. *Science,* 309**,** 264-8.

BRUNKOW, M. E., JEFFERY, E. W., HJERRILD, K. A., PAEPER, B., CLARK, L. B., YASAYKO, S. A., WILKINSON, J. E., GALAS, D., ZIEGLER, S. F. & RAMSDELL, F. 2001. Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. *Nat Genet,* 27**,** 68-73.

BUONOCORE, S., AHERN, P. P., UHLIG, H. H., IVANOV, II, LITTMAN, D. R., MALOY, K. J. & POWRIE, F. 2010. Innate lymphoid cells drive interleukin-23-dependent innate intestinal pathology. *Nature,* 464**,** 1371-5.

CACCAMO, N., LA MENDOLA, C., ORLANDO, V., MERAVIGLIA, S., TODARO, M., STASSI, G., SIRECI, G., FOURNIE, J. J. & DIELI, F. 2011. Differentiation, phenotype, and function of interleukin-17-producing human Vgamma9Vdelta2 T cells. *Blood,* 118**,** 129-38.

CAI, Y., SHEN, X., DING, C., QI, C., LI, K., LI, X., JALA, V. R., ZHANG, H. G., WANG, T., ZHENG, J. & YAN, J. 2011. Pivotal role of dermal IL-17-producing gammadelta T cells in skin inflammation. *Immunity,* 35**,** 596-610.

CARDING, S. R. & EGAN, P. J. 2002. Gammadelta T cells: functional plasticity and heterogeneity. *Nat Rev Immunol,* 2**,** 336-45.

CARLSON, T., KROENKE, M., RAO, P., LANE, T. E. & SEGAL, B. 2008. The Th17-ELR+ CXC chemokine pathway is essential for the development of central nervous system autoimmune disease. *J Exp Med,* 205**,** 811-23.

CARRIZOSA, A. M., NICHOLSON, L. B., FARZAN, M., SOUTHWOOD, S., SETTE, A., SOBEL, R. A. & KUCHROO, V. K. 1998. Expansion by self antigen is necessary for the induction of experimental autoimmune encephalomyelitis by T cells primed with a cross-reactive environmental antigen. *J Immunol,* 161**,** 3307-14.

CHARCOT, J. M. 1877. Lectures on the disease of the nervous system. *delivered at La Salpetrière*.

CHEN, W., JIN, W., HARDEGEN, N., LEI, K. J., LI, L., MARINOS, N., MCGRADY, G. & WAHL, S. M. 2003. Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. *J Exp Med,* 198**,** 1875-86.

CHEN, Y., CHOU, K., FUCHS, E., HAVRAN, W. L. & BOISMENU, R. 2002. Protection of the intestinal mucosa by intraepithelial gamma delta T cells. *Proc Natl Acad Sci U S A,* 99**,** 14338-43.

CHO, J. S., PIETRAS, E. M., GARCIA, N. C., RAMOS, R. I., FARZAM, D. M., MONROE, H. R., MAGORIEN, J. E., BLAUVELT, A., KOLLS, J. K., CHEUNG, A. L., CHENG, G., MODLIN, R. L. & MILLER, L. S. 2010. IL-17 is essential for host defense against cutaneous Staphylococcus aureus infection in mice. *J Clin Invest,* 120**,** 1762-73.

CHUNG, Y., CHANG, S. H., MARTINEZ, G. J., YANG, X. O., NURIEVA, R., KANG, H. S., MA, L., WATOWICH, S. S., JETTEN, A. M., TIAN, Q. & DONG, C. 2009. Critical regulation of early Th17 cell differentiation by interleukin-1 signaling. *Immunity,* 30**,** 576-87.

CIOFANI, M., MADAR, A., GALAN, C., SELLARS, M., MACE, K., PAULI, F., AGARWAL, A., HUANG, W., PARKURST, C. N., MURATET, M., NEWBERRY, K. M., MEADOWS, S., GREENFIELD, A., YANG, Y., JAIN, P., KIRIGIN, F. K., BIRCHMEIER, C., WAGNER, E. F., MURPHY, K. M., MYERS, R. M., BONNEAU, R. & LITTMAN, D. R. 2012. A validated regulatory network for Th17 cell specification. *Cell,* 151**,** 289-303.

CODARRI, L., GYULVESZI, G., TOSEVSKI, V., HESSKE, L., FONTANA, A., MAGNENAT, L., SUTER, T. & BECHER, B. 2011. RORgammat drives production of the cytokine GM-CSF in helper T cells, which is essential for the effector phase of autoimmune neuroinflammation. *Nat Immunol,* 12**,** 560-7.

COMPSTON, A. 2005. *McAlpine's multiple sclerosis,* Philadelphia, Churchill Livingstone Elsevier.

CORREA, I., BIX, M., LIAO, N. S., ZIJLSTRA, M., JAENISCH, R. & RAULET, D. 1992. Most gamma delta T cells develop normally in beta 2-microglobulin-deficient mice. *Proc Natl Acad Sci U S A,* 89**,** 653-7.

CROWLEY, M. P., REICH, Z., MAVADDAT, N., ALTMAN, J. D. & CHIEN, Y. 1997. The recognition of the nonclassical major histocompatibility complex (MHC) class I molecule, T10, by the gammadelta T cell, G8. *J Exp Med,* 185**,** 1223-30.

CUA, D. J., SHERLOCK, J., CHEN, Y., MURPHY, C. A., JOYCE, B., SEYMOUR, B., LUCIAN, L., TO, W., KWAN, S., CHURAKOVA, T., ZURAWSKI, S., WIEKOWSKI, M., LIRA, S. A., GORMAN, D., KASTELEIN, R. A. & SEDGWICK, J. D. 2003. Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain. *Nature,* 421**,** 744-8.

CUA, D. J. & TATO, C. M. 2010. Innate IL-17-producing cells: the sentinels of the immune system. *Nat Rev Immunol,* 10**,** 479-89.

CUROTTO DE LAFAILLE, M. A. & LAFAILLE, J. J. 2009. Natural and adaptive foxp3+ regulatory T cells: more of the same or a division of labor? *Immunity,* 30**,** 626-35.

DARDALHON, V., AWASTHI, A., KWON, H., GALILEOS, G., GAO, W., SOBEL, R. A., MITSDOERFFER, M., STROM, T. B., ELYAMAN, W., HO, I. C., KHOURY, S., OUKKA, M. & KUCHROO, V. K. 2008. IL-4 inhibits TGF-beta-induced Foxp3+ T cells and, together with TGF-beta, generates IL-9+ IL-10+ Foxp3(-) effector T cells. *Nat Immunol,* 9**,** 1347-55.

DO, J. S., FINK, P. J., LI, L., SPOLSKI, R., ROBINSON, J., LEONARD, W. J., LETTERIO, J. J. & MIN, B. 2010. Cutting edge: spontaneous development of IL-17-producing gamma delta T cells in the thymus occurs via a TGF-beta 1-dependent mechanism. *J Immunol,* 184**,** 1675-9.

DO, J. S., VISPERAS, A., DONG, C., BALDWIN, W. M., 3RD & MIN, B. 2011. Cutting edge: Generation of colitogenic Th17 CD4 T cells is enhanced by IL-17+ gammadelta T cells. *J Immunol,* 186**,** 4546-50.

DUAN, J., CHUNG, H., TROY, E. & KASPER, D. L. 2010. Microbial colonization drives expansion of IL-1 receptor 1-expressing and IL-17-producing gamma/delta T cells. *Cell Host Microbe,* 7**,** 140-50.

DUERR, R. H., TAYLOR, K. D., BRANT, S. R., RIOUX, J. D., SILVERBERG, M. S., DALY, M. J., STEINHART, A. H., ABRAHAM, C., REGUEIRO, M., GRIFFITHS, A., DASSOPOULOS, T., BITTON, A., YANG, H., TARGAN, S., DATTA, L. W., KISTNER, E. O., SCHUMM, L. P., LEE, A. T., GREGERSEN, P. K., BARMADA, M. M., ROTTER, J. I., NICOLAE, D. L. & CHO, J. H. 2006. A genome-wide association study identifies IL23R as an inflammatory bowel disease gene. *Science,* 314**,** 1461-3.

EHRENSTEIN, M. R., EVANS, J. G., SINGH, A., MOORE, S., WARNES, G., ISENBERG, D. A. & MAURI, C. 2004. Compromised function of regulatory T cells in rheumatoid arthritis and reversal by anti-TNFalpha therapy. *J Exp Med,* 200**,** 277-85.

EL-BEHI, M., CIRIC, B., DAI, H., YAN, Y., CULLIMORE, M., SAFAVI, F., ZHANG, G. X., DITTEL, B. N. & ROSTAMI, A. 2011. The encephalitogenicity of T(H)17 cells is dependent on IL-1- and IL-23-induced production of the cytokine GM-CSF. *Nat Immunol,* 12**,** 568-75.

FENOGLIO, D., POGGI, A., CATELLANI, S., BATTAGLIA, F., FERRERA, A., SETTI, M., MURDACA, G. & ZOCCHI, M. R. 2009. Vdelta1 T lymphocytes producing IFN-gamma and IL-17 are expanded in HIV-1-infected patients and respond to Candida albicans. *Blood,* 113**,** 6611-8.

FERBER, I. A., BROCKE, S., TAYLOR-EDWARDS, C., RIDGWAY, W., DINISCO, C., STEINMAN, L., DALTON, D. & FATHMAN, C. G. 1996. Mice with a disrupted IFN-gamma gene are susceptible to the induction of experimental autoimmune encephalomyelitis (EAE). *J Immunol,* 156**,** 5-7.

FERRICK, D. A., SCHRENZEL, M. D., MULVANIA, T., HSIEH, B., FERLIN, W. G. & LEPPER, H. 1995. Differential production of interferon-gamma and interleukin-4 in response to Th1- and Th2-stimulating pathogens by gamma delta T cells in vivo. *Nature,* 373**,** 255-7.

FOSSIEZ, F., DJOSSOU, O., CHOMARAT, P., FLORES-ROMO, L., AIT-YAHIA, S., MAAT, C., PIN, J. J., GARRONE, P., GARCIA, E., SAELAND, S., BLANCHARD, D., GAILLARD, C., DAS MAHAPATRA, B., ROUVIER, E., GOLSTEIN, P., BANCHEREAU, J. & LEBECQUE, S. 1996. T cell interleukin-17 induces stromal cells to produce proinflammatory and hematopoietic cytokines. *J Exp Med,* 183**,** 2593-603.

FOWLKES, B. J., EDISON, L., MATHIESON, B. J. & CHUSED, T. M. 1985. Early T lymphocytes. Differentiation in vivo of adult intrathymic precursor cells. *J Exp Med,* 162**,** 802-22.

FRITZ, R. B., CHOU, C. H. & MCFARLIN, D. E. 1983. Induction of experimental allergic encephalomyelitis in PL/J and (SJL/J x PL/J)F1 mice by myelin basic protein and its peptides: localization of a second encephalitogenic determinant. *J Immunol,* 130**,** 191-4.

FUJINAMI, R. S. & OLDSTONE, M. B. 1985. Amino acid homology between the encephalitogenic site of myelin basic protein and virus: mechanism for autoimmunity. *Science,* 230**,** 1043-5.

FUJINO, S., ANDOH, A., BAMBA, S., OGAWA, A., HATA, K., ARAKI, Y., BAMBA, T. & FUJIYAMA, Y. 2003. Increased expression of interleukin 17 in inflammatory bowel disease. *Gut,* 52**,** 65-70.

GIRARDI, M. 2006. Immunosurveillance and immunoregulation by gammadelta T cells. *J Invest Dermatol,* 126**,** 25-31.

GODFREY, D. I., KENNEDY, J., SUDA, T. & ZLOTNIK, A. 1993. A developmental pathway involving four phenotypically and functionally distinct subsets of CD3-CD4-CD8- triple-negative adult mouse thymocytes defined by CD44 and CD25 expression. *J Immunol,* 150**,** 4244-52.

GRAN, B., ZHANG, G. X., YU, S., LI, J., CHEN, X. H., VENTURA, E. S., KAMOUN, M. & ROSTAMI, A. 2002. IL-12p35-deficient mice are susceptible to experimental autoimmune encephalomyelitis: evidence for redundancy in the IL-12 system in the induction of central nervous system autoimmune demyelination. *J Immunol,* 169**,** 7104-10.

GRAY, E. E., RAMIREZ-VALLE, F., XU, Y., WU, S., WU, Z., KARJALAINEN, K. E. & CYSTER, J. G. 2013. Deficiency in IL-17-committed Vgamma4(+) gammadelta T cells in a spontaneous Sox13-mutant CD45.1(+) congenic mouse substrain provides protection from dermatitis. *Nat Immunol,* 14**,** 584-92.

GRAY, E. E., SUZUKI, K. & CYSTER, J. G. 2011. Cutting edge: Identification of a motile IL-17-producing gammadelta T cell population in the dermis. *J Immunol,* 186**,** 6091-5.

GYULVESZI, G., HAAK, S. & BECHER, B. 2009. IL-23-driven encephalo-tropism and Th17 polarization during CNS-inflammation in vivo. *Eur J Immunol,* 39**,** 1864-9.

HAAS, J., HUG, A., VIEHOVER, A., FRITZSCHING, B., FALK, C. S., FILSER, A., VETTER, T., MILKOVA, L., KORPORAL, M., FRITZ, B., STORCH-HAGENLOCHER, B., KRAMMER, P. H., SURI-PAYER, E. & WILDEMANN, B. 2005. Reduced suppressive effect of CD4+CD25high regulatory T cells on the T cell immune response against myelin oligodendrocyte glycoprotein in patients with multiple sclerosis. *Eur J Immunol,* 35**,** 3343-52.

HAAS, J. D., GONZALEZ, F. H., SCHMITZ, S., CHENNUPATI, V., FOHSE, L., KREMMER, E., FORSTER, R. & PRINZ, I. 2009. CCR6 and NK1.1 distinguish between IL-17A and IFN-gamma-producing gammadelta effector T cells. *Eur J Immunol,* 39**,** 3488-97.

HAAS, J. D., RAVENS, S., DUBER, S., SANDROCK, I., OBERDORFER, L., KASHANI, E., CHENNUPATI, V., FOHSE, L., NAUMANN, R., WEISS, S., KRUEGER, A., FORSTER, R. & PRINZ, I. 2012. Development of interleukin-17-producing gammadelta T cells is restricted to a functional embryonic wave. *Immunity,* 37**,** 48-59.

HAINES, C. J., CHEN, Y., BLUMENSCHEIN, W. M., JAIN, R., CHANG, C., JOYCE-SHAIKH, B., PORTH, K., BONIFACE, K., MATTSON, J., BASHAM, B., ANDERTON, S. M., MCCLANAHAN, T. K., SADEKOVA, S., CUA, D. J. &

MCGEACHY, M. J. 2013. Autoimmune memory T helper 17 cell function and expansion are dependent on interleukin-23. *Cell Rep,* 3**,** 1378-88.

HARRINGTON, L. E., JANOWSKI, K. M., OLIVER, J. R., ZAJAC, A. J. & WEAVER, C. T. 2008. Memory CD4 T cells emerge from effector T-cell progenitors. *Nature,* 452**,** 356-60.

HAYDAY, A. C. 2009. Gammadelta T cells and the lymphoid stress-surveillance response. *Immunity,* 31**,** 184-96.

HOFSTETTER, H. H., IBRAHIM, S. M., KOCZAN, D., KRUSE, N., WEISHAUPT, A., TOYKA, K. V. & GOLD, R. 2005. Therapeutic efficacy of IL-17 neutralization in murine experimental autoimmune encephalomyelitis. *Cell Immunol,* 237**,** 123-30.

HOFSTETTER, H. H., SHIVE, C. L. & FORSTHUBER, T. G. 2002. Pertussis toxin modulates the immune response to neuroantigens injected in incomplete Freund's adjuvant: induction of Th1 cells and experimental autoimmune encephalomyelitis in the presence of high frequencies of Th2 cells. *J Immunol,* 169**,** 117-25.

HORWITZ, M. S., BRADLEY, L. M., HARBERTSON, J., KRAHL, T., LEE, J. & SARVETNICK, N. 1998. Diabetes induced by Coxsackie virus: initiation by bystander damage and not molecular mimicry. *Nat Med,* 4**,** 781-5.

HOZUMI, N. & TONEGAWA, S. 1976. Evidence for somatic rearrangement of immunoglobulin genes coding for variable and constant regions. *Proc Natl Acad Sci U S A,* 73**,** 3628-32.

HSIEH, C. S., MACATONIA, S. E., TRIPP, C. S., WOLF, S. F., O'GARRA, A. & MURPHY, K. M. 1993. Development of TH1 CD4+ T cells through IL-12 produced by Listeria-induced macrophages. *Science,* 260**,** 547-9.

HU, C., QIAN, L., MIAO, Y., HUANG, Q., MIAO, P., WANG, P., YU, Q., NIE, H., ZHANG, J., HE, D., XU, R., CHEN, X., LIU, B. & ZHANG, D. 2012. Antigen-presenting effects of effector memory Vgamma9Vdelta2 T cells in rheumatoid arthritis. *Cell Mol Immunol,* 9**,** 245-54.

HU, Y., OTA, N., PENG, I., REFINO, C. J., DANILENKO, D. M., CAPLAZI, P. & OUYANG, W. 2010. IL-17RC is required for IL-17A- and IL-17F-dependent signaling and the pathogenesis of experimental autoimmune encephalomyelitis. *J Immunol,* 184**,** 4307-16.

HUANG, H. & AUERBACH, R. 1993. Identification and characterization of hematopoietic stem cells from the yolk sac of the early mouse embryo. *Proc Natl Acad Sci U S A,* 90**,** 10110-4.

HUANG, W., NA, L., FIDEL, P. L. & SCHWARZENBERGER, P. 2004. Requirement of interleukin-17A for systemic anti-Candida albicans host defense in mice. *J Infect Dis,* 190**,** 624-31.

HUBER, M., BRUSTLE, A., REINHARD, K., GURALNIK, A., WALTER, G., MAHINY, A., VON LOW, E. & LOHOFF, M. 2008. IRF4 is essential for IL-21-mediated induction, amplification, and stabilization of the Th17 phenotype. *Proc Natl Acad Sci U S A,* 105**,** 20846-51.

HUE, S., AHERN, P., BUONOCORE, S., KULLBERG, M. C., CUA, D. J., MCKENZIE, B. S., POWRIE, F. & MALOY, K. J. 2006. Interleukin-23 drives innate and T cell-mediated intestinal inflammation. *J Exp Med,* 203**,** 2473-83.

HUNTER, C. A. 2005. New IL-12-family members: IL-23 and IL-27, cytokines with divergent functions. *Nat Rev Immunol,* 5**,** 521-31.

ITO, Y., USUI, T., KOBAYASHI, S., IGUCHI-HASHIMOTO, M., ITO, H., YOSHITOMI, H., NAKAMURA, T., SHIMIZU, M., KAWABATA, D., YUKAWA, N.,

HASHIMOTO, M., SAKAGUCHI, N., SAKAGUCHI, S., YOSHIFUJI, H., NOJIMA, T., OHMURA, K., FUJII, T. & MIMORI, T. 2009. Gamma/delta T cells are the predominant source of interleukin-17 in affected joints in collagen-induced arthritis, but not in rheumatoid arthritis. *Arthritis Rheum,* 60**,** 2294-303.

IVANOV, II, MCKENZIE, B. S., ZHOU, L., TADOKORO, C. E., LEPELLEY, A., LAFAILLE, J. J., CUA, D. J. & LITTMAN, D. R. 2006. The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells. *Cell,* 126**,** 1121-33.

JAIN, J., MCCAFFREY, P. G., MINER, Z., KERPPOLA, T. K., LAMBERT, J. N., VERDINE, G. L., CURRAN, T. & RAO, A. 1993. The T-cell transcription factor NFATp is a substrate for calcineurin and interacts with Fos and Jun. *Nature,* 365**,** 352-5.

JENSEN, K. D., SU, X., SHIN, S., LI, L., YOUSSEF, S., YAMASAKI, S., STEINMAN, L., SAITO, T., LOCKSLEY, R. M., DAVIS, M. M., BAUMGARTH, N. & CHIEN, Y. H. 2008. Thymic selection determines gammadelta T cell effector fate: antigen-naive cells make interleukin-17 and antigen-experienced cells make interferon gamma. *Immunity,* 29**,** 90-100.

JOHNSON, K. P., BROOKS, B. R., FORD, C. C., GOODMAN, A., GUARNACCIA, J., LISAK, R. P., MYERS, L. W., PANITCH, H. S., PRUITT, A., ROSE, J. W., KACHUCK, N. & WOLINSKY, J. S. 2000. Sustained clinical benefits of glatiramer acetate in relapsing multiple sclerosis patients observed for 6 years. Copolymer 1 Multiple Sclerosis Study Group. *Mult Scler,* 6**,** 255-66.

JOHNSON, R. M., LANCKI, D. W., SPERLING, A. I., DICK, R. F., SPEAR, P. G., FITCH, F. W. & BLUESTONE, J. A. 1992. A murine CD4-, CD8- T cell receptor-gamma delta T lymphocyte clone specific for herpes simplex virus glycoprotein I. *J Immunol,* 148**,** 983-8.

JONES, L. S., RIZZO, L. V., AGARWAL, R. K., TARRANT, T. K., CHAN, C. C., WIGGERT, B. & CASPI, R. R. 1997. IFN-gamma-deficient mice develop experimental autoimmune uveitis in the context of a deviant effector response. *J Immunol,* 158**,** 5997-6005.

KABAT, E. A., WOLF, A. & BEZER, A. E. 1947. The Rapid Production of Acute Disseminated Encephalomyelitis in Rhesus Monkeys by Injection of Heterologous and Homologous Brain Tissue with Adjuvants. *J Exp Med,* 85**,** 117-30.

KAY, A. B., YING, S., VARNEY, V., GAGA, M., DURHAM, S. R., MOQBEL, R., WARDLAW, A. J. & HAMID, Q. 1991. Messenger RNA expression of the cytokine gene cluster, interleukin 3 (IL-3), IL-4, IL-5, and granulocyte/macrophage colony-stimulating factor, in allergen-induced late-phase cutaneous reactions in atopic subjects. *J Exp Med,* 173**,** 775-8.

KELLY, M. N., KOLLS, J. K., HAPPEL, K., SCHWARTZMAN, J. D., SCHWARZENBERGER, P., COMBE, C., MORETTO, M. & KHAN, I. A. 2005. Interleukin-17/interleukin-17 receptor-mediated signaling is important for generation of an optimal polymorphonuclear response against Toxoplasma gondii infection. *Infect Immun,* 73**,** 617-21.

KING, D. P., HYDE, D. M., JACKSON, K. A., NOVOSAD, D. M., ELLIS, T. N., PUTNEY, L., STOVALL, M. Y., VAN WINKLE, L. S., BEAMAN, B. L. & FERRICK, D. A. 1999. Cutting edge: protective response to pulmonary injury requires gamma delta T lymphocytes. *J Immunol,* 162**,** 5033-6.

KISIELOW, J., KOPF, M. & KARJALAINEN, K. 2008. SCART scavenger receptors identify a novel subset of adult gammadelta T cells. *J Immunol,* 181**,** 1710-6.

KOBAYASHI, Y., KAWAI, K., ITO, K., HONDA, H., SOBUE, G. & YOSHIKAI, Y. 1997. Aggravation of murine experimental allergic encephalomyelitis by administration of T-cell receptor gammadelta-specific antibody. *J Neuroimmunol,* 73**,** 169-74.

KOENDERS, M. I., KOLLS, J. K., OPPERS-WALGREEN, B., VAN DEN BERSSELAAR, L., JOOSTEN, L. A., SCHURR, J. R., SCHWARZENBERGER, P., VAN DEN BERG, W. B. & LUBBERTS, E. 2005. Interleukin-17 receptor deficiency results in impaired synovial expression of interleukin-1 and matrix metalloproteinases 3, 9, and 13 and prevents cartilage destruction during chronic reactivated streptococcal cell wall-induced arthritis. *Arthritis Rheum,* 52**,** 3239-47.

KOENECKE, C., CHENNUPATI, V., SCHMITZ, S., MALISSEN, B., FORSTER, R. & PRINZ, I. 2009. In vivo application of mAb directed against the gammadelta TCR does not deplete but generates "invisible" gammadelta T cells. *Eur J Immunol,* 39**,** 372-9.

KOLLS, J. K. & LINDEN, A. 2004. Interleukin-17 family members and inflammation. *Immunity,* 21**,** 467-76.

KOMIYAMA, Y., NAKAE, S., MATSUKI, T., NAMBU, A., ISHIGAME, H., KAKUTA, S., SUDO, K. & IWAKURA, Y. 2006. IL-17 plays an important role in the development of experimental autoimmune encephalomyelitis. *J Immunol,* 177**,** 566-73.

KORN, T., BETTELLI, E., GAO, W., AWASTHI, A., JAGER, A., STROM, T. B., OUKKA, M. & KUCHROO, V. K. 2007a. IL-21 initiates an alternative pathway to induce proinflammatory T(H)17 cells. *Nature,* 448**,** 484-7.

KORN, T., MITSDOERFFER, M. & KUCHROO, V. K. 2010. Immunological basis for the development of tissue inflammation and organ-specific autoimmunity in animal models of multiple sclerosis. *Results Probl Cell Differ,* 51**,** 43-74.

KORN, T. & PETERMANN, F. 2012. Development and function of interleukin 17-producing gammadelta T cells. *Ann N Y Acad Sci,* 1247**,** 34-45.

KORN, T., REDDY, J., GAO, W., BETTELLI, E., AWASTHI, A., PETERSEN, T. R., BACKSTROM, B. T., SOBEL, R. A., WUCHERPFENNIG, K. W., STROM, T. B., OUKKA, M. & KUCHROO, V. K. 2007b. Myelin-specific regulatory T cells accumulate in the CNS but fail to control autoimmune inflammation. *Nat Med,* 13**,** 423-31.

KREYMBORG, K., ETZENSPERGER, R., DUMOUTIER, L., HAAK, S., REBOLLO, A., BUCH, T., HEPPNER, F. L., RENAULD, J. C. & BECHER, B. 2007. IL-22 is expressed by Th17 cells in an IL-23-dependent fashion, but not required for the development of autoimmune encephalomyelitis. *J Immunol,* 179**,** 8098-104.

KRONENBERG, M. & GAPIN, L. 2002. The unconventional lifestyle of NKT cells. *Nat Rev Immunol,* 2**,** 557-68.

KUCHROO, V. K., DAS, M. P., BROWN, J. A., RANGER, A. M., ZAMVIL, S. S., SOBEL, R. A., WEINER, H. L., NABAVI, N. & GLIMCHER, L. H. 1995. B7-1 and B7-2 costimulatory molecules activate differentially the Th1/Th2 developmental pathways: application to autoimmune disease therapy. *Cell,* 80**,** 707-18.

KUCHROO, V. K., MARTIN, C. A., GREER, J. M., JU, S. T., SOBEL, R. A. & DORF, M. E. 1993. Cytokines and adhesion molecules contribute to the ability of myelin proteolipid protein-specific T cell clones to mediate experimental allergic encephalomyelitis. *J Immunol,* 151**,** 4371-82.

KUCHROO, V. K., SOBEL, R. A., YAMAMURA, T., GREENFIELD, E., DORF, M. E. & LEES, M. B. 1991. Induction of experimental allergic encephalomyelitis by myelin

proteolipid-protein-specific T cell clones and synthetic peptides. *Pathobiology,* 59**,** 305-12.

KUESTNER, R. E., TAFT, D. W., HARAN, A., BRANDT, C. S., BRENDER, T., LUM, K., HARDER, B., OKADA, S., OSTRANDER, C. D., KREINDLER, J. L., AUJLA, S. J., REARDON, B., MOORE, M., SHEA, P., SCHRECKHISE, R., BUKOWSKI, T. R., PRESNELL, S., GUERRA-LEWIS, P., PARRISH-NOVAK, J., ELLSWORTH, J. L., JASPERS, S., LEWIS, K. E., APPLEBY, M., KOLLS, J. K., RIXON, M., WEST, J. W., GAO, Z. & LEVIN, S. D. 2007. Identification of the IL-17 receptor related molecule IL-17RC as the receptor for IL-17F. *J Immunol,* 179**,** 5462-73.

KULLBERG, M. C., JANKOVIC, D., FENG, C. G., HUE, S., GORELICK, P. L., MCKENZIE, B. S., CUA, D. J., POWRIE, F., CHEEVER, A. W., MALOY, K. J. & SHER, A. 2006. IL-23 plays a key role in Helicobacter hepaticus-induced T cell-dependent colitis. *J Exp Med,* 203**,** 2485-94.

KURATA, H., LEE, H. J., O'GARRA, A. & ARAI, N. 1999. Ectopic expression of activated Stat6 induces the expression of Th2-specific cytokines and transcription factors in developing Th1 cells. *Immunity,* 11**,** 677-88.

LAIRD, R. M., LAKY, K. & HAYES, S. M. 2010. Unexpected role for the B cell-specific Src family kinase B lymphoid kinase in the development of IL-17-producing gammadelta T cells. *J Immunol,* 185**,** 6518-27.

LANGRISH, C. L., CHEN, Y., BLUMENSCHEIN, W. M., MATTSON, J., BASHAM, B., SEDGWICK, J. D., MCCLANAHAN, T., KASTELEIN, R. A. & CUA, D. J. 2005. IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. *J Exp Med,* 201**,** 233-40.

LAURENCE, A., TATO, C. M., DAVIDSON, T. S., KANNO, Y., CHEN, Z., YAO, Z., BLANK, R. B., MEYLAN, F., SIEGEL, R., HENNIGHAUSEN, L., SHEVACH, E. M. & O'SHEA J, J. 2007. Interleukin-2 signaling via STAT5 constrains T helper 17 cell generation. *Immunity,* 26**,** 371-81.

LIANG, S. C., TAN, X. Y., LUXENBERG, D. P., KARIM, R., DUNUSSI-JOANNOPOULOS, K., COLLINS, M. & FOUSER, L. A. 2006. Interleukin (IL)-22 and IL-17 are coexpressed by Th17 cells and cooperatively enhance expression of antimicrobial peptides. *J Exp Med,* 203**,** 2271-9.

LIBBEY, J. E. & FUJINAMI, R. S. 2011. Experimental autoimmune encephalomyelitis as a testing paradigm for adjuvants and vaccines. *Vaccine,* 29**,** 3356-62.

LININGTON, C., BERGER, T., PERRY, L., WEERTH, S., HINZE-SELCH, D., ZHANG, Y., LU, H. C., LASSMANN, H. & WEKERLE, H. 1993. T cells specific for the myelin oligodendrocyte glycoprotein mediate an unusual autoimmune inflammatory response in the central nervous system. *Eur J Immunol,* 23**,** 1364-72.

LOCHNER, M., PEDUTO, L., CHERRIER, M., SAWA, S., LANGA, F., VARONA, R., RIETHMACHER, D., SI-TAHAR, M., DI SANTO, J. P. & EBERL, G. 2008. In vivo equilibrium of proinflammatory IL-17+ and regulatory IL-10+ Foxp3+ RORgamma t+ T cells. *J Exp Med,* 205**,** 1381-93.

LOCKHART, E., GREEN, A. M. & FLYNN, J. L. 2006. IL-17 production is dominated by gammadelta T cells rather than CD4 T cells during Mycobacterium tuberculosis infection. *J Immunol,* 177**,** 4662-9.

LOHNING, M., HEGAZY, A. N., PINSCHEWER, D. D., BUSSE, D., LANG, K. S., HOFER, T., RADBRUCH, A., ZINKERNAGEL, R. M. & HENGARTNER, H. 2008. Long-lived virus-reactive memory T cells generated from purified cytokine-secreting T helper type 1 and type 2 effectors. *J Exp Med,* 205**,** 53-61.

MACATONIA, S. E., HOSKEN, N. A., LITTON, M., VIEIRA, P., HSIEH, C. S., CULPEPPER, J. A., WYSOCKA, M., TRINCHIERI, G., MURPHY, K. M. & O'GARRA, A. 1995. Dendritic cells produce IL-12 and direct the development of Th1 cells from naive CD4+ T cells. *J Immunol,* 154**,** 5071-9.

MALHOTRA, N., NARAYAN, K., CHO, O. H., SYLVIA, K. E., YIN, C., MELICHAR, H., RASHIGHI, M., LEFEBVRE, V., HARRIS, J. E., BERG, L. J., KANG, J. & IMMUNOLOGICAL GENOME PROJECT, C. 2013. A network of high-mobility group box transcription factors programs innate interleukin-17 production. *Immunity,* 38**,** 681-93.

MANCARDI, G. L., SARDANELLI, F., PARODI, R. C., MELANI, E., CAPELLO, E., INGLESE, M., FERRARI, A., SORMANI, M. P., OTTONELLO, C., LEVRERO, F., UCCELLI, A. & BRUZZI, P. 1998. Effect of copolymer-1 on serial gadolinium-enhanced MRI in relapsing remitting multiple sclerosis. *Neurology,* 50**,** 1127-33.

MANGAN, P. R., HARRINGTON, L. E., O'QUINN, D. B., HELMS, W. S., BULLARD, D. C., ELSON, C. O., HATTON, R. D., WAHL, S. M., SCHOEB, T. R. & WEAVER, C. T. 2006. Transforming growth factor-beta induces development of the T(H)17 lineage. *Nature,* 441**,** 231-4.

MARTIN, B., HIROTA, K., CUA, D. J., STOCKINGER, B. & VELDHOEN, M. 2009. Interleukin-17-producing gammadelta T cells selectively expand in response to pathogen products and environmental signals. *Immunity,* 31**,** 321-30.

MATIS, L. A., FRY, A. M., CRON, R. Q., COTTERMAN, M. M., DICK, R. F. & BLUESTONE, J. A. 1989. Structure and specificity of a class II MHC alloreactive gamma delta T cell receptor heterodimer. *Science,* 245**,** 746-9.

MATUSEVICIUS, D., KIVISAKK, P., HE, B., KOSTULAS, N., OZENCI, V., FREDRIKSON, S. & LINK, H. 1999. Interleukin-17 mRNA expression in blood and CSF mononuclear cells is augmented in multiple sclerosis. *Mult Scler,* 5**,** 101-4.

MCGEACHY, M. J., BAK-JENSEN, K. S., CHEN, Y., TATO, C. M., BLUMENSCHEIN, W., MCCLANAHAN, T. & CUA, D. J. 2007. TGF-beta and IL-6 drive the production of IL-17 and IL-10 by T cells and restrain T(H)-17 cell-mediated pathology. *Nat Immunol,* 8**,** 1390-7.

MCGEACHY, M. J., CHEN, Y., TATO, C. M., LAURENCE, A., JOYCE-SHAIKH, B., BLUMENSCHEIN, W. M., MCCLANAHAN, T. K., O'SHEA, J. J. & CUA, D. J. 2009. The interleukin 23 receptor is essential for the terminal differentiation of interleukin 17-producing effector T helper cells in vivo. *Nat Immunol,* 10**,** 314-24.

MILLER, J. F. & OSOBA, D. 1967. Current concepts of the immunological function of the thymus. *Physiol Rev,* 47**,** 437-520.

MIX, E., OLSSON, T., CORREALE, J., KOSTULAS, V. & LINK, H. 1990. CD4+, CD8+, and CD4- CD8- T cells in CSF and blood of patients with multiple sclerosis and tension headache. *Scand J Immunol,* 31**,** 493-501.

MOENS, E., BROUWER, M., DIMOVA, T., GOLDMAN, M., WILLEMS, F. & VERMIJLEN, D. 2011. IL-23R and TCR signaling drives the generation of neonatal Vgamma9Vdelta2 T cells expressing high levels of cytotoxic mediators and producing IFN-gamma and IL-17. *J Leukoc Biol,* 89**,** 743-52.

MOSELEY, T. A., HAUDENSCHILD, D. R., ROSE, L. & REDDI, A. H. 2003. Interleukin-17 family and IL-17 receptors. *Cytokine Growth Factor Rev,* 14**,** 155-74.

MOSMANN, T. R., CHERWINSKI, H., BOND, M. W., GIEDLIN, M. A. & COFFMAN, R. L. 1986. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J Immunol,* 136**,** 2348-57.

MULLER, M. S., OBEL, L. F., WAAGEPETERSEN, H. S., SCHOUSBOE, A. & BAK, L. K. 2013. Complex actions of ionomycin in cultured cerebellar astrocytes affecting both calcium-induced calcium release and store-operated calcium entry. *Neurochem Res,* 38**,** 1260-5.

MURPHY, C. A., LANGRISH, C. L., CHEN, Y., BLUMENSCHEIN, W., MCCLANAHAN, T., KASTELEIN, R. A., SEDGWICK, J. D. & CUA, D. J. 2003. Divergent pro- and antiinflammatory roles for IL-23 and IL-12 in joint autoimmune inflammation. *J Exp Med,* 198**,** 1951-7.

NAKAE, S., NAMBU, A., SUDO, K. & IWAKURA, Y. 2003. Suppression of immune induction of collagen-induced arthritis in IL-17-deficient mice. *J Immunol,* 171**,** 6173-7.

NARAYAN, K., SYLVIA, K. E., MALHOTRA, N., YIN, C. C., MARTENS, G., VALLERSKOG, T., KORNFELD, H., XIONG, N., COHEN, N. R., BRENNER, M. B., BERG, L. J., KANG, J. & IMMUNOLOGICAL GENOME PROJECT, C. 2012. Intrathymic programming of effector fates in three molecularly distinct gammadelta T cell subtypes. *Nat Immunol,* 13**,** 511-8.

NATH, N., PRASAD, R., GIRI, S., SINGH, A. K. & SINGH, I. 2006. T-bet is essential for the progression of experimental autoimmune encephalomyelitis. *Immunology,* 118**,** 384-91.

NESS-SCHWICKERATH, K. J. & MORITA, C. T. 2011. Regulation and function of IL-17A- and IL-22-producing gammadelta T cells. *Cell Mol Life Sci,* 68**,** 2371-90.

NEUHAUS, O., FARINA, C., YASSOURIDIS, A., WIENDL, H., THEN BERGH, F., DOSE, T., WEKERLE, H. & HOHLFELD, R. 2000. Multiple sclerosis: comparison of copolymer-1- reactive T cell lines from treated and untreated subjects reveals cytokine shift from T helper 1 to T helper 2 cells. *Proc Natl Acad Sci U S A,* 97**,** 7452-7.

NURIEVA, R., YANG, X. O., MARTINEZ, G., ZHANG, Y., PANOPOULOS, A. D., MA, L., SCHLUNS, K., TIAN, Q., WATOWICH, S. S., JETTEN, A. M. & DONG, C. 2007. Essential autocrine regulation by IL-21 in the generation of inflammatory T cells. *Nature,* 448**,** 480-3.

O'BRIEN, R. L., HAPP, M. P., DALLAS, A., PALMER, E., KUBO, R. & BORN, W. K. 1989. Stimulation of a major subset of lymphocytes expressing T cell receptor gamma delta by an antigen derived from Mycobacterium tuberculosis. *Cell,* 57**,** 667-74.

ODYNIEC, A., SZCZEPANIK, M., MYCKO, M. P., STASIOLEK, M., RAINE, C. S. & SELMAJ, K. W. 2004. Gammadelta T cells enhance the expression of experimental autoimmune encephalomyelitis by promoting antigen presentation and IL-12 production. *J Immunol,* 173**,** 682-94.

OPPMANN, B., LESLEY, R., BLOM, B., TIMANS, J. C., XU, Y., HUNTE, B., VEGA, F., YU, N., WANG, J., SINGH, K., ZONIN, F., VAISBERG, E., CHURAKOVA, T., LIU, M., GORMAN, D., WAGNER, J., ZURAWSKI, S., LIU, Y., ABRAMS, J. S., MOORE, K. W., RENNICK, D., DE WAAL-MALEFYT, R., HANNUM, C., BAZAN, J. F. & KASTELEIN, R. A. 2000. Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. *Immunity,* 13**,** 715-25.

OREJA-GUEVARA, C., RAMOS-CEJUDO, J., AROEIRA, L. S., CHAMORRO, B. & DIEZ-TEJEDOR, E. 2012. TH1/TH2 Cytokine profile in relapsing-remitting multiple sclerosis patients treated with Glatiramer acetate or Natalizumab. *BMC Neurol,* 12**,** 95.

PAGET, C., CHOW, M. T., DURET, H., MATTAROLLO, S. R. & SMYTH, M. J. 2012. Role of gammadelta T cells in alpha-galactosylceramide-mediated immunity. *J Immunol,* 188**,** 3928-39.

PANITCH, H. S., HIRSCH, R. L., HALEY, A. S. & JOHNSON, K. P. 1987. Exacerbations of multiple sclerosis in patients treated with gamma interferon. *Lancet,* 1**,** 893-5.

PANTELYUSHIN, S., HAAK, S., INGOLD, B., KULIG, P., HEPPNER, F. L., NAVARINI, A. A. & BECHER, B. 2012. Rorgammat+ innate lymphocytes and gammadelta T cells initiate psoriasiform plaque formation in mice. *J Clin Invest,* 122**,** 2252-6.

PARHAM, C., CHIRICA, M., TIMANS, J., VAISBERG, E., TRAVIS, M., CHEUNG, J., PFLANZ, S., ZHANG, R., SINGH, K. P., VEGA, F., TO, W., WAGNER, J., O'FARRELL, A. M., MCCLANAHAN, T., ZURAWSKI, S., HANNUM, C., GORMAN, D., RENNICK, D. M., KASTELEIN, R. A., DE WAAL MALEFYT, R. & MOORE, K. W. 2002. A receptor for the heterodimeric cytokine IL-23 is composed of IL-12Rbeta1 and a novel cytokine receptor subunit, IL-23R. *J Immunol,* 168**,** 5699-708.

PARK, H., LI, Z., YANG, X. O., CHANG, S. H., NURIEVA, R., WANG, Y. H., WANG, Y., HOOD, L., ZHU, Z., TIAN, Q. & DONG, C. 2005. A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nat Immunol,* 6**,** 1133-41.

PARK, S. G., MATHUR, R., LONG, M., HOSH, N., HAO, L., HAYDEN, M. S. & GHOSH, S. 2010. T regulatory cells maintain intestinal homeostasis by suppressing gammadelta T cells. *Immunity,* 33**,** 791-803.

PENG, M. Y., WANG, Z. H., YAO, C. Y., JIANG, L. N., JIN, Q. L., WANG, J. & LI, B. Q. 2008. Interleukin 17-producing gamma delta T cells increased in patients with active pulmonary tuberculosis. *Cell Mol Immunol,* 5**,** 203-8.

PETERMAN, G. M., SPENCER, C., SPERLING, A. I. & BLUESTONE, J. A. 1993. Role of gamma delta T cells in murine collagen-induced arthritis. *J Immunol,* 151**,** 6546-58.

PETERMANN, F. & KORN, T. 2011. Cytokines and effector T cell subsets causing autoimmune CNS disease. *FEBS Lett,* 585**,** 3747-57.

PETERSON, J. W., BO, L., MORK, S., CHANG, A. & TRAPP, B. D. 2001. Transected neurites, apoptotic neurons, and reduced inflammation in cortical multiple sclerosis lesions. *Ann Neurol,* 50**,** 389-400.

POLLINGER, B. 2012. IL-17 producing T cells in mouse models of multiple sclerosis and rheumatoid arthritis. *J Mol Med (Berl),* 90**,** 613-24.

POWELL, B. R., BUIST, N. R. & STENZEL, P. 1982. An X-linked syndrome of diarrhea, polyendocrinopathy, and fatal infection in infancy. *J Pediatr,* 100**,** 731-7.

POWOLNY-BUDNICKA, I., RIEMANN, M., TANZER, S., SCHMID, R. M., HEHLGANS, T. & WEIH, F. 2011. RelA and RelB transcription factors in distinct thymocyte populations control lymphotoxin-dependent interleukin-17 production in gammadelta T cells. *Immunity,* 34**,** 364-74.

RACKE, M. K., BONOMO, A., SCOTT, D. E., CANNELLA, B., LEVINE, A., RAINE, C. S., SHEVACH, E. M. & ROCKEN, M. 1994. Cytokine-induced immune deviation as a therapy for inflammatory autoimmune disease. *J Exp Med,* 180**,** 1961-6.

RAIFER, H., MAHINY, A. J., BOLLIG, N., PETERMANN, F., HELLHUND, A., KELLNER, K., GURALNIK, A., REINHARD, K., BOTHUR, E., HUBER, M., BAUER, S., LOHNING, M., KISS, E. A., GANAL, S. C., DIEFENBACH, A., KORN, T. & LOHOFF, M. 2012. Unlike alphabeta T cells, gammadelta T cells, LTi cells and NKT cells do not require IRF4 for the production of IL-17A and IL-22. *Eur J Immunol,* 42**,** 3189-201.

RAJAN, A. J., ASENSIO, V. C., CAMPBELL, I. L. & BROSNAN, C. F. 2000. Experimental autoimmune encephalomyelitis on the SJL mouse: effect of gamma delta T cell depletion on chemokine and chemokine receptor expression in the central nervous system. *J Immunol,* 164**,** 2120-30.

RAJAN, A. J., GAO, Y. L., RAINE, C. S. & BROSNAN, C. F. 1996. A pathogenic role for gamma delta T cells in relapsing-remitting experimental allergic encephalomyelitis in the SJL mouse. *J Immunol,* 157**,** 941-9.

RAJAN, A. J., KLEIN, J. D. & BROSNAN, C. F. 1998. The effect of gammadelta T cell depletion on cytokine gene expression in experimental allergic encephalomyelitis. *J Immunol,* 160**,** 5955-62.

REBOLDI, A., COISNE, C., BAUMJOHANN, D., BENVENUTO, F., BOTTINELLI, D., LIRA, S., UCCELLI, A., LANZAVECCHIA, A., ENGELHARDT, B. & SALLUSTO, F. 2009. C-C chemokine receptor 6-regulated entry of TH-17 cells into the CNS through the choroid plexus is required for the initiation of EAE. *Nat Immunol,* 10**,** 514-23.

REGE, T. A. & HAGOOD, J. S. 2006. Thy-1 as a regulator of cell-cell and cell-matrix interactions in axon regeneration, apoptosis, adhesion, migration, cancer, and fibrosis. *FASEB J,* 20**,** 1045-54.

RIBOT, J. C., CHAVES-FERREIRA, M., D'OREY, F., WENCKER, M., GONCALVES-SOUSA, N., DECALF, J., SIMAS, J. P., HAYDAY, A. C. & SILVA-SANTOS, B. 2010. Cutting edge: adaptive versus innate receptor signals selectively control the pool sizes of murine IFN-gamma- or IL-17-producing gammadelta T cells upon infection. *J Immunol,* 185**,** 6421-5.

RIBOT, J. C., DEBARROS, A., PANG, D. J., NEVES, J. F., PEPERZAK, V., ROBERTS, S. J., GIRARDI, M., BORST, J., HAYDAY, A. C., PENNINGTON, D. J. & SILVA-SANTOS, B. 2009. CD27 is a thymic determinant of the balance between interferon-gamma- and interleukin 17-producing gammadelta T cell subsets. *Nat Immunol,* 10**,** 427-36.

RIVERS, T. M. & SCHWENTKER, F. F. 1935. Encephalomyelitis Accompanied by Myelin Destruction Experimentally Produced in Monkeys. *J Exp Med,* 61**,** 689-702.

ROARK, C. L., FRENCH, J. D., TAYLOR, M. A., BENDELE, A. M., BORN, W. K. & O'BRIEN, R. L. 2007. Exacerbation of collagen-induced arthritis by oligoclonal, IL-17-producing gamma delta T cells. *J Immunol,* 179**,** 5576-83.

ROBINSON, D. S., HAMID, Q., YING, S., TSICOPOULOS, A., BARKANS, J., BENTLEY, A. M., CORRIGAN, C., DURHAM, S. R. & KAY, A. B. 1992. Predominant TH2-like bronchoalveolar T-lymphocyte population in atopic asthma. *N Engl J Med,* 326**,** 298-304.

SASAKI, K. & SONODA, Y. 2000. Histometrical and three-dimensional analyses of liver hematopoiesis in the mouse embryo. *Arch Histol Cytol,* 63**,** 137-46.

SCHIRMER, L., ROTHHAMMER, V., HEMMER, B. & KORN, T. 2013. Enriched CD161high CCR6+ gammadelta T cells in the cerebrospinal fluid of patients with multiple sclerosis. *JAMA Neurol,* 70**,** 345-51.

SHIBATA, K., YAMADA, H., HARA, H., KISHIHARA, K. & YOSHIKAI, Y. 2007. Resident Vdelta1+ gammadelta T cells control early infiltration of neutrophils after Escherichia coli infection via IL-17 production. *J Immunol,* 178**,** 4466-72.

SHIBATA, K., YAMADA, H., SATO, T., DEJIMA, T., NAKAMURA, M., IKAWA, T., HARA, H., YAMASAKI, S., KAGEYAMA, R., IWAKURA, Y., KAWAMOTO, H., TOH, H. & YOSHIKAI, Y. 2011. Notch-Hes1 pathway is required for the development of IL-17-producing gammadelta T cells. *Blood,* 118**,** 586-93.

SHIMONKEVITZ, R., COLBURN, C., BURNHAM, J. A., MURRAY, R. S. & KOTZIN, B. L. 1993. Clonal expansions of activated gamma/delta T cells in recent-onset multiple sclerosis. *Proc Natl Acad Sci U S A,* 90**,** 923-7.

SMITH, R. L., WARREN, R. B., EYRE, S., HO, P., KE, X., YOUNG, H. S., GRIFFITHS, C. E. & WORTHINGTON, J. 2008. Polymorphisms in the IL-12beta and IL-23R genes are associated with psoriasis of early onset in a UK cohort. *J Invest Dermatol,* 128**,** 1325-7.

SMITH, S. S. & BARNUM, S. R. 2008. Differential expression of beta 2-integrins and cytokine production between gammadelta and alphabeta T cells in experimental autoimmune encephalomyelitis. *J Leukoc Biol,* 83**,** 71-9.

SPAHN, T. W., ISSAZADAH, S., SALVIN, A. J. & WEINER, H. L. 1999. Decreased severity of myelin oligodendrocyte glycoprotein peptide 33 - 35-induced experimental autoimmune encephalomyelitis in mice with a disrupted TCR delta chain gene. *Eur J Immunol,* 29**,** 4060-71.

STARNES, T., ROBERTSON, M. J., SLEDGE, G., KELICH, S., NAKSHATRI, H., BROXMEYER, H. E. & HROMAS, R. 2001. Cutting edge: IL-17F, a novel cytokine selectively expressed in activated T cells and monocytes, regulates angiogenesis and endothelial cell cytokine production. *J Immunol,* 167**,** 4137-40.

STINISSEN, P., VANDEVYVER, C., MEDAER, R., VANDEGAER, L., NIES, J., TUYLS, L., HAFLER, D. A., RAUS, J. & ZHANG, J. 1995. Increased frequency of gamma delta T cells in cerebrospinal fluid and peripheral blood of patients with multiple sclerosis. Reactivity, cytotoxicity, and T cell receptor V gene rearrangements. *J Immunol,* 154**,** 4883-94.

SUTTON, C., BRERETON, C., KEOGH, B., MILLS, K. H. & LAVELLE, E. C. 2006. A crucial role for interleukin (IL)-1 in the induction of IL-17-producing T cells that mediate autoimmune encephalomyelitis. *J Exp Med,* 203**,** 1685-91.

SUTTON, C. E., LALOR, S. J., SWEENEY, C. M., BRERETON, C. F., LAVELLE, E. C. & MILLS, K. H. 2009. Interleukin-1 and IL-23 induce innate IL-17 production from gammadelta T cells, amplifying Th17 responses and autoimmunity. *Immunity,* 31**,** 331-41.

TAM, S., KING, D. P. & BEAMAN, B. L. 2001. Increase of gammadelta T lymphocytes in murine lungs occurs during recovery from pulmonary infection by Nocardia asteroides. *Infect Immun,* 69**,** 6165-71.

TARTAKOFF, A. M. & VASSALLI, P. 1977. Plasma cell immunoglobulin secretion: arrest is accompanied by alterations of the golgi complex. *J Exp Med,* 146**,** 1332-45.

TEUNISSEN, M. B., KOOMEN, C. W., DE WAAL MALEFYT, R., WIERENGA, E. A. & BOS, J. D. 1998. Interleukin-17 and interferon-gamma synergize in the enhancement of proinflammatory cytokine production by human keratinocytes. *J Invest Dermatol,* 111**,** 645-9.

THEDREZ, A., SABOURIN, C., GERTNER, J., DEVILDER, M. C., ALLAIN-MAILLET, S., FOURNIE, J. J., SCOTET, E. & BONNEVILLE, M. 2007. Self/non-self discrimination by human gammadelta T cells: simple solutions for a complex issue? *Immunol Rev,* 215**,** 123-35.

TOH, M. L., GONZALES, G., KOENDERS, M. I., TOURNADRE, A., BOYLE, D., LUBBERTS, E., ZHOU, Y., FIRESTEIN, G. S., VAN DEN BERG, W. B. & MIOSSEC, P. 2010. Role of interleukin 17 in arthritis chronicity through survival of synoviocytes via regulation of synoviolin expression. *PLoS One,* 5**,** e13416.

TOSELLO BOARI, J., AMEZCUA VESELY, M. C., BERMEJO, D. A., RAMELLO, M. C., MONTES, C. L., CEJAS, H., GRUPPI, A. & ACOSTA RODRIGUEZ, E. V. 2012. IL-17RA signaling reduces inflammation and mortality during Trypanosoma cruzi

infection by recruiting suppressive IL-10-producing neutrophils. *PLoS Pathog,* 8**,** e1002658.

TOY, D., KUGLER, D., WOLFSON, M., VANDEN BOS, T., GURGEL, J., DERRY, J., TOCKER, J. & PESCHON, J. 2006. Cutting edge: interleukin 17 signals through a heteromeric receptor complex. *J Immunol,* 177**,** 36-9.

TRAPP, B. D., PETERSON, J., RANSOHOFF, R. M., RUDICK, R., MORK, S. & BO, L. 1998. Axonal transection in the lesions of multiple sclerosis. *N Engl J Med,* 338**,** 278-85.

TRUSHIN, S. A., PENNINGTON, K. N., ALGECIRAS-SCHIMNICH, A. & PAYA, C. V. 1999. Protein kinase C and calcineurin synergize to activate IkappaB kinase and NF-kappaB in T lymphocytes. *J Biol Chem,* 274**,** 22923-31.

TSUJI, S., MATSUMOTO, M., TAKEUCHI, O., AKIRA, S., AZUMA, I., HAYASHI, A., TOYOSHIMA, K. & SEYA, T. 2000. Maturation of human dendritic cells by cell wall skeleton of Mycobacterium bovis bacillus Calmette-Guerin: involvement of toll-like receptors. *Infect Immun,* 68**,** 6883-90.

TURCHINOVICH, G. & HAYDAY, A. C. 2011. Skint-1 identifies a common molecular mechanism for the development of interferon-gamma-secreting versus interleukin-17-secreting gammadelta T cells. *Immunity,* 35**,** 59-68.

UHLIG, H. H., MCKENZIE, B. S., HUE, S., THOMPSON, C., JOYCE-SHAIKH, B., STEPANKOVA, R., ROBINSON, N., BUONOCORE, S., TLASKALOVA-HOGENOVA, H., CUA, D. J. & POWRIE, F. 2006. Differential activity of IL-12 and IL-23 in mucosal and systemic innate immune pathology. *Immunity,* 25**,** 309-18.

VAN AMELSFORT, J. M., JACOBS, K. M., BIJLSMA, J. W., LAFEBER, F. P. & TAAMS, L. S. 2004. CD4(+)CD25(+) regulatory T cells in rheumatoid arthritis: differences in the presence, phenotype, and function between peripheral blood and synovial fluid. *Arthritis Rheum,* 50**,** 2775-85.

VAN BELLE, A. B., DE HEUSCH, M., LEMAIRE, M. M., HENDRICKX, E., WARNIER, G., DUNUSSI-JOANNOPOULOS, K., FOUSER, L. A., RENAULD, J. C. & DUMOUTIER, L. 2012. IL-22 is required for imiquimod-induced psoriasiform skin inflammation in mice. *J Immunol,* 188**,** 462-9.

VAN DER FITS, L., MOURITS, S., VOERMAN, J. S., KANT, M., BOON, L., LAMAN, J. D., CORNELISSEN, F., MUS, A. M., FLORENCIA, E., PRENS, E. P. & LUBBERTS, E. 2009. Imiquimod-induced psoriasis-like skin inflammation in mice is mediated via the IL-23/IL-17 axis. *J Immunol,* 182**,** 5836-45.

VAVROVA, J. & PETYREK, P. 1988. Effect of short-term splitting of gamma-ray doses on changes in haemopoietic stem cell number in bone marrow and on mortality of whole-body irradiated mice. *Folia Biol (Praha),* 34**,** 399-408.

VELDHOEN, M., HOCKING, R. J., ATKINS, C. J., LOCKSLEY, R. M. & STOCKINGER, B. 2006. TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. *Immunity,* 24**,** 179-89.

VERMEIRE, K., HEREMANS, H., VANDEPUTTE, M., HUANG, S., BILLIAU, A. & MATTHYS, P. 1997. Accelerated collagen-induced arthritis in IFN-gamma receptor-deficient mice. *J Immunol,* 158**,** 5507-13.

VIGLIETTA, V., BAECHER-ALLAN, C., WEINER, H. L. & HAFLER, D. A. 2004. Loss of functional suppression by CD4+CD25+ regulatory T cells in patients with multiple sclerosis. *J Exp Med,* 199**,** 971-9.

WEAVER, C. T., HATTON, R. D., MANGAN, P. R. & HARRINGTON, L. E. 2007. IL-17 family cytokines and the expanding diversity of effector T cell lineages. *Annu Rev Immunol,* 25**,** 821-52.

WEHRENS, E. J., MIJNHEER, G., DUURLAND, C. L., KLEIN, M., MEERDING, J., VAN LOOSDREGT, J., DE JAGER, W., SAWITZKI, B., COFFER, P. J., VASTERT, B., PRAKKEN, B. J. & VAN WIJK, F. 2011. Functional human regulatory T cells fail to control autoimmune inflammation due to PKB/c-akt hyperactivation in effector cells. *Blood,* 118**,** 3538-48.

WEI, L., LAURENCE, A., ELIAS, K. M. & O'SHEA, J. J. 2007. IL-21 is produced by Th17 cells and drives IL-17 production in a STAT3-dependent manner. *J Biol Chem,* 282**,** 34605-10.

WEINTRAUB, B. C., JACKSON, M. R. & HEDRICK, S. M. 1994. Gamma delta T cells can recognize nonclassical MHC in the absence of conventional antigenic peptides. *J Immunol,* 153**,** 3051-8.

WIEKOWSKI, M. T., LEACH, M. W., EVANS, E. W., SULLIVAN, L., CHEN, S. C., VASSILEVA, G., BAZAN, J. F., GORMAN, D. M., KASTELEIN, R. A., NARULA, S. & LIRA, S. A. 2001. Ubiquitous transgenic expression of the IL-23 subunit p19 induces multiorgan inflammation, runting, infertility, and premature death. *J Immunol,* 166**,** 7563-70.

WILLENBORG, D. O., FORDHAM, S., BERNARD, C. C., COWDEN, W. B. & RAMSHAW, I. A. 1996. IFN-gamma plays a critical down-regulatory role in the induction and effector phase of myelin oligodendrocyte glycoprotein-induced autoimmune encephalomyelitis. *J Immunol,* 157**,** 3223-7.

WOHLER, J. E., SMITH, S. S., ZINN, K. R., BULLARD, D. C. & BARNUM, S. R. 2009. Gammadelta T cells in EAE: early trafficking events and cytokine requirements. *Eur J Immunol,* 39**,** 1516-26.

WUCHERPFENNIG, K. W., NEWCOMBE, J., LI, H., KEDDY, C., CUZNER, M. L. & HAFLER, D. A. 1992. Gamma delta T-cell receptor repertoire in acute multiple sclerosis lesions. *Proc Natl Acad Sci U S A,* 89**,** 4588-92.

YANG, X. O., CHANG, S. H., PARK, H., NURIEVA, R., SHAH, B., ACERO, L., WANG, Y. H., SCHLUNS, K. S., BROADDUS, R. R., ZHU, Z. & DONG, C. 2008a. Regulation of inflammatory responses by IL-17F. *J Exp Med,* 205**,** 1063-75.

YANG, X. O., PAPPU, B. P., NURIEVA, R., AKIMZHANOV, A., KANG, H. S., CHUNG, Y., MA, L., SHAH, B., PANOPOULOS, A. D., SCHLUNS, K. S., WATOWICH, S. S., TIAN, Q., JETTEN, A. M. & DONG, C. 2008b. T helper 17 lineage differentiation is programmed by orphan nuclear receptors ROR alpha and ROR gamma. *Immunity,* 28**,** 29-39.

YAO, Z., FANSLOW, W. C., SELDIN, M. F., ROUSSEAU, A. M., PAINTER, S. L., COMEAU, M. R., COHEN, J. I. & SPRIGGS, M. K. 1995. Herpesvirus Saimiri encodes a new cytokine, IL-17, which binds to a novel cytokine receptor. *Immunity,* 3**,** 811-21.

YE, P., RODRIGUEZ, F. H., KANALY, S., STOCKING, K. L., SCHURR, J., SCHWARZENBERGER, P., OLIVER, P., HUANG, W., ZHANG, P., ZHANG, J., SHELLITO, J. E., BAGBY, G. J., NELSON, S., CHARRIER, K., PESCHON, J. J. & KOLLS, J. K. 2001. Requirement of interleukin 17 receptor signaling for lung CXC chemokine and granulocyte colony-stimulating factor expression, neutrophil recruitment, and host defense. *J Exp Med,* 194**,** 519-27.

YEN, D., CHEUNG, J., SCHEERENS, H., POULET, F., MCCLANAHAN, T., MCKENZIE, B., KLEINSCHEK, M. A., OWYANG, A., MATTSON, J., BLUMENSCHEIN, W., MURPHY, E., SATHE, M., CUA, D. J., KASTELEIN, R. A. & RENNICK, D. 2006. IL-23 is essential for T cell-mediated colitis and promotes inflammation via IL-17 and IL-6. *J Clin Invest,* 116**,** 1310-6.

YONG, T., MEININGER, G. A. & LINTHICUM, D. S. 1993. Enhancement of histamine-induced vascular leakage by pertussis toxin in SJL/J mice but not BALB/c mice. *J Neuroimmunol,* 45**,** 47-52.

YOSHIDA, S. & PLANT, S. 1992. Mechanism of release of Ca2+ from intracellular stores in response to ionomycin in oocytes of the frog Xenopus laevis. *J Physiol,* 458**,** 307-18.

YU, J. J., RUDDY, M. J., WONG, G. C., SFINTESCU, C., BAKER, P. J., SMITH, J. B., EVANS, R. T. & GAFFEN, S. L. 2007. An essential role for IL-17 in preventing pathogen-initiated bone destruction: recruitment of neutrophils to inflamed bone requires IL-17 receptor-dependent signals. *Blood,* 109**,** 3794-802.

ZHANG, Z., ZHENG, M., BINDAS, J., SCHWARZENBERGER, P. & KOLLS, J. K. 2006. Critical role of IL-17 receptor signaling in acute TNBS-induced colitis. *Inflamm Bowel Dis,* 12**,** 382-8.

ZHENG, W. & FLAVELL, R. A. 1997. The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells. *Cell,* 89**,** 587-96.

ZHENG, Y., DANILENKO, D. M., VALDEZ, P., KASMAN, I., EASTHAM-ANDERSON, J., WU, J. & OUYANG, W. 2007. Interleukin-22, a T(H)17 cytokine, mediates IL-23-induced dermal inflammation and acanthosis. *Nature,* 445**,** 648-51.

8 Appendix

8.1 List of figures

8.2 List of tables

8.3 Attributions

The data shown in Figure 5.7 and Figure 5.8 were generated by Dr. Lorena Riol-Blanco (Department of Pathology, Harvard Medical School, Boston, USA).

The Real-time PCR experiment depicted in Figure 5.6C was performed by Jan D. Haas (Institute for Immunology, Medical School Hannover, Hannover, Germany).

8.4 Abbreviations

Actb	Beta-Actin
APC	Antigen presenting cell
BBB	Blood brain barrier
Blk	B lymphoid kinase
BMC	Bone marrow chimera
BSA	Bovine serum albumin
CCL	Chemokine ligand
CCR	Chemokine (C-C motif) receptor
CXCR	Chemokine (C-X-C motif) receptor
CD	Cluster of differentiation
cDNA	Complementary DNA
CFA	Complete Freund's adjuvant
CIA	Collagen-induced arthritis
CNS	Central nervous system
CSF	Cerebrospinal fluid
DAG	Diacylglycerol
DC	Dendritic cell
DETC	Dendritic epidermal T cell
dLN	Draining lymph node
DMEM	Dulbecco's modified Eagle's medium
DN	Double negative
DNA	Desoxyribonucleic acid
DNAse	Desoxyribonuclease
EAE	Experimental autoimmune encephalomyelitis
EDTA	Ethylenediaminetetraacetic acid
e.g.	For example (*exempli gratia*)
Egr2	Early growth response protein 2
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum

Foxp3	Forkhead box p3
FRET	Förster resonance energy transfer
γδT1	IFN-γ producing γδ T cells
γδT17	IL-17 producing γδ T cells
GA	Glatiramer acetate
GATA3	GATA binding protein 3
GFP	Green fluorescent protein
GM-CSF	Granulocyte macrophage colony-stimulating factor
HIV	Human immunodeficiency virus
IBD	Inflammatory bowel disease
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
i.p.	Intraperitoneal
IPEX	Immune dysregulation, polyendocrinopathy, enteropathy, X-linked
IPP	Isopentenyl pyrophosphate
IRF4	Interferon regulatory factor 4
iTreg cell	Induced regulatory T cell
i.v.	Intravenous
kb	Kilobase
KI	Knock-in
KO	Knock-out
LN	Lymph node
mAB	Monoclonal antibody
MACS	Magnetic cell separation
MBP	Myelin basic protein
MCP-1	Monocyte chemoattractant protein-1
MHC	Major histocompatibility complex
MIP-2	Macrophage inflammatory protein 2
MOG	Myelin oligodendrocte glycoprotein
MS	Multiple sclerosis
NFAT	Nuclear factor of activated T cells

NF-κB	Nuclear factor κB
nTreg cell	Natural regulatory T cell
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PKC	Protein kinase C
PLP	Proteolipid protein
PMA	Phorbol myristate acetate
PRR	Pattern recognition receptor
R	Receptor
RA	Rheumatoid arthritis
Rag	Recombination-activating gene
RNA	Ribonucleic acid
Rorγt	Retinoic acid-related orphan receptor γt
s.c.	Subcutaneous
SD	Standard deviation
SEM	Standard error of the mean
SMAD	Sma and mad-related protein
Sox13	Sex determining region Y-box 13
SPL	Spleen
STAT	Signal transducer and activator of transcription
TAE	Tris-acetate-EDTA
T-bet	T-box expressed in T cells
Tbx21	T-box 21
TCR	T cell receptor
TGF-β	Transforming growth factor β
Th cell	T helper cell
THY	Thymus
TLR	Toll like receptor
TNF	Tumor necrosis factor
Treg cell	Regulatory T cell

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