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IL-12 antagonizes IL-23 in autoimmune inflammation by Blimp1-dependent programming of $CD4^+$ effector T cells into suppressive IL-10 producers

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Summary

Organ specific autoimmune diseases like multiple sclerosis are strongly regulated by the balance of pro-inflammatory and anti-inflammatory T helper cells. An uncontrolled activation of pro-inflammatory T cells such asTh17 and Th1 results in tissue injury and partly irreversible tissue damage. In contrast, anti-inflammatory T cells diminish the inflammation and prevent the CNS form destruction through autoimmune inflammation. Cytokines play an important role both as differentiation factors for pathogenic or tolerogenic T cell subsets and as effector molecules of these T cells. The signals and molecular switches that control the induction of anti-inflammatory cytokines like IL-10 in $CD4^+$ effector T cells during autoimmune inflammation are not well understood. Yet, the plasticity and re-programming of pathogenic T cell subsets into anti-inflammatory T cells receives increasing attention – also in the context of immunotherapeutic strategies.

The goal of this work was to expand the knowledge about the biological function of IL-12 cytokine family members during the generation of pathogenic T cell responses in autoimmune inflammation like multiple sclerosis. Using a micro array approach pro-inflammatory Th17 cells were compared with anti-inflammatory IL-10 producers, Tr-1 cells, to identify differently expressed transcription factors. Here, the transcriptional regulator Blimp1 (*Prdm1*) was detected to be expressed in Tr-1 cells but not in Th17 cells. In contrast to IL-23, IL-12 was a strong inducer of Blimp1 expression. Notably, IL-12/Stat4 induced IL-10 in pre-committed Tr-1 cells and also in Th17 cells in a Blimp1 dependent manner. Thus, while IL-23 stabilized the Th17 phenotype, IL-12 promoted the re-programming of Th17 cells into IL-10 producing Tr-1 like cells.

These *in vitro* findings were validated *in vivo*. Blimp1-deficient effector T cells and IL-12R deficient T cells failed to produce IL-10 in the central nervous system (CNS) and T cell conditional Blimp1 deficient mice were hyper-susceptible to experimental autoimmune encephalomyelitis (EAE). In line with this observation, ablation of *Il10* and *Ifng*, the effector molecules of Tr-1 cells, reversed the resistance to EAE of IL-23R deficient animals suggesting that the resistance of *Il23r*$^{-/-}$ mice to EAE is – at least in part – due to an exaggerated IL-12/Blimp1/IL-10 pathway.

In conclusion, the data presented in this thesis show that the balance of IL-23 and IL-12 signals into $CD4^+$ effector T cells determines whether tissue inflammation is perpetuated or resolved. This work demonstrates that Blimp1 acts as transcriptional switch, which is required for IL-10 production in committed $CD4^+$ T cells. This is an important aspect for the plasticity of T cells in terms of re-programming of pro-inflammatory T cells into anti-inflammatory IL-10 producers in the late phase of inflammatory processes.

Zusammenfassung

Autoimmunerkrankungen wie die Multiple Sklerose entstehen wahrscheinlich aufgrund einer gestörten Balance von pro- und anti-inflammatorischen T-Helferzellen. Eine unkontrollierte Aktivierung von pro-inflammatorischen T-Zellen, wie Th17 und Th1 Zellen, führt zu einer Gewebeschädigung mit teilweise irreversiblen Folgen. Im Gegensatz dazu wirken anti-inflammatorische T-Zellen entzündungssupprimierend und schützen so das Zentral-nervensystem (ZNS) vor Zerstörung durch Autoimmunreaktionen. Zytokine dienen den pathogenen und tolerogenen T-Zelluntergruppen dabei sowohl als Differenzierungsfaktoren als auch als Effektormoleküle. Jedoch sind bislang die Signale und molekularen Schalter unbekannt, die während der autoimmunen Pathogenese die Induktion von anti-inflammatorischen Zytokinen, wie IL-10 in $CD4^+$ Effektor-T-Zellen steuern. Zugleich bekommt die Plastizität und das Umprogrammieren von pathogenen T-Zelluntergruppen in anti-inflammatorische T-Zellen eine immer größere Bedeutung, besonders im Zusammenhang mit immuntherapeutischen Strategien.

Das Ziel dieser Arbeit war es, biologische Funktionen von IL-12 Zytokin-Familienmitgliedern während der Generierung von pathogenen T-Zellantworten in autoimmunen Entzündungen, wie Multiple Sklerose, besser zu verstehen. Mit Hilfe eines Microarrays wurden pro-inflammatorische Th17 Zellen mit anti-inflammatorischen IL-10 Produzenten, den sogenannten Tr-1 Zellen, verglichen, um unterschiedlich aktivierte Transkriptionsfaktoren zu identifizieren. Es konnte festgestellt werden, dass der regulatorische Transkriptionsfaktor Blimp1 (*Prdm1*) in Tr-1 Zellen aktiviert ist, in Th17 Zellen hingegen nicht. Im Gegensatz zu IL-23 ist IL-12 ein starker Aktivator der Blimp1 Expression. IL-12 induziert über einen Stat4 Signalweg Blimp1-abhängig IL-10 in Tr-1 Zellen als auch in Th17 Zellen. Somit fördert IL-12 das Umprogrammieren von Th17 Zellen in Tr-1- ähnliche IL-10 Produzenten. IL-23 hingegen stabilisiert den pro-inflammatorischen Th17 Phänotyp.

Die Resultate aus den *in vitro* Experimenten konnten *in vivo* bestätigt werden. Blimp1-defiziente Effektor-T-Zellen und IL-12R-defiziente T-Zellen verlieren die Fähigkeit, IL-10 im Zielorgan ZNS zu produzieren. Zusätzlich zeigen Blimp1-defiziente Mäuse eine Hyper-Sensibilität gegenüber EAE Induktion. In guter Übereinstimmung mit diesem Ergebnis zeigte sich, dass die genetische Depletion der Tr-1-Zell-Effektorzytokine *Il10* und *Ifng* die Resistenz der IL-23R KO Tiere gegenüber der EAE Induktion aufhebt. Daraus kann geschlussfolgert werden, dass die Resistenz der IL-23R KO Mäuse gegenüber EAE Induktion zumindest zum Teil auf eine übersteigerte Aktivierung des IL-12/Blimp1/IL-10 Signalweges zurückzuführen ist.

Zusammenfassend zeigen die Daten dieser Arbeit, dass die Balance von IL-23 und IL-12 Signalen in $CD4^+$ Effektor-T-Zellen entscheidet, ob Entzündungen im Gewebe aufrechterhalten oder aufgelöst werden. Es konnte gezeigt werden, dass Blimp1 als transkriptionaler Schalter wirkt und essenziell für die IL-10 Produktion in $CD4^+$ T-Zellen ist. Dies unterstreicht die wichtige Funktion von Blimp1 bei der Plastizität von T-Zellen hinsichtlich der Umprogrammierung von pro-inflammatorischen T-Zellen zu anti-inflammatorischen IL-10 Produzenten während der späten Phase von Entzündungsprozessen.

1 Introduction

1.1 Immune system

The immune system presents a defense system that protects the host against possibly harmful substances from the outside and damage from the inside by recognizing and responding to antigens. Microorganisms like bacteria or viruses are restrained to enter the body by mechanistic barriers. Nevertheless, should pathogens move in, they are recognized, marked and eventually eliminated by a variety of immune cell types and molecules. Thus, the function of host tissues is maintained or re-established. The immune system can be divided into two parts, the innate and the adaptive immune system. The innate system is phylogenetically old and represents the first line of defense. It destroys antigens in a non-specific manner and is based on physical and chemical barriers (skin and mucous membranes), cells (granulocytes, macrophages and natural killer cells (NK cells)), and the complement system. Cells of the innate immune system identify pathogens via small molecular structures or motifs of pathogens, so called pathogen-associated molecular patterns (PAMPs), which in turn are recognized by Toll-like receptors (TLRs) and other pattern recognition receptors (PRRs). The identification of pathogens by several receptors of highly conserved soluble and membrane-bound structures (like protein, DNA molecules or lipids), which are unique to microbes, results in a rapid trigger of inflammatory responses. These include secretion of chemical factors or mediators (like cytokines), activation of complement cascades and recruitment of phagocytic immune cells followed by activation of the adaptive immune system. In comparison to the adaptive immune system which is very flexible and constantly under development, the innate system is not adaptable but extremely fast. The adaptive immune system includes two parallel and mutually supportive immune reactions, the humoral response mediated by immunoglobulins (Ig) secreted by plasma cells and the cell-mediated response which is based on antigen-specific T cells. During the humoral immune response, B lymphocytes recognize antigens in a soluble form with their B cell receptor (BCR). The full activation of B cell requires a co-simulative trigger from an activated T cell. Activated B cells differentiate into plasma cells and produce soluble immunoglobulins. Some antigen specifically activated T cells and B cells develop into memory T or B cells and are thus able to mount a faster antigen specific immune response upon second exposure to the priming antigen.

1.2 Autoimmunity

Autoimmunity is an excessive immune response against a self-antigen, which results in “aseptic” tissue damage by inflammatory processes. Auto-antigen can be present locally (organ specific) or systemically. A small frequency of self-reactive lymphocytes is present in the circulation and the lymphoid tissues. To limit the generation and the activation of these cells with subsequent autoimmune inflammation, several mechanisms to keep immune tolerance are operational. For example due to immunologic ignorance, autoreactive lymphocytes never “see” their antigen because it is sequestrated behind anatomical barriers (e.g. the blood-brain-barrier). Furthermore, central and peripheral immune tolerance are processes to limit the frequency of autoreactive lymphocytes and to keep in check those autoreactive clones that “escaped” deletional tolerance mechanisms in the thymus and the bone marrow. The central immune tolerance of T cells include the first selection step during lymphocyte development, at which cells of the thymus epithelium present self-antigens on major histocompatibility complex (MHC) -I and MHC-II molecules. During positive selection, $CD4^+CD8^+$ (CD = cluster of differentiation) double positive precursor cells which do not bind MHC molecules do not receive survival signals (death by neglection). In addition, during negative selection, i. e. thymocytes, which bind autoantigen/MHC molecules on thymic medullary epithelial cells with excessive affinity are programmed for apoptotic cell death and are thus purged from the repertoire. As a result, only 1 to 2 % of the $CD4^+$ and $CD8^+$ single positive thymocytes mature into T cells that leave the thymus and populate peripheral lymphoid tissues. However, negative selection is leaky and some autoreactive clones escape thymic deletional tolerance (Anderson et al., 2000; Kuchroo et al., 2002). In the peripheral immune compartment, further tolerance mechanisms are aberrant leading to activation induced cell death or anergy and suppression of autoreactive T cells by regulatory T cells.

In case of failure of central and peripheral tolerance mechanisms, autoimmune diseases may occur. Autoimmune diseases are common diseases in industrialized countries and their incidence is increasing suggesting that environmental factors contribute to breaching immune tolerance. Genetic and environmental influences, infections, too few demands of the immune system as well as defects or disorder of immune tolerance are possible causes of autoimmune diseases. At the moment, it is assumed that multifactor cooperation of several factors is the beginning of immune mediated tissue damages.

1.3 Multiple sclerosis and EAE

Multiple sclerosis (MS) is the most common autoimmune inflammatory disease of the central nervous system (CNS) in adult humans, with prevalence of 0.15% (Kobelt et al., 2000). It affects young individuals between 15 to 45 years with a female predominance (Disanto et al., 2012; Minagar, 2013). The inflammation affects both, white and grey matter of CNS and results in demyelination and degeneration of several neurons and axons, which explains the multitude of various symptoms in MS. Clinically, the disease can be distinguished into four patterns: relapsing remitting (RR-MS), primary and secondary progressive (PP-MS and SP-MS), and relapsing progressive (RP-MS). During the remitting course, the attacks usual recover completely (Minagar, 2013). The progressive course is characterized by creeping and proceeding exacerbation of the symptoms. Many hypotheses exist on the etiology of MS, including genetic and environmental factors, involvement of the immune system and infections (Korn, 2008; Sadovnick et al., 2000). Several pieces of evidence from genome wide association studies, animal models or therapeutic strategies argue in favor of the idea that the myelin sheath is attacked by auto-antigen specific immune cells (Petermann and Korn, 2011). Immunological tolerance of auto-reactive T cells can be broken (Gold et al., 2006; Korn et al., 2010) due to many reasons including aberrant activation of autoreactive T cells by foreign antigens (molecular mimicry) and failure of regulatory T cells. These auto-reactive T cells are triggered in the periphery, cross the blood-brain barrier, are re-activated in the perivascular compartment, infiltrate into the CNS parenchyma, recognize target antigens and start lesion development (Becher et al., 2006; Korn et al., 2010; Petermann and Korn, 2011).

Most of the present knowledge about CNS inflammation dates back from animal models. Experimental autoimmune encephalomyelitis (EAE) has provided the most coherent pathogenetic concept of CNS autoimmunity and serves as a well accepted model for human MS. EAE reflects key histo-pathological features of MS and is usually used to simulate autoimmune inflammation and demyelination in the CNS (Petermann and Korn, 2011; Wekerle et al., 1994). With this prototype of organ-specific autoimmune diseases, the development of effector lymphocytes and the involved cytokine network during inflammatory immune reaction in the CNS can be investigated. Depending on the protocol to induce EAE, the participation of B cells can be studied. A major advantage of EAE is the induction of several disease courses by immunization of different susceptible mouse strains (Rodgers and Miller, 2012). A common method to induce active EAE in mice is the application of an emulsion of myelin proteins (MBP, PLP or MOG) or peptides in complete Freund's adjuvant (CFA) (Amor et al., 1994; Ben-Nun et al., 1981; Fritz et

al., 1983; Kuchroo et al., 1991; Linington et al., 1993). The adjuvant contains mycobacteria (M. tuberculosis), which activates and enhances the immune reaction. Whether the additional application of pertussis toxin (PTx) is necessary to induce clinical signs of EAE, is dependent on the mouse strain, which is used. One hypothesis for the effect of PTx is that it makes the blood brain barrier permeable. Recent work suggested that PTx increased adhesion molecule expression that initiated leukocyte infiltration into the brain (Kerfoot et al., 2004). Other reports showed that PTx induced the maturation of dendritic cells (Hou et al., 2003; Wakatsuki et al., 2003), or that it suppressed $CD4^+CD25^+$ T regulatory cells (Chen et al., 2006). Nevertheless, the whole function of PTx as component of the EAE induction is still under discussion. The current hypothesis of EAE processing is that after immunization, dendritic cells (DCs) are activated in the lymph nodes by Toll-like receptors (TLR) and present myelin antigens to naïve T cells. After antigen specific sensitization in draining lymph nodes, myelin specific T cells enter the blood stream and invade the CNS compartment. Infiltrating T cells detect their cognate antigen and become reactivated by local antigen presenting cells (APCs). T cell expansion and cytokines release and recruitment of other immune cells promote tissue damage including demyelination and axonal degeneration (Figure 1) (Fletcher et al., 2010). The first EAE symptoms are detectable 10 days after induction. The ascending paralysis begins in the tail proceeding to the hind legs and to the front legs (Korn et al., 2007c). The MOG_{35-55} induced EAE in C57BL/6 mice is monophasic and self-remitting.

A second approach to induce EAE is by adoptive transfer of activated myelin specific $CD4^+$ T helper cells, which substantiates the critical role of T cells in the development of MS (Holda et al., 1983; Langrish et al., 2005; Pettinelli and McFarlin, 1981). The transfer of activated myelin-specific T cells into naïve animals is sufficient to induce EAE (Bettelli et al., 2003; Wekerle et al., 1994). Thus, myelin specific T helper cells are necessary and (together with auxiliary cells like macrophages) sufficient to induce inflammatory demyelination in the CNS.

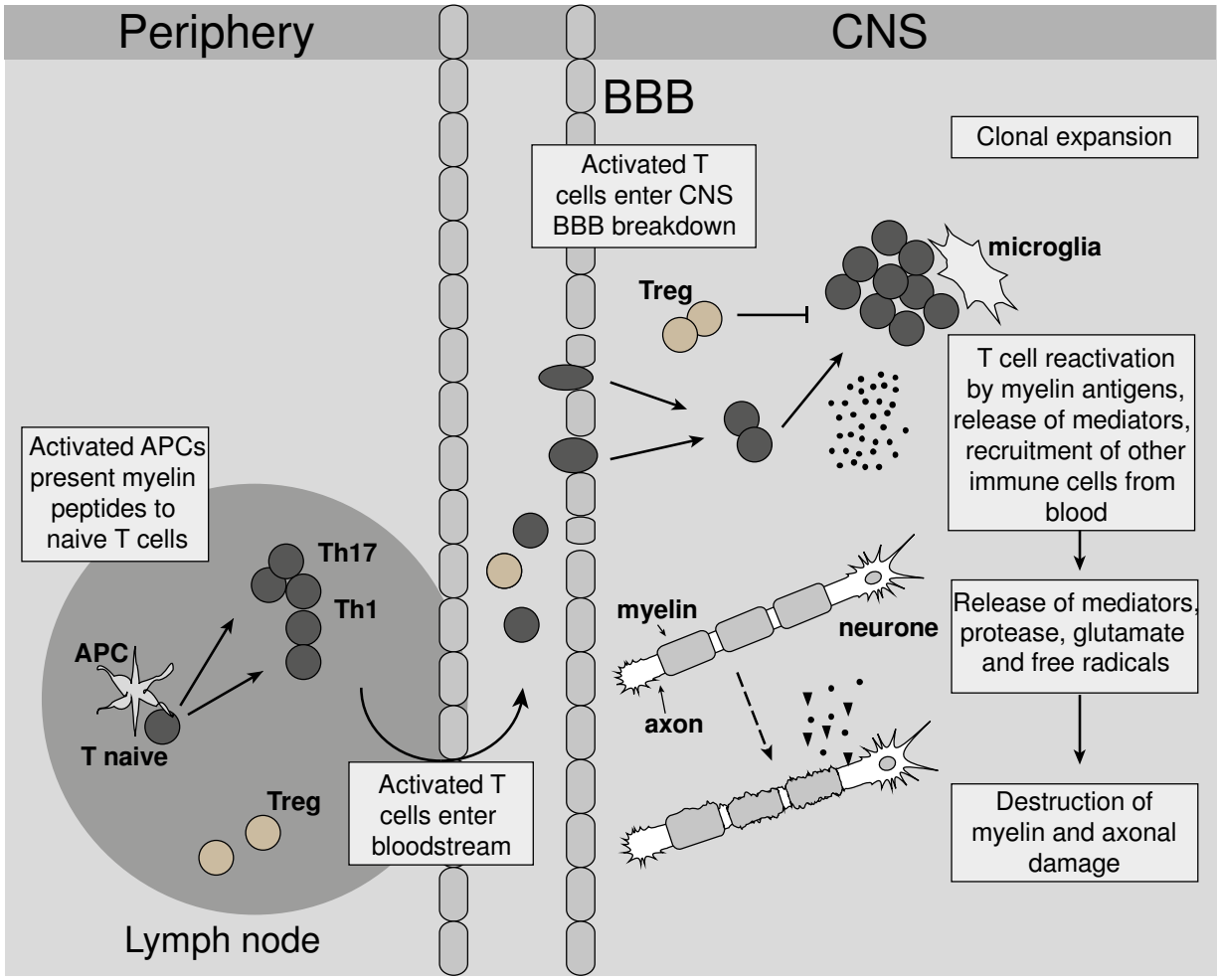

Figure 1: Schematic diagram of the progress of a neurological inflammation.

1.4 T helper cells

Naïve $CD4^+$ T cells circulate in the blood and lymphoid tissues and recognize antigens presented by antigen presenting cells (APCs) in the context of MHC class II molecules. For full activation, T helper cells must receive a costimulatory signal in addition to the engagement of their T cell receptor (TCR). The antigens, on the MHC-complexes, were detected by TCR in cooperation with co-receptors CD4 and CD8. $CD4^+$ T cells recognize MHC-II-molecules in contrast to $CD8^+$ T cells which detect MHC-I-molecules (Stoeckle and Tolosa, 2010). Major co-stimulatory signals are conveyed by CD28/B7 or CD154/CD40 (Abe, 2005). A third signal that contributes to the activation of T helper cells consists in cytokines produced by APCs or other auxiliary cells. These cytokine cues are sensed by T helper cells and guide their commitment to

distinct effector T cell subsets (see Figure 2). Activated T helper cells expand and execute their function by secretion of cytokines and by providing help to other immune cells by direct interaction. For example interaction of CD154 (on T cells) and CD40 (on B cells) is required for B cells to induce class switch recombination of their immunoglobulin genes.

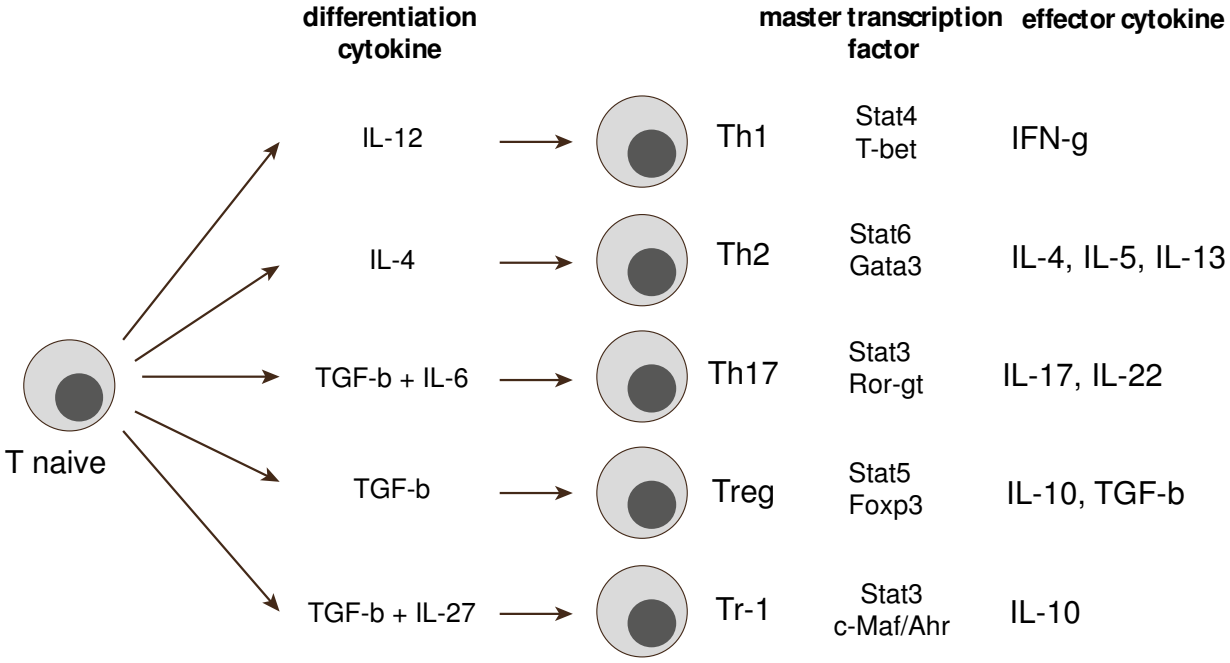

Figure 2: Selected T cell subsets. Naïve T cells can differentiate into T helper type 1 (Th1), Th2, Th17 and regulatory T cells, like Treg and Tr-1 cells, depending on the cytokine milieu upon antigen recognition. At the moment, master transcription factors and effector cytokines served as specific characteristics of the discrimination into T helper subsets.

1.4.1 Th1 and Th2 cells

Mosmann and Coffman were the first to suggest that T helper cells can be distinguished according to a distinct (and non-overlapping) cytokine profile that conincides with distinct functional properties of T helper cell subsets (Mosmann et al., 1986). In this work they described so-called Th1 and Th2 cells. Th2 cells secrete IL-4, IL-5, IL-13 and IL-10, but not IFN-γ (Ho and Glimcher, 2002). In the presence of IL-4 and TCR stimulation, naïve T cells develop into Th2 cells by activation of Signal Transducers and Activators of Transcription 6 (Stat6), which translocates into the nucleus and induces the expression of trans-acting T-cell-specific transcription factor GATA-3 (Gata3), the master transcription factor of Th2 cells (Kurata et al., 1999; Ouyang et al., 1998; Zheng and Flavell, 1997). In addition to Gata3, avian musculoa-

poneurotic fibrosarcoma v-maf (c-Maf), a member of the b-ZIP (basic-region leucin-zipper) protein family, was also identified as a Th2-cell-specific transcription factor that regulates the expression of IL-4 (Ho et al., 1996). Th2 cells are imported for elimination of helminthic parasites and are responsible for the immunity against extracellular pathogens as well as for the pathogenesis of asthma and allergy (Kay et al., 1991; Robinson et al., 1992). Th2 cells have been believed to have “regulatory” role in organ specific autoimmunity.

In contrast to Th2 cells, Th1 cells produce large amounts of IFN-γ and are important for protection against intracellular pathogens, thus responsible for cell-mediated immunity. After TCR signaling in presence of small amount of IFN-γ (e.g.: derived from innate immune cells or already differentiated Th1 cells), naïve $CD4^+$ T cell develop into Th1 cells by up-regulation of the master transcription factor, T-bet (T-box expressed in T cells). The expression of T-bet is Stat1 dependent and induces the expression of IL-12 receptor (IL-12R). IL-12 was discovered in 1989 and is a disulfide-linked heterodimer molecule composed of p35 (IL12A) and p40 (IL12B) subunits (Kastelein et al., 2007; Schoenhaut et al., 1992) (Figure 3).

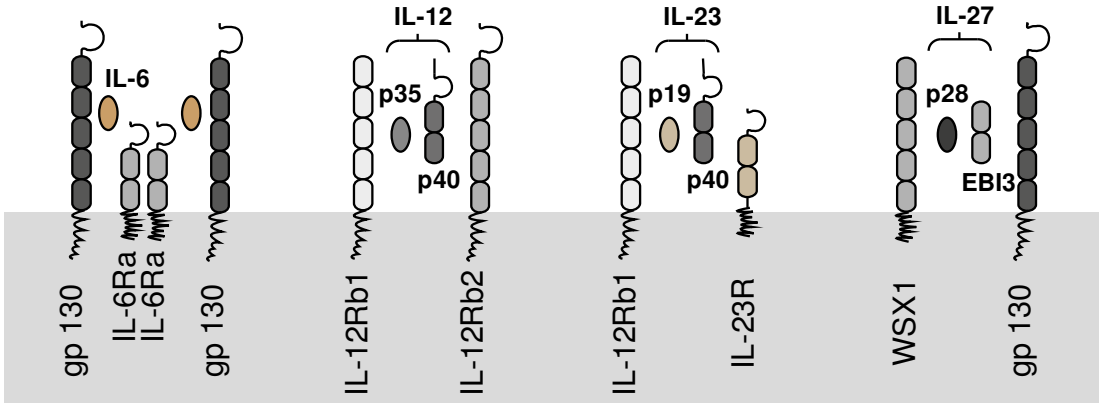

Figure 3: Cytokines and their receptors from IL-6 and the IL-12 cytokine family. Besides IL-12 and IL-23, IL-27 is also a member of the IL-12 cytokine family. In addition to the relationship of the cytokines and there receptors, the IL-6 receptor and the IL-12 cytokines family receptors are all type 1 cytokine receptors and are defined by structural motifs, similar properties and their immune modulatory functions (Figure 3). The identification of some members of the family is based on the mixing and matching of ligands and receptor units due to the same heterodimer structures. It is possible that additional heterodimer complexes exist (Kastelein et al., 2007).

The p40 subunit is expressed in 10 to 1000-fold excesses over p35 and can also act as monomer (Abdi, 2002; Kastelein et al., 2007). APCs produce IL-12, which has pleiotropic functions (Schoenhaut et al., 1992). Signals from IL-12 are transduced through the Stat4

pathway to induce the expression of further IL-12 receptor subunit IL-12Rβ2 (Morinobu et al., 2002; Wu et al., 2000). Together with IL-12Rβ1 subunit, the receptor can bind IL-12 which results in a Jak2-dependent phosphorylation of the receptor and creation of a binding site for Stat4 molecules. Stat4 is phosphorylated, translocates in the nucleus and binds the IFN-γ promoter (Park et al., 2004; Yamamoto et al., 1999), which determines full commitment of T cells to the Th1 lineage (Afkarian et al., 2002; Morinobu et al., 2002; Murphy et al., 2000; Park et al., 2004). Th1 cells have been associated with immunopathology in organ specific autoimmune diseases and in particular in MS because myelin specific Th1 cells induce EAE upon adoptive transfer into naive recipient mice. Moreover, Th1 associated cytokines were detected in lesions of MS patients (Domingues et al., 2010; Yura et al., 2001). Several findings support the important role of Th1 and their cytokines in the development of EAE: mice which were deficient for T-bet (*Tbx21*$^{-/-}$) and IL-12p40 (*Il12p40*$^{-/-}$) were resistant to the induction of EAE, treatment with a neutralizing antibody to p40 subunit of IL-12 reduced severity in EAE and IFN-γ levels correlate with disease severity in EAE (Becher et al., 2002; Bettelli et al., 2004; Gran et al., 2002; Kennedy et al., 1992; Leonard et al., 1995; Nath et al., 2006). However, mice deficient for IFN-γ (*Ifng*$^{-/-}$), IFN-γ receptor (*Ifngr*$^{-/-}$) and the second subunit of IL-12 p35 (*Il12p35*$^{-/-}$) were susceptible to EAE. These observations refuted the concept that EAE pathogenesis was exclusively dependent on Th1 cells (Becher et al., 2002; Chu et al., 2000; Ferber et al., 1996; Willenborg et al., 1996).

Nearly ten years after the initial description of IL-12, IL-23 as new member of the cytokine family was discovered. IL-23 is also a heterodimeric protein composed of two subunits, p19 and p40 (Oppmann et al., 2000). The IL-23 receptor is a combination of the IL-23R and IL-12Rβ1 subunits (Parham et al., 2002), in which the binding of IL-23 activates the transcription factor Stat3 (Cho et al., 2006; Harris et al., 2007) (Figure 3). The discovery of IL-23 and the fact that p19 KO mice (*Il23p19*$^{-/-}$) were resistant to EAE like p40 KO mice (*Il12p40*$^{-/-}$), indicated that IL-23 rather than IL-12 was essential for the pathogenesis of organ-specific autoimmunity and the disease induction in EAE (Awasthi et al., 2009; Cua et al., 2003; Langrish et al., 2005; Oppmann et al., 2000). IL-23 does not induce Th1 cells rather promotes the secretion of IL-17 in activated $CD4^+$ T cells. IL-17 does not belong to either the Th1 or Th2 cytokine profile and thus promoted the notion that IL-17 producing T cells might constitute a novel T helper cell subset independent of Th1 or Th2 cells.

1.4.2 Th17 cells

Th17 cells were proposed as the “third” lineage of effector T helper cells besides Th1 and Th2 cells. In addition to IL-17 (IL-17a), Th17 cells produce IL-17f, IL-21, IL-22 and GM-CSF (Korn et al., 2007b; Liang et al., 2006; Starnes et al., 2001). Th17 cells have been regarded as independent of Th1 and Th2 cells because they can be generated from naive T cells in the genetic absence of Th1 and Th2 master transcription factors. IL-17 production can be induced in naive T cells by TCR ligation in the presence of TGF-β and IL-6 (Bettelli et al., 2006; Mangan et al., 2006; Veldhoen et al., 2006). IL-21 can also induce Th17 differentiation in the absence of IL-6. Interestingly, Th17 cells produce IL-21 themselves. Thus, IL-21 stabilizes Th17 lineage commitment in an autocrine manner (Korn et al., 2007b; Nurieva et al., 2007; Zhou et al., 2007). Both IL-6 and IL-21 activate Stat3 which induces the master transcription factor of Th17 cells, retinoid-related orphan receptor (Ror)-γt (Ivanov et al., 2006; Yang et al., 2008). Stat3 and Ror-γt cooperate to drive the expression of IL-17a, IL-17f and GM-CSF (Codarri et al., 2011; Ghoreschi et al., 2010). Although naive T cells do not express IL-23R, the combination of TGF-β and IL-6 strongly induces IL-23R expression and IL-23 plays a prominent role in the expansion and stabilization of pathogenic Th17 cells (Bettelli et al., 2006; Bettelli et al., 2007; Mangan et al., 2006; Thakker et al., 2007; Veldhoen et al., 2006). The Th17 differentiation with TGF-β in combination with IL-6 (in the absence of IL-23) supports IL-10 production, which has been shown to be downregulated by IL-23. In fact, suppression of IL-10 in Th17 cells was proposed to be a major T cell intrinsic function of IL-23 in terms of generating pathogenic Th17 cells (McGeachy and Cua, 2007). Other transcription factors such as Ror-α, c-Maf and IRF4 are also believed to be involved in the Th17 commitment by stabilization or regulation of the Th17 associated cytokines expression profile (Bauquet et al., 2009; Ciofani et al., 2012; Huber et al., 2013; Lee et al., 2012; Yang et al., 2008)

Functionally, Th17 cells are involved in the clearance of bacterial and fungal infections, but also play a major role in autoimmune pathology, including MS (Cua et al., 2003; Langrish et al., 2005; Lubberts et al., 2004). The following findings consolidated the hypothesis of pathogenic Th17 cells in autoimmune diseases: IL-17 mRNA was increased in PBMCs from MS patients and was found in MS lesions (Lock et al., 2002; Matusevicius et al., 1999; Tzartos et al., 2008); IL-17 deficient mice showed decreased EAE severity and delayed onset of EAE (Komiyama et al., 2006) and administration of IL-17 neutralizing antibodies reduced the severity of EAE (Hofstetter et al., 2005). Since IL-23p19 KO mice show deficiencies in Th17 development (Langrish et al., 2005), the hypothesis was raised that IL-23 drives the Th17 differentiation or Th17 lineage stabilization *in vivo*.

1.4.3 Regulatory T cells

In contrast to pro-inflammatory Th subsets, regulatory T (Treg) cells inhibit autoimmunity and modulate activation and effector functions of autoimmune T cells throughout the life of an individual (also called peripheral tolerance) (McGeachy et al., 2005; Sakaguchi, 2004; Zou, 2006). Prior to the identification of a Treg-specific transcription factor, surface expression of the alpha chain of the IL-2 receptor (CD25) was the only possibility to identify Tregs, although activated effector T cells also express CD25 (Asseman and von Herrath, 2002). The identification of the master transcription factor of Tregs (Foxp3) (Brunkow et al., 2001; Kasprowicz et al., 2005) and the description as distinct T cell lineage were related to the discovery of a fatal multi-organ autoimmunity (Fontenot et al., 2003; Hori et al., 2003; Kim et al., 2007). The severe disorder, immunodysregulation, polyendocrinopathy and enterophathy X-linked syndrome (IPEX syndrome) in humans (Bennett et al., 2001; Powell et al., 1982; Wildin et al., 2001) and the scurfy phenotype in mice (Brunkow et al., 2001; Godfrey et al., 1991; Lahl et al., 2007; Schubert et al., 2001) were caused by a mutation of the *Foxp3* gene, which results in an perturbed generation of regulatory T cells. The pool of Tregs in the periphery consist of natural Tregs (nTregs), generated in the thymus, and induced Tregs (iTregs), generated in the periphery. Both express Foxp3 as well as CD25 and are not distinguishable by phenotype. Naturally occurring Tregs were generated in presence of TCR triggering and co-stimulation (Fontenot et al., 2005b; Tai et al., 2005), however TGF-β, IL-2 and Foxp3 are dispensable for the thymic selection (Fontenot et al., 2005a; Gavin et al., 2007; Marie et al., 2005). The population of nTegs in the periphery is 6 to 10% of the peripheral $CD4^+$ T cell population and can only increase by generation of iTregs in an inflammatory environment (Korn et al., 2007a). TGF-β signaling, TCR stimulation, co-stimulation and low dose administration of antigens induced the differentiation of $CD4^+CD25^-$ T cells into $CD4^+CD25^+$ Tregs (Apostolou and von Boehmer, 2004; Chen et al., 2003; Korn et al., 2007a). TGF-β acts as pleiotropic cytokine with multiple functions in the induction of $Foxp3^+$ T cells as well as at the differentiation of Th17 cells from naïve T cells. The Th17 inducers IL-6 and IL-21 suppress the generation of regulatory T cells (Bettelli et al., 2006; Korn et al., 2007b). IL-6 is one of the switch factors in the dichotomy of Th17 and Treg cell that re-directs T cell differentiation from the Treg pathway into the Th17 pathway (Korn et al., 2007a). The suppressing function of regulatory T cells seems to be a potentially attractive therapeutic approach for autoimmune diseases. The function of regulatory T cells during autoimmunity was characterized by suppressing of pathogenic effector T cells. The absence or reduction of these suppression mechanisms results in an excessive reaction of the immune system with tissue injury, e.g. in multiple sclerosis, type I diabetes or rheumatoid

arthritis (Ehrenstein et al., 2004; Kohm et al., 2002; Viglietta et al., 2004). Performing EAE experiments in *Foxp3gfp*.knock-in (KI) reporter mice show that antigen-specific Foxp3$^+$ Treg cells accumulate in the CNS but fail to inhibit antigen-specific effector T cells at the acute phase of disease. CNS derived effector T cells were also resistant to the Treg cell-mediated suppression whereas spleen derived cells were not (Korn et al., 2007c).

Besides Foxp3$^+$ iTregs, other suppressive T cells, generated in the periphery, act to terminate immune responses and prevent autoimmune pathology. These cells are CD4$^+$ Th3 cells, CD8$^+$ Tregs, regulatory typ1 T cells (Tr-1) cells, and NK T cells (Gol-Ara et al., 2012). In the recent years, the Tr-1 cell subset attracted some attention because Tr-1 cells were antigen specific and their suppressive activity was highly related to the production of IL-10. Tr-1 cells are Foxp3$^-$ and are characterized by the production of large amounts of IL-10 but also low levels of IL-4 like Th2 cells. Tr-1 cells express also variable amount of IFN-γ (Battaglia et al., 2006; Groux et al., 1997). It has been established that Tr-1 cells and Foxp3$^+$ Tregs are two distinct populations with different biological properties, including different mechanisms of actions and responsiveness to cytokines. Tr-1 cells are inducible in an antigen-specific manner, their suppressive function is independent of Foxp3 (Roncarolo et al., 2006). Tr-1 cells were first described in severely combined immuno-deficient (SCID) patients who had developed long-term tolerance to stem cell allografts (Bacchetta et al., 1994). These and other studies suggested that Tr-1 cells suppressed immune responses and that the suppressive activity is dependent on their IL-10 production (Astier et al., 2006; Bacchetta et al., 1994; Groux et al., 1997). A clear dissociation of Tr-1 cells from other T cell subsets and the establishment of Tr-1 cells as a T helper cell lineage of their own has not (yet) been successful (Pot et al., 2010). Many studies tried to identify specific markers for Tr-1 cells. Gagliani et al. defined Tr-1 cells in human and mouse by co-expression of CD49b, a α2 integrin subunit of VLA-2 and LAG-3 (CD223), a CD4 homolog (Gagliani et al., 2013). Tr-1 cells were described to suppress Th1 and Th2-mediated immune responses (Cottrez et al., 2000; Groux et al., 1997) but the suppressive effects of Tr-1 cells by IL-10 secretion or a cell-cell contact are still not fully understood (Groux et al., 1997; Magnani et al., 2011; Zhang et al., 2004).

The conditions the lead to the differentiation of CD4$^+$ T cells into Tr-1 cells remain to be determined. In early work, IL-10 itself was proposed to induce the differentiation of naive T cells into Tr-1 cells. However, more recently, IL-27 (together with TGF-β) has been shown to promote the generation of Tr-1 cells *in vitro* (Awasthi et al., 2007; Fitzgerald et al., 2007; Stumhofer et al., 2007). Like IL-12 and IL-23, IL-27 (p28/EBI3) is a member of the IL-12 family of cytokines (Hall et al., 2012; Hunter, 2005; Hunter and Kastelein, 2012; Pflanz et al., 2002). Studies report that

IL-27 drives IL-21 production, which acts as an autocrine growth factor for Tr-1 cells and induces the expression of c-Maf and ICOS (Pot et al., 2009). The IL-27 receptor forms a heterodimer containing gp130 (a subunit of the IL-6 receptor) and a type 1 cytokine receptor WSX-1 (Pflanz et al., 2004) (Figure 3). The ligand IL-27 is also a heterodimer protein complex based on p28 and Epstein-Barr virus-induced molecule 3 (EBI3) (Pflanz et al., 2002). The binding of the ligand to the receptor starts a signal through Stat1(Hibbert et al., 2003). IL-27 was initially described as pro-inflammatory cytokine, based on the findings that IL-27 increases the proliferation of naïve $CD4^+$ T cells. It is permissive for IL-12 sensing by T cells, mediates IFN-γ production and induces T-bet expression (Hibbert et al., 2003; Lucas et al., 2003; Takeda et al., 2003). In addition, other reports described that IL-27 induce naïve $CD4^+$ T cells to the Th1 polarizing effects of IL-12 and have a critical role in the early events that influence T cell activation (Kastelein et al., 2007; Villarino et al., 2003). However, mice deficient for IL-27 or the receptor of IL-27, failed to up-regulate IL-10 in the CNS and failed to resolve inflammation showing severe EAE (Batten et al., 2006; Diveu et al., 2009). Further, administration of recombinant IL-27 was able to reduce the frequency of pro-inflammatory Th17 cells (Fitzgerald et al., 2007). Thus, IL-27 has been shown to be important for the induction of IL-10 in T cells and *in vivo* rather plays a regulatory than a pro-inflammatory role. Microarray analysis data of IL-27 activated T cells suggested that IL-27 induced the transcription factor c-Maf (Pot et al., 2009). C-Maf is a leucine zipper protein and was first proposed to be essential for IL-4 production and differentiation of Th2 cells (Ho et al., 1996). Later, reports disclosed that c-Maf is upregulated in Th17 cells (Bauquet et al., 2009) and directly trans-activated the *il10* promoter (Xu et al., 2009) as well as the *il21* promoter (Pot et al., 2009). Interestingly, c-Maf might cooperate with the transcription factor Aryl hydrocarbon receptor (Ahr) to maintain IL-10 production in Tr-1 cells (Apetoh et al., 2010). The fact that all findings are not unique for Tr-1 cells opens up new scope for research of the complex transcriptional network for anti-inflammatory cytokine production in the different T cell subsets.

1.5 Regulation of inflammation

The autoimmune inflammation depends on a fine balance between regulatory and inflammatory T cells. During onset and peak of disease inflammatory T cells such Th1 and Th17 cell dominate the pathologic process. Regulatory T cells like Treg and Tr-1 cells lead to resolve the inflammation. Several studies focus on suppressing immune pathogenic T cells by regulatory T cells including Tr-1 cells. The regulatory effect acts via expression of inhibitory cell-surface

molecules, induction of apoptotic processes and/or by the production of immunoregulatory cytokines, such as IL-10 and TGF-β (Buc, 2013; Groux, 2003). But also other studies elevate the role of the local inflammatory cytokine milieu in modeling regulatory and effector T cell function in autoimmunity.

1.5.1 IL-10 as regulatory cytokine

IL-10 was first described as a soluble factor produced by mouse Th2 cells able to inhibit the activation of Th1 cells and their cytokine production. IL-10 were originally named 'cytokine synthesis inhibitory factor (CSIF)' (Moore et al., 1990; Mosmann and Coffman, 1989). Currently, IL-10 is considered as soluble factor that plays a central role in controlling inflammatory processes, suppressing T cell responses and maintaining immunological tolerance (Moore et al., 2001). IL-10 indirectly suppresses T cell responses by inhibiting the antigen-presenting capacity of antigen-presenting cells (APCs), down-regulates the expression of MHC class II molecules as well as co-stimulatory molecules and inhibits the secretion of cytokines and chemokines produced by APCs, which influence T cell differentiation, proliferation and migration (Groux, 2003; Moore et al., 2001; Pestka et al., 2004). On the other hand, IL-10 directly regulates T cells by inhibiting their ability to produce cytokines and to proliferate (Groux, 2003; Groux et al., 1997; Pestka et al., 2004; Roncarolo et al., 2001). IL-10 is expressed by many cells of the adaptive immune system, including Th1 cells, Th2 cells, Th17 cells, Tregs, $CD8^+$ T cells and B cells (Moore et al., 2001). Many different regulator molecules of the IL-10 production are under discussion. For example, Gata3 regulates the IL-10 expression in mouse Th2 cells (Chang et al., 2007; Shoemaker et al., 2006), c-Maf controls the IL-10 production in Th17 cells as well as in IL-10-producing $Foxp3^-$ regulatory T cells (Tr-1 cells) (Pot et al., 2009; Xu et al., 2009). Stat4 plays an important role in the IL-10 production by mouse Th1 cells (Saraiva et al., 2009). IL-27 and IL-6 induce IL-10 in a Stat1 and/or Stat3 dependent manner (Batten et al., 2008; Stumhofer et al., 2007; Xu et al., 2009). In contrast, T-bet, B cell lymphoma 3 (Bcl3) and Bcl6 might have a role in the negative regulation of IL-10 expression by T cells and macrophages (Kusam et al., 2003; Riemann et al., 2005; Sullivan et al., 2005). IL-21 and IL-2 together with other cytokines induce IRF4, Stat3 and Stat5 which results in an enhanced expression of B lymphocyte induced maturation protein-1 (Blimp1). Blimp1 is required for B cell development, it drives mature B cells towards the antibody-secreting cell-fate (Angelin-Duclos et al., 2000; Kallies et al., 2004; Shapiro-Shelef et al., 2003), but also has an impacts in the T cell homeostasis and function (Kallies et al., 2006; Martins et al., 2006). The IL-10 production in T cells subsets, including $CD4^+$, $CD8^+$ T cells and Treg were also associated with Blimp1 expression (Cui et al., 2011;

Iwasaki et al., 2013; Lin et al., 2013; Sun et al., 2011). The activity of several transcription factors to regulate the IL-10 production seems to depend on the cell type and the type of stimuli. All the findings suggest that IL-10 is not regulated in a simple and linear manner.

1.5.2 TGF-β as regulatory cytokine

Beside IL-10, TGF-β has also suppressing activity. Based on the pleiotropic role of TGF-β, many controversial discussed studies exist, which want to evaluate the function of TGF-β during T cell development (Oh and Li, 2013; Shevach, 2009). TGF-β binding triggers receptor serin/ threonine kinase activity. Signaling pathways are mostly Smad-dependent, but also Smad-independent pathways are described. In combination with TCR interaction, co-stimulation and cytokines, TGF-β controls the development of thymus-derived $CD4^+CD25^+Foxp3^+$ regulatory T cells (Liu et al., 2008). TGF-β could promote development of the Th17 lineage by inhibiting the differentiation of other helper subsets together with IL-6 (Bettelli et al., 2006; Mangan et al., 2006; Veldhoen et al., 2006). Nakamura et al. discussed that TGF-β produced from the regulatory T cells bind to the cell surface and mediate the suppression in a cell contact-depending manner (Nakamura et al., 2001). An explanation would be that latent TGF-β on the surface mediates the conversion of $Focp3^-$ T cells into $Focp3^+$ Tregs (Andersson et al., 2008). This is confirmed with the data from *in vivo* experiments, which show that a treatment with anti-TGF-β prevent the conversion of $CD4^+Foxp3^+$ IL-10-producing T cells (Maynard et al., 2007). This stands in contrast to other reports, which could not detect the latent TGF-β or/ and could not find the production of TGF-β (Piccirillo et al., 2002). The pleiotropic functions of TGF-β are not well understood at the moment. Another suppressing effect of regulatory T cells on T cells is explained by preventing priming and effector cell expansion by blocking IL-2 production (de la Rosa et al., 2004; Shevach, 2009).

1.5.3 Plasticity of T cells as cell intrinsic regulatory mechanism

Natural occurring Tregs may not be sufficient for the regulation of massive inflammation (Korn et al., 2007c). In particular, T cellular sources of IL-10 other than $Foxp3^+$ Tregs were observed to be crucial for controlling inflammation in autoimmune diseases (Moore et al., 2001; Moore et al., 1990). Thus, the acquisition of IL-10 production by effector T cell subsets like Th1 or Th17 cells that are involved in immunopathology of organ specific autoimmune diseases might be a means to down-modulate inflammation in an “effector T cell intrinsic” manner. In viral infections, IL-10 production by virus specific effector T cells has long been known as a mechanism to prevent exaggerated immunopathology (Sher et al., 1991; Stewart and Rooney, 1992). In particular Th17

cells are increasingly being considered as plastic as to their cytokine expression profile. Th17 cells are able to produce IFN-γ (Hirota et al., 2011; Kurschus et al., 2010) at sites of inflammation. *In vitro*, IL-4 even appears to skew Th17 cells into IL-4 production (Lexberg et al., 2008). It is reported that TGF-β, an important cytokine in the differentiation of Th17 and Treg cells, "reprograms" Th2 cells into a IL-9 secreting T cell lineage, also called Th9 cells (Veldhoen et al., 2008). Th2 cells can acquire the ability to produce IFN-γ and T-bet together with GATA3 can be stably co-expressed (Hegazy et al., 2010). T-bet and Bcl6, the "master transcription factors" of Th1 and Tfh cells can also be expressed simultaneously (Nakayamada et al., 2011; Oestreich et al., 2012). Tfh cells might have also overlappings and distinct contributions because they can make Th1 and Th2 cytokines like IL-21, IFN-γ, IL-4, IL-17 or IL-10 but also contribute specifically to antibody formation and are important for providing B cell help (Crotty, 2011; Hirahara et al., 2013). Foxp3$^+$ Treg cells can lose Foxp3 expression and convert to Th cells in response to certain extrinsic signals (Hori, 2010; Hori, 2011). Co-expression of Foxp3 with T-bet, GATA3, Rorγt or Bcl6 are also documented in Treg cells (Chung et al., 2011; Koch et al., 2012; Koch et al., 2009; Wohlfert et al., 2011; Yang et al., 2008). It is unclear if only a fraction or all of *in vivo* generated T cells can convert. For Th17 cells observations were made that only a fraction can reprogram whereby the majority maintained their profile (Lexberg et al., 2008). In addition to the research of plasticity, the search of intrinsic and extrinsic factors that drive specification and the mechanisms of flexibility of T cells were more and more focused. These factors could be classic T cell differentiation factors like cytokines or transcription factors. For example cytokines, which were produced by many cells of the immune system (also during inflammation) and have showed clear modulatory functions, were cytokines from the IL-12 cytokine family.

It will a challenge to determine the cytokine cues that drive the "re-programming" of effector T helper cells e.g. into IL-10 producing Tr-1 like cells in the context of autoimmune inflammation.

2 Aim of the study

Autoimmune diseases based on the uncontrolled activation of T helper cells result in partly irreversible tissue damage. T cell mediated autoimmunity can be conceptualized as a disorder of tolerance mechanisms which results in an imbalance of regulatory T cells vs. effector T cells. In the present study, I focused on effector T cell intrinsic re-programming events (from pathogenic T cells to Tr-1 like cells). The aim of the study was to characterize which transcriptional modules are activated by IL-12 cytokine family members in pre-committed Th17 cells. I compared pro- and anti-inflammatory T cell subsets, Th17 and Tr-1 cells, respectively, to identify transcription factors that were differentially expressed and in addition modulated by IL-23 and other IL-12 cytokine family members. The functional relevance of Tr-1 associated transcription factors should be tested *in vivo* in EAE by modulating the expression of these factors in pathogenic Th17 cells. Finally, I sought to address the question which biological functions of IL-23 are most relevant for the generation of pathogenic T helper cell species and to extend the knowledge about the plasticity of T helper cells as a result of intrinsic regulatory.

3 Material and methods

3.1 Material

3.1.1 Reagents

2-Propanol	Sigma-Aldrich (St. Louis, USA)
Agarose	Sigma-Aldrich (St. Louis, USA)
Arlacel A (Mannide Monooleate)	GERBU (Wieblingen, Germany)
Aqua (sterile)	B.Braun (Melsungen, Germany)
β-mercaptoethanol	Thermo Fisher (Waltham, USA)
Baytril (2.5 %)	Bayer HealthCare (Leverkusen, Germany)
Bovine serum albumin (BSA)	Sigma-Aldrich (St. Louis, USA)
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich (St. Louis, USA)
Diphtheria toxin (DTx)	Merck Millipore (Darmstadt, Germany)
Dulbecco`s modified Eagle medium (DMEM)	PAA (Pasching, Australian)
DNA ladder (100 bp and 1 kb)	Life Technologies (Carlsbad, USA)
EDTA	Merck Millipore (Darmstadt, Germany)
Developer – G153 solution A and B	AGFA Healthcare (Mortsel, Belgium)
Ethanol	Merck Millipore (Darmstadt, Germany)
Ethidiumbromide	Sigma-Aldrich (St. Louis, USA)
Fetal calf serum (FCS)	Sigma-Aldrich (St. Louis, USA)
Fixer G354	AGFA Healthcare (Mortsel, Belgium)
Fixation and permeabilization solution (BD Cytofix/Cytoperm)	BD Biosciences (Heidelberg, Germany)
Glycerol	Sigma-Aldrich (St. Louis, USA)
Glycine	Applichem (Darmstadt, Germany)
Golgi Stop (including Monensin)	BD Biosciences (Heidelberg, Germany)
H37Ra Mycobacterium tuberculosis	BD Biosciences (Heidelberg, Germany)
HEPES	Sigma-Aldrich (St. Louis, USA)
Hydrochloric acid (HCl)	Roth (Karlsruhe, Germany)
Incidin Plus	EcoLAB (Wien, Austria)
Isoflurane – IsoFlo	Albrecht GmbH (Aulendorf, Germany)

Ionomycin calcium salt (from Streptomyces conglobatus)	Sigma-Aldrich (St. Louis, USA)
L- Arginine	Sigma-Aldrich (St. Louis, USA)
L-Asparagine	Sigma-Aldrich (St. Louis, USA)
Methanol	Merck Millipore (Darmstadt, Germany)
MEM vitamin solution	Sigma-Aldrich (St. Louis, USA)
Milk powder	Roth (Karlsruhe, Germany)
MOG_{35-55} (MEVGWYRSPFSRVVHLYRNGK)	Auspep (Tullamarine, Australian)
Nitrocellulose membrane (Sandwich; 0,2 µm)	Life Technologies (Carlsbad, USA)
Non-essential amino acids	Life Technologies (Carlsbad, USA)
Nuclease free water	Life Technologies (Carlsbad, USA)
NuPAGE MOPS SDS running buffer (20x)	Life Technologies (Carlsbad, USA)
NuPAGE MES SDS running buffer (20x)	Life Technologies (Carlsbad, USA)
NuPAGE LDS sample buffer (4x)	Life Technologies (Carlsbad, USA)
NuPAGE Sample reducing agent (10x)	Life Technologies (Carlsbad, USA)
NuPAGE 4-12% Bis-Tris gel	Life Technologies (Carlsbad, USA)
Orthovanadate	Santa Cruz (Dalles, USA)
Paraffin oil	Sigma-Aldrich (St. Louis, USA)
Paraformaldehyde (PFA)	Merck Millipore (Darmstadt, Germany)
Percoll	GE Healthcare (Munich, Germany)
Penicillin/Streptomycin solution	Life Technologies (Carlsbad, USA)
Permeabilization/wash buffer (BD Perm/Wash)	BD Biosciences (Heidelberg, Germany)
Phenol:Chloroform:Isoamylalcohol	Roth (Karlsruhe, Germany)
Phenyl methyl sulfonyl fluoride (PMSF)	Applichem (Darmstadt, Germany)
Pertussis toxin (PTx)	Sigma-Aldrich (St. Louis, USA)
Phorbol 12-myristate 13- acetate (Oppmann et al.)	Sigma-Aldrich (St. Louis, USA)
Phosphate-buffed saline (PBS)	PAA (Pasching, Australian)
Protein maker (SeeBlue®Plus2)	Life Technologies (Carlsbad, USA)
Red blood cell lysis Puffer (BD PharmLyse)	BD Biosciences (Heidelberg, Germany)
RIPA buffer	Sigma-Aldrich (St. Louis, USA)
RNase surface decontamination solution (RNaseZap)	Sigma-Aldrich (St. Louis, USA)

Scintillation solution– Betaplate SCINT	PerkinElmer (Waltham, USA)
Scintillation membrane – Filtermat A	PerkinElmer (Waltham, USA)
Sodium acetate	Sigma-Aldrich (St. Louis, USA)
Sodium dodecyl sulfate (SDS)	Sigma-Aldrich (St. Louis, USA)
Sodium hydroxide (NaOH)	Sigma-Aldrich (St. Louis, USA)
Sulphuric asid (N2)	Roth (Karlsruhe, Germany)
TaqMan Fast Universal PCR master mix (2x)	Life Technologies (Carlsbad, USA)
TMB peroxidase substrate solution	KPL (Gaithersburg, USA)
Trizma Base	Calbiochem (Darmstadt, Germany)
Trypan blue	Life Technologies (Carlsbad, USA)
Thymidine [methyl ^{3}H]	PerkinElmer
Tween 20	Sigma-Aldrich (St. Louis, USA)
Vitamins solution	Life Technologies (Carlsbad, USA)

3.1.2 Enzymes

Collagenase D	Roche (Mannheim, Germany)
DNase I	Roche (Mannheim, Germany)
Dream Taq PCR Master Mix	Thermo Fisher (Waltham, USA)
Green Taq	Thermo Fisher (Waltham, USA)
Platinum PCR Super Mix	Life Technologies (Carlsbad, USA)
Proteinase K	Sigma-Aldrich (St. Louis, USA)
RNase Inhibitor 20U/µl	Life Technologies (Carlsbad, USA)
Protease Inhibitor (Western Blot)	Sigma-Aldrich (St. Louis, USA)
Protease Inhibitor (P8340)	Sigma-Aldrich (St. Louis, USA)
Benzonase Nuclease	Sigma-Aldrich (St. Louis, USA)

3.1.3 Kits

Ambion WT Expression Kit	Life Technologies (Carlsbad, USA)
Clarity Western ECL Substrate Kit	Bio-Rad (Hercules, USA)
GeneChip WT Terminal Labeling Kit	Affymetrix (Santa Clara, USA)
Intracellular Foxp3 Staining Kit	eBioscience (San Diego, USA)
LIVE/DEAD Fixable Dead Cell Stain Kit	Life Technologies (Carlsbad, USA)

Novex ECL/ HRP Substrate Kit	Life Technologies (Carlsbad, USA)
PCR Product Purification Kit	Qiagen (Hilden, Germany)
Reverse Transcription Reagents	Life Technologies (Carlsbad, USA)
RNeasy Mini Kit	Qiagen (Hilden, Germany)
RNeasy FFPE Kit	Qiagen (Hilden, Germany)
SyBr Select Master Mix	Life Technologies (Carlsbad, USA)

3.1.4 Magnetic Beads

CD4 (L3T4)	Miltenyi Biotech (Bergisch Gladbach, Germany)
$CD4^+$ T cell Isolation Kit II	Miltenyi Biotech (Bergisch Gladbach, Germany)
$CD4^+CD62L^+$ Isolation Kit	Miltenyi Biotech (Bergisch Gladbach, Germany)
CD90.2 Microbeads	Miltenyi Biotech (Bergisch Gladbach, Germany)
Dynabeads Protein A	Life Technologies (Carlsbad, USA)
Dynabeads Protein G	Life Technologies (Carlsbad, USA)
Mouse IL-17 Secretion Assay	Miltenyi Biotech (Bergisch Gladbach, Germany)

3.1.5 Cytokines

Mouse recombinant TGF-β	R&D (Minneapolis, USA)
Mouse recombinant IL-6	R&D (Minneapolis, USA)
Mouse recombinant IL-12	R&D (Minneapolis, USA)
Mouse recombinant IL-23	R&D (Minneapolis, USA)
Mouse recombinant IL-27	R&D (Minneapolis, USA)
Mouse recombinant IL-2	R&D (Minneapolis, USA)
Mouse recombinant IFN-γ	R&D (Minneapolis, USA)

3.1.6 Antibodies

The following antibodies are used for T cell differentiation and cell culture:

Antigen	Conjugate	Clone	Manufacturer
CD3	unconjugated	145-C11	BioXCell (West Lebanon, USA)
CD28	unconjugated	PV-1	BioXCell (West Lebanon, USA)

IFN-γ	unconjugated	R4-6A2	BioXCell (West Lebanon, USA)
IL-4	unconjugated	11B11	BioXCell (West Lebanon, USA)
TGF-β 1-3	unconjugated	1D11	R&D (Minneapolis, USA)

Antibodies and reagents for flow cytometry:

Antigen	Conjugate	Clone	Manufacturer
CD16/CD32	unconjugated	2.4G2	BD Biosciences (Heidelberg, Germany) (Fc block)
IgG	APC		R&D (Minneapolis, USA)
IgG1 k	APC		eBioscience (San Diego, USA)
IgG2a k	APC		BD Biosciences (Heidelberg, Germany)
CD3	APC	145-2C11	BD Biosciences (Heidelberg, Germany)
CD4	APC	RM4-5	BD Biosciences (Heidelberg, Germany)
CD8	APC	53-6.7	BD Biosciences (Heidelberg, Germany)
CD25	APC	PC61.5	BD Biosciences (Heidelberg, Germany)
CD44	APC	IM7	BD Biosciences (Heidelberg, Germany)
CD62L	APC	MEL-14	BD Biosciences (Heidelberg, Germany)
IL-10	APC	JES5-16E3	BD Biosciences (Heidelberg, Germany)
IL-17 A	APC	TC11-18H10.1	BioLegend (San Diego, USA)
Foxp3	APC	FJK-16s	eBioscience (San Diego, USA)
CD4	APC-eFluor 780	RM4-5	eBioscience (San Diego, USA)
CD45.2	APC-eFluor 780	104	eBioscience (San Diego, USA)
Vα3.2 [b,c]	FITC	RR3-16	BD Biosciences (Heidelberg, Germany)
CD3	FITC	145-2C11	BD Biosciences (Heidelberg, Germany)
CD4	FITC	GK1.5	eBioscience (San Diego, USA)
CD3	PcBl	500A2	BD Biosciences (Heidelberg, Germany)
CD3	V450	500A2	BD Biosciences (Heidelberg, Germany)
CD4	PcBl	RM4-5	BD Biosciences (Heidelberg, Germany)
CD25	PcBl	PC61.5	Life Technologies (Carlsbad, USA)
CD45.2	PcBl	104	BioLegend (San Diego, USA)
pStat1	PcBl	14/p-Stat1	BD Biosciences (Heidelberg, Germany)
Vβ11 TCR	PE	RR3-15	BD Biosciences (Heidelberg, Germany)
IgG	PE		Santa Cruz (Dalles, USA)

IgG1	PE		eBioscience (San Diego, USA)
IgG1 a	PE		BD Biosciences (Heidelberg, Germany)
IgG2a	PE		eBioscience (San Diego, USA)
IgG2b k	PE		BD Biosciences (Heidelberg, Germany)
IgG2 k	PE		BD Biosciences (Heidelberg, Germany)
CD3	PE	145-2C11	BD Biosciences (Heidelberg, Germany)
CD4	PE	RM4-5	BD Biosciences (Heidelberg, Germany)
CD25	PE	7D4	BD Biosciences (Heidelberg, Germany)
CD25	PE	PC61.5	eBioscience (San Diego, USA)
CD44	PE	IM7	BD Biosciences (Heidelberg, Germany)
IL-10	PE	JES5-16E3	BD Biosciences (Heidelberg, Germany)
IL-17 A	PE	TC11-18H10	BD Biosciences (Heidelberg, Germany)
GM-CSF	PE	MP1-22E9	BD Biosciences (Heidelberg, Germany)
Foxp3	PE	FJK-16s	eBioscience (San Diego, USA)
pStat4	PE	38/p-Stat4	BD Biosciences (Heidelberg, Germany)
CD45.1	PE-Cy7	A20	eBioscience (San Diego, USA)
IFN-γ	PE-Cy7	XMG1.2	eBioscience (San Diego, USA)
CD3	PerCP	145-2C11	BD Biosciences (Heidelberg, Germany)
CD4	PerCP	RM4-5	BD Biosciences (Heidelberg, Germany)
CD8	PerCP	OX-8	BD Biosciences (Heidelberg, Germany)
SA	PerCP		BD Biosciences (Heidelberg, Germany)
7-AAD	PerCP		BD Biosciences (Heidelberg, Germany)
IL-17 A	PerCP-Cy5.5	TC11-18H10	BD Biosciences (Heidelberg, Germany)

Antibodies used for Western Blot:

Antigen	Comment	Clone	Manufacturer
Blimp1		5E7	Santa Cruz (Dalles, USA)
Stat1		Polyclonal rabbit	Cell Signaling (Danvers, USA)
Stat3		124H6	Cell Signaling (Danvers, USA)
Stat4		Polyclonal rabbit	Santa Cruz (Dalles, USA)
b-Actin		Polyclonal rabbit	Abcam (Cambridge, UK)
pStat1	Y701	Polyclonal rabbit	Cell Signaling (Danvers, USA)

pStat3	Y705	D3A7	Cell Signaling (Danvers, USA)
pStat4	Y693	Polyclonal rabbit	Life Technologies (Carlsbad, USA)
GaH	POD		Jackson ImmunoResearch (West Grove, USA)
GaR	POD		Jackson ImmunoResearch (West Grove, USA)
GaM	POD		Jackson ImmunoResearch (West Grove, USA)
GaRAT	POD		Jackson ImmunoResearch (West Grove, USA)

3.1.7 TaqMan probes

All TaqMan probes (FAM labeled) and the b-actin probe (VIC labeled) were obtained from Life Technologies (Carlsbad, USA).

Gene	Exon boundary	Amplification length	Order number
Actin		115	NM_007393.1
Ahr	3-4	61	Mm00478932_m1
Blimp1	2-3	72	Mm00476128_m1
Blimp1	5-6	75	Mm01187286_g1
c-Maf	1-1	154	Mm02581355_s1
gp130	30-31	64	Mm00511512_m1
Ifng	1-2	101	Mm00801778_m1
IL-10	1-2	79	Mm00439614_m1
IL-17	1-2	80	Mm00439618_m1
Il6ra	6-7	98	Mm00439653_m1
Il12rb1	13-14	60	Mm00434189_m1
Il12rb2	12-13	74	Mm00434200_m1
Il23r	8-9	72	Mm00519943_m1
Il23r	10-11	113	Mm01186163_m1
Il27ra	6-7	69	Mm00497259_m1
Rorc	8-9	77	Mm00441144_g1
T-bet	1-2	69	Mm00450960_m1

3.1.8 Buffers and solutions

Blocking buffer:

5 % milk powder in TBS-T

ChIP lysis buffer:

1 % SDS, 10 mM EDTA, 50 mM Tris-HCl, protease Inhibitor (1:25) and 1 mM PMSF

ChIP incubation buffer:

1 % Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl (pH 8.1)

ChIP reverse buffer:

0.1 M Na_2CO_3, 1 % SDS, 1.5 µg/µl Pronase

Clone medium:

DMEM, 10 % FCS, 50 U/ml Penicillin, 50 µg/ml Streptomycin, non-essential amino acids (MEM-NEAA, final concentration: 1x), vitamins solution (MEM Vitamin Solution, 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 D, and 1 mg/ml DNase I

FACS buffer:

PBS with 2 % FCS, sterile filtered

IP lysis buffer:

50 mM Tris-HCl, 150 mM NaCl, 1% Maltoside, fresh addition of 25 µl Protease Inhibitor (Sigma P8340) and 4 µl Benzonase Nuclease (Sigma; 250 U/µl) in 10 ml IP lysis buffer

Complete Freund's adjuvant:

17 ml Paraffin oil, 3 ml Arlacel A and 100 µg Mycobacterium tuberculosis (pastled)

Tail lysis buffer:

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.

10x TBS buffer:

24.2 g Tris Base, 80 g NaCl, ad 1 l aqua dest. , pH 7.6 adjusted with HCl

TBS-T buffer:

100 ml 10x TBS-Puffer, 1 ml Tween 20, ad 1 l aqua dest.

10x transfer buffer:

30.3 g Tris Base, 144.1 g Glycine, ad 1 l aqua dest.

1x transfer-Puffer:

100 ml 10x transfer buffer, 200 ml Methanol, and ad 1 l aqua dest.

Stripping buffer:

100 mM Glycine (pH 2.0)

3.1.9 Laboratory equipment and consumables

Agarose gel electrophoresis system	Bio-Rad (Hercules, USA)
Blood lancets	Megro (Wesel, Germany)
Cell harvester - MicroBeta FilterMate-96	PerkinElmer (Waltham, USA)
Cell strainer	BD Biosciences (Heidelberg, Germany)
Cell culture plates	Greiner Bio-One (Frickenhausen, Germany)
Cell sorter, FACSAria II	BD Biosciences (Heidelberg, Germany)
Cell sorter, high speed-MoFlo	Beckman Coulter (Brea, USA)
Centrifuge 5417R	Eppendorf (Hamburg, Germany)
Centrifuge 5424	Eppendorf (Hamburg, Germany)
Centrifuge 5810 R	Eppendorf (Hamburg, Germany)
Centrifuge – Galaxy Mini	VWR (Radnor, USA)
Centrifuge-Multifuge 3SR+	Thermo Scientific (Waltham, USA)
CO_2 Incubator- APT.line C 150	Binder (Tuttlingen, Germany)
Conical centrifuge tubes, 15 and 50 ml	Greiner Bio-One (Frickenhausen, Germany)
Cryogenic storage vials	Greiner Bio-One (Frickenhausen, Germany)
Cryostorage system	Taylor-Wharton (Minneapolis, USA)
Rotator	neoLab (Heidelberg, Germany)
Developer unit – Cawomat 2000 IR	WIROM AG (Niederscherli, Switzerland)
ELISA plates – Costa (96-well)	Sigma-Aldrich (St. Louis, USA)
ELISA reader - Genios	Tecan (Männedorf, Switzerland)
Flow cytometer– CyAn ADP	Beckman Coulter (Brea, USA)
Fusion SL	Peqlab (Erlangen, Germany)
Gel chamber - Novex Mini Cell	Life Technologies (Carlsbad, USA)
Gel documentation – Felix 1010	Biotec-Fischer (Reiskirchen, Germany)
Horizontal shaker - IKA-VIBRAX-VXR	IKA (Staufen, Germany)
Irradiation unit – D3-225	Gulmay (Buford, USA)
Irradiation cage	Workshop, Klinikum Rechts der Isar (Munich, Germany)
Magnetic mixer and stirrer- IKAMAG RCT	IKA (Staufen, Germany)

Magnetic tube rack	Life Technologies (Carlsbad, USA)
Microwave R-2V14	Sharp (Munich, Germany)
Mini centrifuge – Spectrafuge	Labnet interational (Edison, USA)
Mouse ear tags and ear tag applier	Fine Science Tools (Heidelberg, Germany)
Nanodrop-NP-1000	PEQLAB (Erlangen, Germany)
Neubauer chamber (improved)	Sigma-Aldrich (St. Louis, USA)
Overhead shaker – Reax 2	Heidolph (schwabach, Germany)
PCR Machine – Thermal cycler-2720	Life Technologies (Carlsbad, USA)
Pipettes (P10, P200, P1000)	Eppendorf (Hamburg, Germany)
Pipette tips	Eppendorf (Hamburg, Germany)
Power Supply – PowerPac Basic	Bio-Rad (Hercules, USA)
Quadro MACS Multistand	Miltenyi Biotech (Bergisch Gladbach, Germany)
Reaction tubes 0.2 ml, 0.5 ml, 1.5 ml and 2 ml	SARSTEDT (Nümbrecht, Germany)
Reaction tubes 8 strip	SARSTEDT (Nümbrecht, Germany)
Real-Time PCR system – StepOne	Life Technologies (Carlsbad, USA)
Scalpel-Feather	Electron Microscopy Sciences (Hatfield, USA)
Scintillation solution – Betaplate scint	PerkinElmer (Waltham, USA)
Scintillation counter – MicroBeta TriLux	PerkinElmer (Waltham, USA)
Syringe filter unit – Millex-GS	Merck Millipore (Darmstadt, Germany)
Syringes and needles of different sizes	B.Braun (Melsungen, Germany)
TaqMan 96-well plates - MicroAmp Fast optical	Life Technologies (Carlsbad, USA)
TaqMan plastic sheet (optical adhesive film)	Life Technologies (Carlsbad, USA)
Thermo mixer	Eppendorf (Hamburg, Germany)
Ultrasonic device/homogenizer – Sonopuls	Bandelin electronic (Berlin, Germany)
Vortex Mixer	VWR (Radnor, USA)
Water bath	Memmert (Schwabach, Germany)
Western Blot chamber – Xcell II Blot Module	Life Technologies (Carlsbad, USA)
X-ray film – Amesham Hyperfilm ECL	GE Healthcare (Munich, Germany)
X-ray film – Kodak Biomax MR Film	Sigma-Aldrich (St. Louis, USA)

3.1.10 Software

Endnote	Thomson Reuters (New York, USA)
Excel	Microsoft (Redmond, USA)
Fusion	Peqlab (Erlangen, Germany)
FloJo	FlowJo LLC / Tree Star Inc. (Ashland, USA)
GenePattern	Broad institute (Harvard, USA)
Illustrator	Adobe Systems Incorporated (San Jose, USA)
Magellan	Tecan (Männedorf, Switzerland)
Photoshop	Adobe Systems Incorporated (San Jose, USA)
Prism	GraphPad Software (San Diego, USA)
StepOne	Life Technologies

3.2 Methods

3.2.1 Protein- and biochemistry methods

3.2.1.1 Western Blot

The Western Blot method is used to detect single or several proteins from mixtures like cell lysates. After gel electrophoretic separation based on the weight and electrical properties the proteins are transferred onto a nitrocellulose membrane using electrical voltages. The separated proteins are accessible for antibody binding and further detections.

To generate cell lysates, differentiated T cells were washed with PBS and lysed with RIPA buffer including Protease inhibitor (1:25). When phosphorylated proteins were detected, 2 mM orthovanadate were additionally added to the RIPA/Protease inhibitor buffer. After 5 min of incubation on ice cells were disrupted using 3 Freeze/Thaw cycles with liquid nitrogen. The supernatants were collected after centrifugation (8000 g, 10 min) and stored at -80°C. For SDS gel electrophoresis cell lysates were mixed with LDS sample buffer and reducing Agent (NuPAGE, Life Technologies) and boiled 10 min at 70°C. Electrophoretic separation was performed using 4-12% Bis-Tris NuPAGE mini gels with MOPS SDS or MES SDS running buffer, respectively and constant Voltage of 200V. Gel, nitrocellulose membrane and filter paper were equilibrated with 1x transfer buffer before assembly of the blotting sandwich according to the manufacturer's instructions (Life Technologies) followed by a transfer at constant voltage of 30 V for 1 h. Free binding sites on the membrane were blocked by incubation of the membrane in blocking buffer for not less than 1 h (shaking, RT). After incubation with the primary antibody (dilution in blocking buffer) over night at 4°C, the membrane was washed (15 min, RT) with TBS-T buffer three times. The secondary antibody, coupled with peroxidase, was diluted in blocking buffer and incubated with the membrane for 1 h by shaking (RT), followed by washing with TBS-T (two times) and TBS (once). The incubation of the membrane with ECL substrate (Bio-Rad or Life Technologies) was performed according to the manufacturer’s instructions. The chemiluminescence signal was detected using either x-ray film exposure or by an imaging system from PeqLab.

3.2.1.2 Immunoprecipitation (IP)

To analyze protein-protein interactions, an antibody which is linked to a solid substrate like sepharose A/B or magnetic beads captures a specific antigen including potential interaction

partners out of a solution of proteins. In the present work IP was used to enrich Stat proteins to detect phosphorylated Stat signals.

Five to ten million cells were washed with cold PBS, lysed with IP Lysis buffer for 2 h at 4°C under rotation and centrifuged (14000 rpm; 4°C; 30 min) to collect the supernatant. For specific binding of the antibodies to the antigen, 500 µg soluble protein were incubated with 1 µg antibody in 200 µl IP Lysis buffer overnight at 4 °C under constant rotation. For precipitation of the antibody-antigen-complex, magnetic sepharose A/B beads were added to the cell lysate (using manufacturer's instructions, Invitrogen) and incubated 1 ½ h at 4°C under constant rotation. With a magnetic tube rack the sepharose A/B beads including the antibody-antigen-complex could be separated from remaining cell lysate. The binding complex was washed with IP Lysis buffer without Protease Inhibitor and Benzonase Nuclease. To remove proteins from the beads, NuPAGE LDS sample buffer and reducing Agent (Life Technologies) were added. After boiling at 95°C for 5 min, and centrifugation, the supernatant was collected and stored at -80°C for further analysis.

3.2.1.3 Chromatin immunoprecipitation (ChIP)

The chromatin immunprecipitation (ChIP) is a specific IP method to analyze interaction between protein and DNA in general. Specific protein binding regions on the DNA as well as global protein complexes like transcription complex binding on the DNA can be analyzed. For this purpose cells were fixed and disrupted using ultrasonics which leads to fragmentation of chromatin but keeps the interaction between DNA and protein. Antibodies capture and precipitate a specific protein out of the solution and DNA fragments bound to the precipitated protein can be detected.

After polyclonal stimulation with IL-12 for 2 h, approximately 10^7 T cells were washed with cold PBS and fixed with 1% paraformaldehyde (PFA) for 15 min at RT. After stopping fixation by adding 125 mM glycine for 5 min cells were pelleted and resuspended in 2 ml ChIP Lysis buffer. Cells were disrupted by 6 ultrasonic pulses à 1 min at 60 % power (Brandolin) with constant cooling on ice. To collect the containing chromatin supernatant, the cell lysate was centrifuged (15 min, 13000 rpm, 4°C) and stored at -80°C. Before specific precipitation with the antibody, a preclearance only with magnetic sepharose A/B beads was performed to minimize unspecific binding of the protein-chromatin-complex to the magnetic beads. Therefore, 200 µl lysate was incubated with magnetic sepharose A/B beads in 1 ml with ChIP incubation buffer for 4 h at 4°C under rotation. Using a magnetic tube rack, the magnetic sepharose A/B beads with unspecifically bound proteins were separated from the remaining cell lysate. 10% of this

precleared chromatin was labeled as INPUT and stored at -80°C. 1 µg antibody and magnetic sepharose A/B beads were added to the remaining precleared chromatin fractions followed by incubation overnight at 4°C and constant rotation. The bead-protein-chromatin-complexes were pelleted and washed 2x with TE buffer by using the magnetic tube rack. To disrupt the complex, the protein and the sepharose beads, 100 µl of ChIP reverse buffer was added and incubated at 65°C for 6 h. After centrifugation the DNA within the supernatant was purified using the PCR fragment purification kit (Qiagen).

3.2.2 Cell biology methods

3.2.2.1 Preparation of lymphocytes

After isoflurane inhalation mice were killed by cervical dislocation and organs were dissected. To obtain single-cell suspensions, tissues were passed through a 70 µm pore size nylon mesh (cell strainer, BD) and washed with DMEM. Spleen cells were lysed to reduce red blood cells (Pharm Lyse, BD). To determine the number of absolute live cells, cells were mixed with trypan blue and counted using a Neubauer improved heamocytometer. After washing, the cells were resuspended in Clone Medium.

To prepare the CNS, mice were narcotized by isoflurane inhalation and perfused through the left cardiac ventricle with 10 ml PBS. The forebrain and cerebellum were dissected and spinal cords flushed out with PBS by hydrostatic pressure. CNS tissue was cut into pieces and incubated in 1 ml CNS digestion solution at 37°C for 45 min. After passing CNS tissue through a cell strainer, cells were resuspended in 5 ml 70% Percoll. To perform a gradient centrifugation, an upper layer of 37 % Percoll (5 ml) was added followed by centrifugation without brake (20 min, 1800 rpm; RT). Mononuclear cells were removed from the interphase, washed with DMEM and the cell numbers were determined. The cells were resuspended in Clone Medium and stored on ice until further processing.

3.2.2.2 Flow cytometry

Flow cytometry is a laser-based technology to dimension, count, sort and analyze cells. A large number of cells can be analyzed simultaneously for multiple parameters like size, structure and specific characteristics in a short time (thousands of particles per second). The method relies on the scattering of the cell itself and the emission of fluorescent light when cells have been stained with fluorochrome-labelled antibodies and passed a couple of focused laser-beams. The

antibodies can be specific for characteristic surface or intracellular protein markers. *Foxp3gfp.*KI mice (Bettelli et al., 2006) and *Il23r.gfp.*KI mice (Awasthi et al., 2009) contain fluorescence reporter signals -in our case GFP- which are detectable by the focused laser-beams and can visualized without additional antibodies. The fluorescence signals as well as the forward and sideward scatter (FSC and SSC) were detected by photodetectors and deliver additional information concerning size, granularity, structures of the cells and size of the nucleus.

To analyze surface protein markers, up to $2x10^6$ cells were incubated with Fc-blocking antibodies and specific surface marker antibodies (1:100 dilution) for 30 min at 4°C. The Fc-blocking antibodies protect from unspecific binding of the specific antibodies on the Fc receptor. To exclude dead cells a Live/Dead dye 7-AAD or a LIVE⁄DEAD Fixable Cell Stain Kit (Life Technologies) were used. The staining takes place in a round-bottom 96-well plate in a total volume of 100 µl. Afterwards the cells were washed in FACS buffer, transferred into FACS-tubes and analyzed by CyAn flow cytometer (BeckmanCoulter). For analysis of intracellular protein markers, cells were incubated with restimulation medium for 3 h at 37°C. The containing PMA (Phorbol-12-myristat-13-acetat) and Ionomycin activate the protein kinase C (PKC) and increase the intracellular Ca^{2+} concentration, (Muller et al., 2013; Yoshida and Plant, 1992) which results in cytokine production. The cytokine production is specific for Th subsets and helps during classification and verification of the efficiency of T cell differentiation. The component Monensin (GolgiStop, BD Biosciences) blocks the intracellular protein transport followed by an accumulation of all proteins within the Golgi apparatus and thus allows staining cytokines intracellularly (Tartakoff and Vassalli, 1977). After restimulation, the cells were stained with surface antibodies (see above), fixed with 4% paraformaldehyde (Cytofix, BD) for 5 to 20 min at 4°C and permeabilized with saponine containing permeabilization/wash buffer (Perm/Wash Buffer, BD Biosciences), followed by staining with monoclonal antibodies specific for cytokines (dilution 1:100 in Perm/Wash buffer) for 30 min at 4°C overnight. To exclude dead cells, the LIVE⁄DEAD Fixable Cell Stain Kit (Life Technologies) was used according to the specifications of the manufacturer in combination with the surface staining. Afterwards, the cells were washed in FACS buffer, transferred to FACS-tubes and analyzed by a CyAn FACS machine (BeckmanCoulter). To stain the transcription factors within the cell nucleus the Intacellullar-Foxp3-stain-Kit (eBioscience) was used according to the manufacturer's instructions.

To analyze flow cytometry data, the software FlowJo was used. Lymphocytes were first characterized according to their size and granularity by FSC and SSC. Cell aggregates were excluded and the further analyses were based on fluorescence signals and were analyzed by a gating strategy.

3.2.2.3 Cell sorting

If cells are sorted after flow cytometry, the method is called fluorescence activated cell sorting (FACS). All FACS analyses were performed by members of the core facility under the direction of Dr. Mathias Schiemann, Institute for Medical Microbiology, Immunology and Hygiene TU Munich. Both freshly isolated (native) and fixed cells were stained with the explained methods and were sorted with FACSAria II or high speed-MoFlo.

Another sorting strategy is to isolate cells from a cell solution using magnetic beads which is called magnetic cell separation (MACS). Cells were labeled with antibodies linked to magnetic beads. Using a magnetic field, labeled cells can be separated from unlabeled cells. Labeled cells were washed and eluated from the column by removing the magnetic field. All magnetic cell separations were performed according to the specifications of the manufacturer (Miltenyi). Naïve T cells and $CD4^+$ T cells were isolated with $CD4^+CD62L^+$ Isolation Kit and CD4 L3T4 or $CD4^+$ T cell Isolation Kit II, respectively. To deplete CD90.2 T cells out of bone marrow cell suspension and to enriched IL-17^+ producing T cells Magnetic beads Kits from Miltenyi were used, too.

3.2.2.4 Cell cultures and T cell differentiation

For *in vitro* T cell differentiation, spleen and lymph nodes from naïve mice were isolated and naïve T cells were either sorted by MACS or FACS. The purity of MACS sorted naïve T cells ($CD4^+CD62L^+$ Isolation Kit Miltenyi) was analyzed by flow cytometry and was detected between 80% and 90%. Higher purity was obtained when cells from *Foxp3gfp*.KI or Bl6 mice were isolated and sorted by FACS ($CD4^+Foxp3^-CD44^-$ or $CD4^+CD25^-CD44^-$, respectively). After cell sorting, cells were washed, counted and transferred into wells of 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 (2 µg/ml) was added to the stimulation medium. This polyclonal stimulation results in an antigen independent activation of the T cells. For the differentiation of T helper cell subsets, the following cytokines (obtained from R&D Systems) were added exogenously into the indicated concentrations.

Th0	Without exogenous cytokines
Th1	IL-12 (10 ng/ml) and αIL-4 (10 µg/ml)
Th17	TGF-β (2 ng/ml) and IL-6 (50 ng/ml)
Tr-1	TGF-β (1 ng/ml) and IL-27 (100 ng/ml)

After 16 to 18 h, Th1 cells were transferred from the anti-CD3-coated to an uncoated 96-well plate to avoid an over activation of the T cells. Cells were harvested to analyze the mRNA by TaqMan, protein expression by Western Blot and/or intracellular cytokine staining (ICCS) after 3 days of T cell differentiation. Supernatants of the differentiation cultures were analyzed for secreted cytokines by ELISA.

To analyze the effect of cytokine stimulation on pre-committed Th cells, T cells from the first differentiation round (R1) were subjected to a second stimulation round (R2) after a resting phase of 5 to 6 days in IL-2 medium (2 ng/ml) without TCR stimulation. Rested cells were transferred to a CD3 coated flat-bottom 96-well plate and were polyclonally stimulated without exogenously added cytokines or with IL-12 (10 ng/ml) or IL-23 (10 ng/ml) for 3 days. Cells and the supernatant were harvested for analyses of the mRNA, protein expression or cytokine production on day 3 of the second stimulation round. To generate protein lysates for Western Blot analyses of phosphorylated signals, cells from day 3 of R1 or after resting phase were polyclonally stimulated with cytokines for only 3 h.

3.2.3 Molecular biology methods

3.2.3.1 Isolation of genomic DNA from tail biopsies

To obtain crude genomic DNA, tail biopsies were taken from narcotized mice and boiled for 30 min in 180 µl tail lysis buffer at 99°C. After cooling down, 180 µl neutralization buffer was added and mixed well by vortexing. To obtain purified genomic DNA, tail biopsies were digested in proteinase K buffer (0.5 mg/ml) overnight at 55°C. 350 µl Phenol:Chloroform:Isoamylalcohol (25:24:1) was added subsequently, vortexed and centrifuged (13000 rpm; 4°C; 15 min). The aqueous phase was transferred into a new reaction tube and mixed with an equal volume of 2-Propanol followed by DNA precipitation with 3 M sodiumacetat (1/10 volume). After centrifugation (13000 rpm; 4°C; 5 min), the supernatant was discarded and the precipitate DNA was washed with 1 ml 70% ethanol. The DNA pellet was air-dried and resuspended in 100 µl dest. Aqua.

3.2.3.2 Isolation of RNA from native or fixed cells

To isolate RNA from native or fixed cells, the RNeasy Kits (RNeasy Mini Kit or RNeasy FFPE Kit), both from Qiagen were used following the manufacturer's instructions.

3.2.3.3 PCR, real-Time PCR and RT-PCR

The polymerase chain reaction (PCR) is a technology for exponential amplification of specific DNA sequences by the enzyme DNA polymerase. For the last years, many advances of the basic methods have been established. The real time PCR or the quantitative PCR (RT-PCR) allow the comparison of gene expression between two or more groups as well as absolute quantification with standard curves. For basic quantification several fluorescence reagents exist. Most of them are inactive fluorescent dyes, intercalating in the DNA like ethidium bromide or SYBR Green. A more state of the art variant is based on the transformation of the inactive fluorescence reagents to active reagents by an enzymatic reaction of the DNA polymerase. The amount of intercalation as well as conversion of the fluorescence reagents is directly proportional to the amount of amplificated DNA. In this work TaqMan probes were used which are a modification of FRET-probes (Förster resonance energy transfer). FRET is based on a fluorescent reporter (most FAM or VIC) on one end of the probe and a quencher of the fluorescence on the other. They are located in close proximity to each other so that the fluorescence signal gets quenched. The probe binds to specific DNA sequences. Upon DNA amplification, the DNA polymerase degrades the probe which leads to the separation of quencher and fluorescent reporter and subsequent fluorescence emission because the fluorescence signal is no longer suppressed by the quencher. Another PCR method is the reverse transcription (rt-PCR) in which RNA is transcribed in complementary DNA (cDNA) using the RNA dependent DNA polymerase. The cDNA is free of intron sequences and is the basis of gene expression analyses.

In this work genotyping was performed using general PCR methods (see section 3.2.4.3). To analyze gene expression of differentiated T cells, the mRNA was isolated from native or fixed cells and transcribed in cDNA with the reverse transcription kit (Life Technologies). In the following PCR program 1 µg total RNA was added to the mixture (100 µl) as template: 10 min 25°C; 2 h 37°C; 5 sec 85°C and stored at 4°C. After reverse transcription, 4 µl of the cDNA was added to the real-time PCR (RT-PCR), together with 10 µl 2xTaqMan Fast universal PCR Mix (Life Technologies), 5 µl dest. aqua and 1 µl genespecific TaqMan-probe (Life Technologies). A StepOnePlus machine (Life Technologies) with the following program was used for the PCR:

Real-Time PCR program:

Number of circles		**Temperature**	**Duration**
1	pre-denaturation	95 °C	5 min
60	denaturation annealing elongation	95 °C 58 °C 72 °C	15 sec 15 sec 15 sec
1	last elongation	72 °C	7 min
	storage	4 °C	∞

The obtained C_t-value is based on the calculation of the first cycle that shows a fluorescence signal above a defined threshold. To get the Δ C_t- value, normalization with a reference gene (β-Actin) was performed. The ΔΔ C_t- value was obtained by calculation of a control group and the target group. If no other information is denoted, all gene expressions are referred to gene expression of naïve T cells. The relative gene expression is calculated as follows: $2^{-\Delta\Delta Ct}$.

To quantify the ChIP assay, the DNA intercalator SYBR Green was used allowing to quantify amplification in general (without amplicon-specific fluorescence reporter/quencher probe). Thus, the isolated DNA (PCR fragment purification kit, Qiagen) was mixed with SYBR Green PCR Master Mix (Life Technologies) by manufacturer's specifications and the following PCR program and primers were used:

PCR program:

Number of circles		**Temperature**	**Duration**
1		50 °C	2 min
1	pre-denaturation	95 °C	2 min
55	denaturation annealing & elongation	95 °C 60 °C	10 sec 30 sec

Primer:

PCR	**Primer**	**sequence (5´- 3´)**	**Aplicon lengh**
Blimp1 A	forward	CATTAGGTTGGGTCTGGGTG	71 bp
	reverse	CACACGGGGCATACACACC	
Blimp1 B	forward	GAACATCTCTGTGTTGTGAGG	78 bp
	reverse	ACTGCTTTCTCAACCTGAGC	

Il4	forward	TGATTCTGAGGTCATATGAGG	78 bp
	reverse	TTTGGTCCGAGTCCTAGAGC	
Tbx21	forward	CACCATCTCGCTTTCCGCT	63 bp
	reverse	TCCCCCTGGCTGACTTTTC	

To calculate the fold enrichment, at first an adjustment between the C_t- values of specific Stat4 binding and unspecific binding of a control IgG antibody to obtain the ΔC_t- value were performed. Finally, the fold enrichment was depicted as follows: $2^{-\Delta Ct}$.

3.2.3.4 DNA microarray

The microarray is a multiplex method to automatically and completely analyze the smallest amounts of fluidity on one chip. Mostly, differentially expressed gene activation was compared using thousand single analyses. The including RNA was transcribed to cDNA by simultaneous labeling with fluorescent dye. The real GeneChip, a glass plate with a multiplicity of genes to be analyzed in form of single strain DNA was spotted on the surface of the chip and was loaded with single strain sample DNA which binds (hybridation) to matching sites on the chip. Therefore specific sites of the Chip were labeled with fluorescence. Based on the signal strength a calculation and comparison of the gene activation between groups are possible.

The RNA was isolated from Th0, Th17 and Tr-1 cells and was used for cDNA syntheses (Ambion WT expression Kit), followed by fragmentation in single strain cDNA and simultaneous labeling with biotin-allonamide-triphosphate (GeneChip WT terminal labeling Kit). For hybridization with the GeneChip (GeneChip Mouse Gene 1.0 ST Array, Affymetrix) 5 µg single strain cDNA was used. The synthesis and performing of the microarray was done by collaborators of the Institute for Medical Microbiology, Immunology and Hygiene on TU Munich (Prof. Dr. Thomas Buch). The data were analyzed with the free software GenePattern (www.genepattern.broadinstitute.org).

3.2.4 Animal experiments

3.2.4.1 Housing and breeding conditions

Mice were kept and bred under specific pathogen free (SPF) conditions in the animal facility at the Klinikum rechts der Isar of the Technical University Munich under standardized conditions in individually ventilated cages (IVC´s) at a temperature of 20 to 22°C, humidity of 40 to 50% and a

12/12-hour light/dark cycle. Food and water was provided ad libitum. For breeding, one > 6 week old male mouse was paired with two > 8 week old female mice (harem mating). Pups at an age of 3 to 4 weeks were gender-specifically separated and tagged with earmarks. For geno- or phenotyping tail biopsies or blood from the retrobulbar venous plexus, the cheek pouch or the lateral tail vein were taken. All mice used for interventions and experiments were 6 to 10 weeks old. All experiments were performed in accordance with the guidelines prescribed by the Bavarian state authorities (Az 55.2-1-54-2531-88-08 und Az 55.2-1-54-2532-164-11).

3.2.4.2 Mouse strains

C57BL/6 (stock number (SN):000664), *CD45.1* (SN:002014), *Stat4*$^{-/-}$ (SN:002826), Balb/c (SN:000651), *Il12rb2*$^{-/-}$ (SN:003248), *Il10*$^{-/-}$ (SN:002251), *Ifng*$^{-/-}$ (SN: 002287), *Rag1*$^{-/-}$ (SN:002216), *Il27r*$^{-/-}$ (SN:018078) and Blimp1 CKO (SN:008100) mice were obtain from Jackson Laboratories (Bar Harbor). Except *Stat4*$^{-/-}$ which are Balb/c background, all other mice were crossed into Bl6 background. CD45.1 mice feature one of two identifiable alleles of CD45, which were described as congenic marker CD45.1 (Ly5.1), in contrast to CD45.2 (Ly5.2). Based on the two types of CD45, cells were functionally identical but distinguishable. *Foxp3gfp*.KI mice (Bettelli et al., 2006), *Il23r.gfp*.KI mice (Awasthi et al., 2009) and MOG TCR transgenic - 2D2 (Bettelli et al., 2003) mice were provided by Prof. Vijay Kuchroo (Harvard Medical School, Boston, USA). P13 (gp61-80) TCR transgenic mice – SMARTA (Oxenius et al., 1998); T cell conditional Blimp1 KO mice - *Lck*^{Cre} or *CD4*^{Cre} *x Blimp1*$^{flox/flox}$ (Kallies et al., 2009; Lee et al., 2001) as well as Blimp1 reporter mice - *Blimp1*$^{gfp/+}$ (Kallies et al., 2004) were provided by Dr. Axel Kallies (Walter and Eliza Hall Institute, Parkville, Australia). T cell conditional Stat3 KO mice - CD4^{Cre} x Stat3$^{flox/flox}$ (Takeda et al., 1998) were obtained as described.

2D2 and SMARTA mice were transgenic mouse strains in which most of all T cells have a genetically generated T cell receptor (TCR) for a specific antigen (MOG_{35-55} or LCMV (lymphocytic Choriomeningitis Virus) epitope gp61-80). The IL-23 reporter mice (*Il23r*$^{gfp/+}$) have an IRES-GFP cassette in the IL23r allele. Therefore IL23R^{+} cells are detectable by the GFP signal. Heterozygote mice (*Il23r*$^{gfp/+}$) are reporter mice in contrast to homozygote mice (*Il23r*$^{gfp/gfp}$) which were deficient for the IL-23 receptor and were called as *Il23r*$^{-/-}$ mice. In *Foxp3gfp*.KI mice the EGFP was induced in the endogenic Foxp3 locus, whereby the analysis of Foxp3 expressing cells is possible by detecting the GFP signal.

3.2.4.3 Geno- and Phenotyping

For Genotyping, DNA was isolated from the mouse tail biopsies like described in chapter 3.2.3. The genomic DNA was amplified with specific primers and analyzed by gel electrophoresis (2% agarose gel in 1x TAE buffer). Unless otherwise noted, the genotyping protocols from Jackson Laboratory (www.jax.org) were used. For genotyping of *Foxp3gfp* and *Il23rgfp.*KI mice the following primers and PCR programs were applied.

Primer:

PCR	**Primer**	**Sequence (5´- 3´)**	**Amplicon lengh**
Foxp3gfp	forward	ACG CCC CAA CAA GTG CTC CAA T	WT: 450 bp Mutant: 200 bp
	WT reverse	GTG TGA GTC AGT AGG ACT GCA G	
	Mutant reverse	ACC CCT AGG AAT GCT CGT CAA G	
*Il23rgfp.*KI	WT forward	GAT CAT CTT ATG GCT GGT CCT C	WT: 300 bp Mutant: 400 bp
	WT 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:

Number of circles		**Temperature**	**Duration**
1	pre-denaturation	94 °C	2 -10 min
40	denaturation annealing elongation	94 °C 55 °C 72 °C	30 sec 30 sec 30 sec
1	last elongation	72 °C	10 min
	storage	4 °C	∞

For phenotyping of the mouse strains CD45.1 or 2D2, blood was collected in 2 mM EDTA/PBS solution. After short lysis of the red blood cells (PharmLyse, BD), the cells were stained with surface antibodies for CD4, CD45.1, CD45.2, Vα3.2 or Vβ11, respectively.

3.2.4.4 Induction of experimental autoimmune encephalomyelitis (EAE)

The experimental autoimmune encephalomyelitis (EAE) is a murine model of the autoimmune disease Multiple sclerosis (MS) reflecting partial aspects of the disease. To induce EAE with components of the CNS (e.g. myeline) in combination with inflammatory substances (adjuvants), the analysis of infiltrating T cells, which trigger the destruction of myelin followed by damages of axons, is very easy and comfortable.

Active EAE was induced in mice with MOG_{35-55} peptide emulsified in complete Freund`s Adjuvant (CFA) with simultaneous application of Pertussis Toxin (PTx). A 1:1 emulsion was prepared directly in syringes containing 1 mg/ml MOG_{35-55} peptide in PBS and 2.5 mg/ml Mycobacteria tuberculosis (H37Ra) in CFA by using a three way stopcock. The emulsion was freshly prepared and stored on ice until using. 200 µl of the emulsion was applicated subcutaneously at the two sites at the base of the tail. In addition, 200 ng PTx in 100 µl PBS were injected intravenously (i.v.) on the day of immunization and two days later. By using the suboptimal immunization protocol, only 50 ng PTx in PBS (instead of 200 ng PTx in a volume of 100 µl) were injected. Mice were monitored daily to detect first signs of disease development. This was usually around day 9. Sick mice were once to twice a day weighted and scored with the following criterions: **0** – no disease signs; **0.5** – starting of tail paralysis, tail tip slumped on the ground; **1** – complete paralysis of the tail; **1.5** – impairment of the hold/grip reflex of the hind limps; **2** – hind limp weakness; **2.5** – one paralyzed hind leg; **3** – complete hind limp paralysis; **3.5** – weak front limp paralysis; **4** – front and hind limp paralysis (tetraplegia); **5** – moribund. Euthanasia was required when more than 20% of the initial body weight was lost or a score of 4 was reached.

3.2.4.5 Adoptive T cell transfer experiments

For adoptive T cell transfer experiments, naïve T cells from 2D2 x WT or 2D2 x Il12rb2 deficient mice were differentiated in presence of polyclonal stimulation and TGF-β, IL-6 or IL-27 respectively into Th17 or Tr-1 cells for 3 days. 10 Million of pre-committed MOG specific T cells were transferred intravenously into congenic mice on day 6 after immunization.

3.2.4.6 Bone marrow chimeras

For the generation of (mixed) bone marrow chimeras (mBMC), *Rag1*$^{-/-}$ recipients were lethally irradiated 2x 3.3 Gy in an interval of 3 hours by using an X-ray device (Gulmay Medical D3-225). The recipients were treated with antibiotic-water (Baytril; 0.1 mg/ml) one week before until two

weeks after irradiation. To isolate donor cells from IL-12RKO, T cell conditional Blimp1 KO *Lck*^{Cre} *x Blimp1*$^{flox/flox}$ and CD45.1 mice, femurs and humeruses were prepared. Bones were dissected and the bone marrow was flushed out the bone with 10 ml DMEM. Cells were passed through a 70 µm cell strainer, followed by a lysis of the red blood cells (PharmLyse, BD). To deplete T cells out of the bone marrow cell suspension the CD90.2 MicroBeads Kit from Miltenyi was used. The cells were washed several times with PBS and injected intravenously into the recipients within a maximum volume of 150 µl. The reconstitution was controlled by flow cytometric analysis of cells in the blood. 4 to 6 weeks after cell transfer and a successful reconstitution, active EAE was induced in the chimeric mice.

3.2.5 Statistical analyses

Statistical evaluation of two groups (cell frequency, cell numbers, mRNA or protein levels) was performed with the unpaired, two-tailed Student`s T test. A continuous probability distribution (normal distribution of data) was assumed. Multiple comparisons were performed with one-way-ANOVA followed by a post-hoc test (Turkey). EAE scores between groups were analyzed as disease burden per individual day with one one-way-ANOVA and a post-hoc test as indicated. Generally, p-values < 0.05 were considered significant.

4 Results

4.1 Blimp1 is differentially expressed in Th cell subsets

4.1.1 Tr-1 cells express Blimp1

Effector T helper cell lineages were identified based on their master transcription factors, signal cytokine profiles and partially on their surface marker expression. Th1 and Th2 cells are the first described Th subsets and are believed to be relatively stable. In contrast, recently described Th subsets, e.g. Th17, Th9, or Tr-1 cells are more plastic and flexible. Interestingly, it appears that TGF-β is an essential constituent of the cytokine milieu that results in the differentiation of those more plastic T cell subsets. TGF-β is a pleiotropic molecule, which – among others – inhibits premature commitment of naïve $CD4^+$ T cells and promotes, together with T cell extrinsic cytokines, like IL-6 or IL-27, the differentiation of pro-inflammatory Th17 cells (that are characterized by the signature cytokine IL-17) or anti-inflammatory Tr-1 cells (that are characterized by the production of IL-10 and variable amounts of IFN-γ). Both IL-6 and IL-27 activate Stat3 (Awasthi et al., 2007; Bettelli et al., 2006), while the resultant Th subsets are fundamentally different. Transcription factors control fate decisions during the differentiation of T helper cell subsets. Thus, the first question was to analyze the gene expression profile of Th17 and Tr-1 cells and look for differentially expressed transcription factor genes. Therefore, Th17 and Tr-1 cells were differentiated for 3 days, using TGF-β plus IL-6 and TGF-β plus IL-27 as differentiation cocktail, respectively. RNA was isolated and transcribed into cDNA. Differentially expressed genes were identified by microarray expression analysis in comparison to Th0 cells. Four transcription factors, *Irf1*, *Irf8*, *Prdm1* (termed *Blimp1* hereafter) and *Tbx21* (termed also T-bet hereafter), were 3-fold higher expressed in Tr-1 cells than in Th17 cells (Figure 4A; Supplemental Tables 1 and 2). *Irf8* has previously been reported to be a transcriptional repressor of the Th17 cell program and to be important in silencing Th17 responses (Ouyang et al., 2011). *Irf1* and *Tbx21* are essential for Th1 cell differentiation by increasing the responsiveness to IL-12 by enhancing IL-12Rβ2 and IL-12Rβ1 expression, respectively (Kano et al., 2008). B lymphocyte induced maturation protein-1 (*Blimp1*) was first described to be essential for B cell maturation into antibody producing plasma cells (Kallies and Nutt, 2007; Shapiro-Shelef et al., 2003). Blimp1 is also required for the expression of IL-10 in $CD8^+$ T cells and Treg cells (Cretney et al., 2011; Sun et al., 2011). Since IL-10 is the hallmark effector

cytokine of Tr-1 cells (Roncarolo et al., 2006), I hypothesized that Blimp1 expression in Tr-1 cells might be functionally relevant for their production of IL-10.

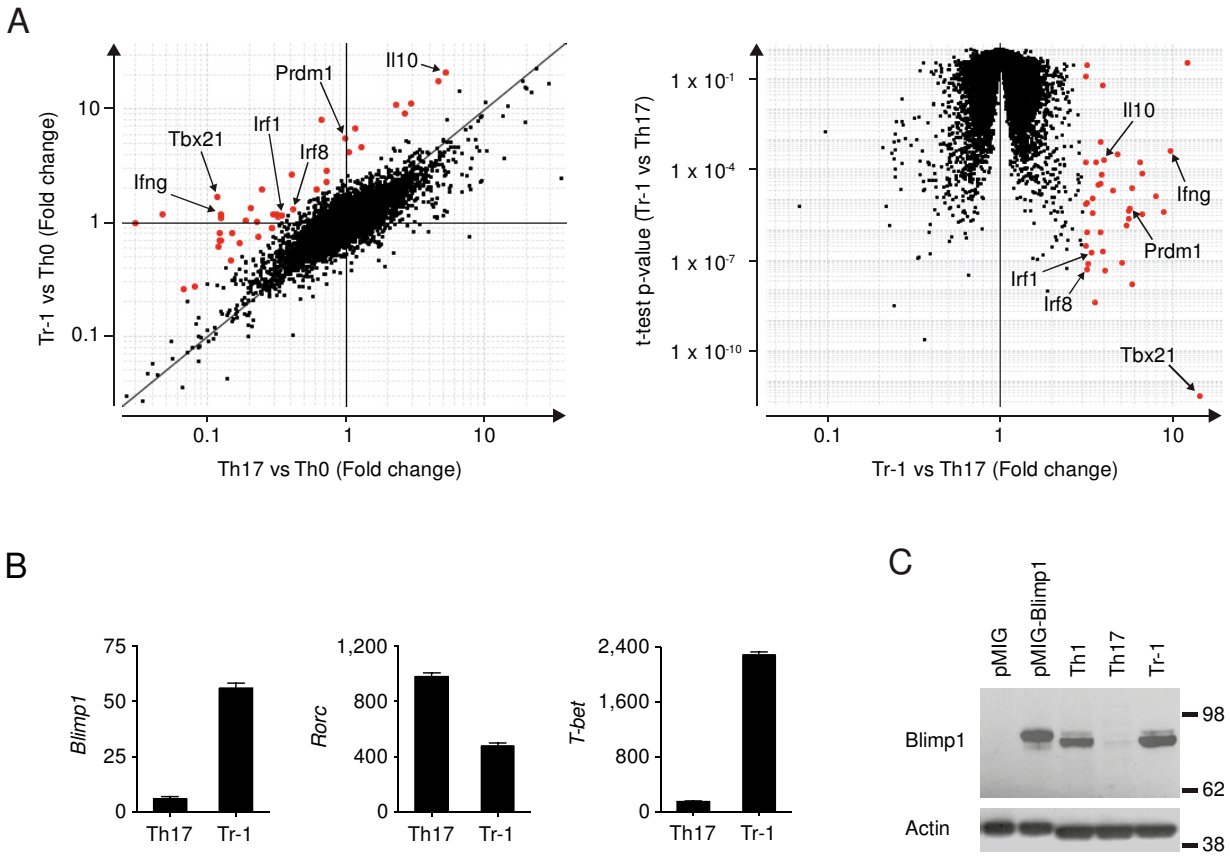

Figure 4: Blimp1 is differentially expressed in Tr-1 and Th17 cells. (A) Naïve CD4$^+$ T cells were differentiated into Th0, Th17 or Tr-1 by polyclonal stimulation for 3 days. RNA was isolated and transcribed into cDNA which was used as source for microarray analysis. Left, mean expression values of five independent experiments are depicted as fold change Tr-1/Th0 (Y-axis) vs. fold change Th1/Th0 (X-axis), and fold change Tr-1/Th17 vs. T-test p-value (volcano blot) right. Genes with at least 3-fold higher expression in Tr-1 cells as compared to Th17 cells are highlighted in red. (B) Relative mRNA amount of Blimp1, Rorc and T-bet in Th17 cells vs. Tr-1 cells as measured by quantitative RT-PCR. (C) Blimp1 protein expression in Th1, Th17 and Tr-1 cells as measured by Western Blot. Overexpressed Blimp1 in lysates of Th0 cells retrovirally transduced with Blimp1/pMIG or an empty vector pMIG served as positive or negative control.

To validate the microarray data, Th17 and Tr-1 cells were differentiated *in vitro* and the mRNA and protein levels of Blimp1 were compared. The quantitative RT-PCR analysis confirmed that Tr-1 cells expressed Blimp1 significantly higher than Th17 cells and also expressed the Th1 master transcription factor T-bet, that controls the expression of the Th1 hallmark cytokine, IFN-γ (Figure 4B). Western blot analysis confirmed that Blimp1 was detectable in similar amounts in

protein lysates from Th1 and Tr-1 cells whereas Th17 lacked Blimp1 (Figure 4C). Collectively, the data indicated that Blimp1 is expressed in Tr-1 cells but not in Th17 cells.

4.1.2 The IL-27-mediated IL-10 induction is dependent on Blimp1

The first results showed that anti-inflammatory Tr-1 cells differentiated with TGF-β plus IL-27 expressed high amounts of Blimp1. In contrast, Th17 cells that were differentiated in the presence of TGF-β plus IL-6 lacked Blimp1 expression. Although both T cell differentiation programs are initiated by TGF-β plus a Stat3 inducing signal (IL-6 or IL-27), the outcome in terms of the effector cytokine profile of the committed T cells is quite different. Next, we investigated the impact of TGF-β on the expression of Blimp1 and other lineage determining transcription factors, which were described to be important during differentiation of anti-inflammatory Tr-1 cells and production of anti-inflammatory cytokines, like IL-10 and IFN-γ. C-Maf and Ahr are two transcription factors, which have previously been proposed to promote differentiation of Tr-1 cells and to be essential for the IL-10 production by Tr-1 cells (Apetoh et al., 2010). We designed a differentiation experiment of Tr-1 cells and Th17 cells with fixed concentrations of IL-27 or IL-6 together with increasing concentrations of TGF-β and analyzed the expression of the transcription factors Blimp1, c-Maf, Ahr in addition to the cytokine expression of IL-10 and IFN-γ (Figure 5A and Figure 5B). I included a condition with IL-6 or IL-27 plus a neutralizing antibody against TGF-β to inhibit trace amounts of TGF-β in the medium, which contained fetal bovine serum.

I found that IL-27 was able to induce Blimp1 in naive sorted $CD4^+$ T cells in the absence of TGF-β. Low concentration of TGF-β enhanced the IL-27 induced expression of Blimp1. In contrast, high TGF-β concentration inhibited the expression of Blimp1 but induced the expression of c-Maf and Ahr in a dose dependent manner. Notably, high concentrations of TGF-β did not suppress but rather sustained IL-10 expression whereas the expression of IFN-γ was reduced with increasing TGF-β (Figure 5A). In contrast to IL-27, IL-6 alone failed to induce Blimp1 (Figure 5B). Addition of TGF-β to IL-6 did not rescue Blimp1 expression but at high concentrations induced c-Maf and Ahr, which coincided with increasing expression of IL-10.
These data suggested that IL-10 expression in $CD4^+$ T cells was dependent on Blimp1 in a milieu with no TGF-β or low concentrations of TGF-β. When high amounts of TGF-β were available, IL-10 expression was no longer associated with Blimp1 expression. Instead, c-Maf and Ahr appeared to correlate with the maintenance of IL-10 expression in $CD4^+$ T cells under

conditions of high TGF-β concentrations in the ambient milieu. These data indicate that the induction of IL-10 in activated CD4$^+$ T cells is controlled by different pathways depending on the availability of TGF-β.

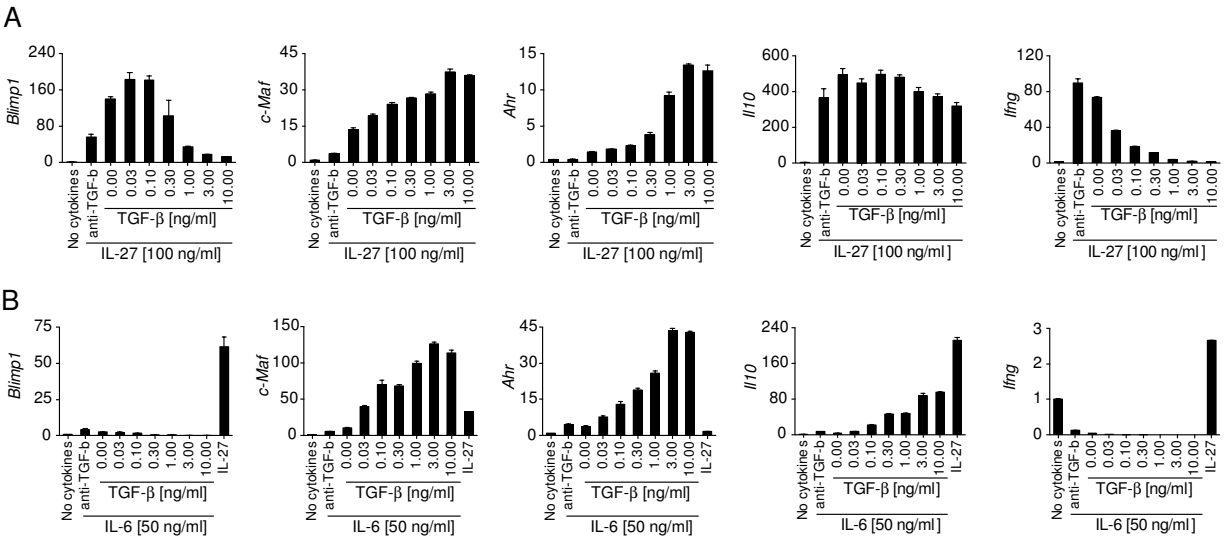

Figure 5: **The availability of TGF-β in the milieu decides on Blimp1-dependent or –independent IL-10 production in Tr-1 cells.** FACS sorted naïve CD4$^+$CD44$^-$Foxp3(GFP)$^-$ T cells were polyclonally stimulated for 3 days and the quantification of gene expression was performed by quantitative RT-PCR. T naïve cells served as reference. (A) Naïve T cells were differentiated into Tr-1 cells in the presence of IL-27 (100 ng/ml) and increasing concentrations of TGF-β (from 0 to 10 ng/ml) or anti-TGF-β (20 µg/ml). (B) Naïve T cells were differentiated into Th17 cells in the presence of IL-6 (50 ng/ml) and increasing concentrations of TGF-β (from 0 to 10 ng/ml) or anti-TGF-β (20 µg/ml). Control conditions were without exogenous cytokines or with IL-27 (100 ng/ml). All RT-PCR data are depicted as the fold change to naïve T cells (naïve T cells =1).

To study the individual impact of IL-27 in the presence or absence of TGF-β, we differentiated Tr-1 cells with increasing IL-27 concentrations together with either a blocking antibody for TGF-β or a high fixed concentration of TGF-β (Figure 6).

Without TGF-β, low concentrations of IL-27 induce Blimp1, which cannot be enhanced by increasing IL-27. With high concentrations of TGF-β, IL-27 alone is no longer required for the expression of Blimp1. Notably, TGF-β per se is not able to induce Blimp1. In contrast, the expression of c-Maf and Ahr were strictly TGF-β-dependent and were enhanced by IL-27.

In summary, at low concentrations of TGF-β, IL-27 induced IL-10 expression, which was associated with Blimp1 expression. With increasing TGF-β in the milieu, the IL-10 expression appeared to be c-Maf and Ahr dependent and Blimp1-independent. Thus, different pathways to induce IL-10 in CD4$^+$ T cells seem to be operational depending on the ambient cytokine milieu.

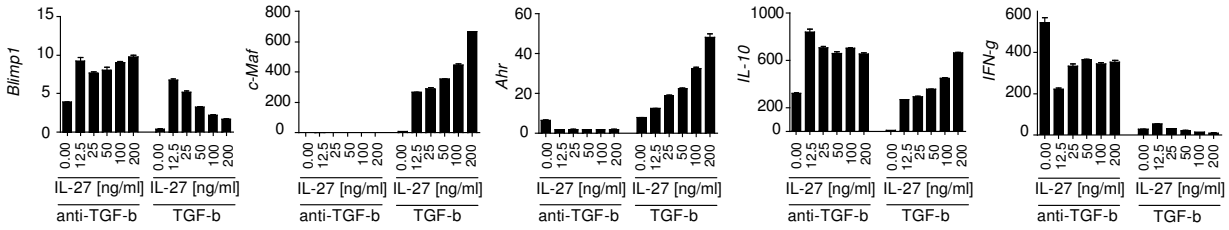

Figure 6: **IL-27 promotes the TGF-β-independent but Blimp1-dependent IL-10 expression.** Naïve T cells were polyclonally stimulated for 3 days into Tr-1 cells with increasing concentrations of IL-27 (0 to 200 ng/ml) either in presence of anti-TGF-β (20 µg/ml) or with 3 ng/ml TGF-β. Quantification of gene expression was performed by quantitative RT-PCR. T naïve cells served as reference. Data are representative of at least three independent experiments.

Next, we wanted to validate the functional relevance of Blimp1 for the IL-10 production in Tr-1 cells. While Blimp1 expression was accompanied by IL-10 expression when naive $CD4^+$ T cells were stimulated with IL-27, this is not a formal proof that IL-10 is actually dependent on the expression of Blimp1 in $CD4^+$ T cells. To verify the Blimp1-dependency of IL-10 production in Tr-1 cells, naïve T cells from wild type mice and mice with conditional deletion of Blimp1 in T cells (*$CD4^{Cre}$ x $Blimp1^{flox/flox}$*, termed Blimp1 CKO) were differentiated into Tr-1 cells in the presence and absence of TGF-β. On day 3, cells were collected and stimulated with PMA/Iono for the assessment of intracellular cytokine production. The cells were stained with antibodies for CD4 and cytokines IL-10 and IFN-γ to analyze the single producing T cells as well as the double producing T cells. In the presence of TGF-β, the IL-27 driven IL-10 production was not completely Blimp1 dependent. Only a reduction of 40% $IL\text{-}10^+CD4^+$ T cells was detectable in Blimp1 deficient T cells in comparison to WT cells. However, IL-10 secretion was almost entirely absent (reduction of 85%) in Blimp1 CKO cells when the cells were stimulated with IL-27 in the absence of TGF-β (Figure 7). In addition, intracellular cytokine staining demonstrated that most of the Blimp1 dependent IL-10 producing T cells were actually IL-10/IFN-γ double producing T cells. These protein data were replicated on the mRNA level as assessed by real time PCR comparing IL-27 stimulated wild type or Blimp1 CKO T cells. In fact, IL-27 failed to induce Il10 mRNA in Blimp1 CKO T cells (Figure 7).

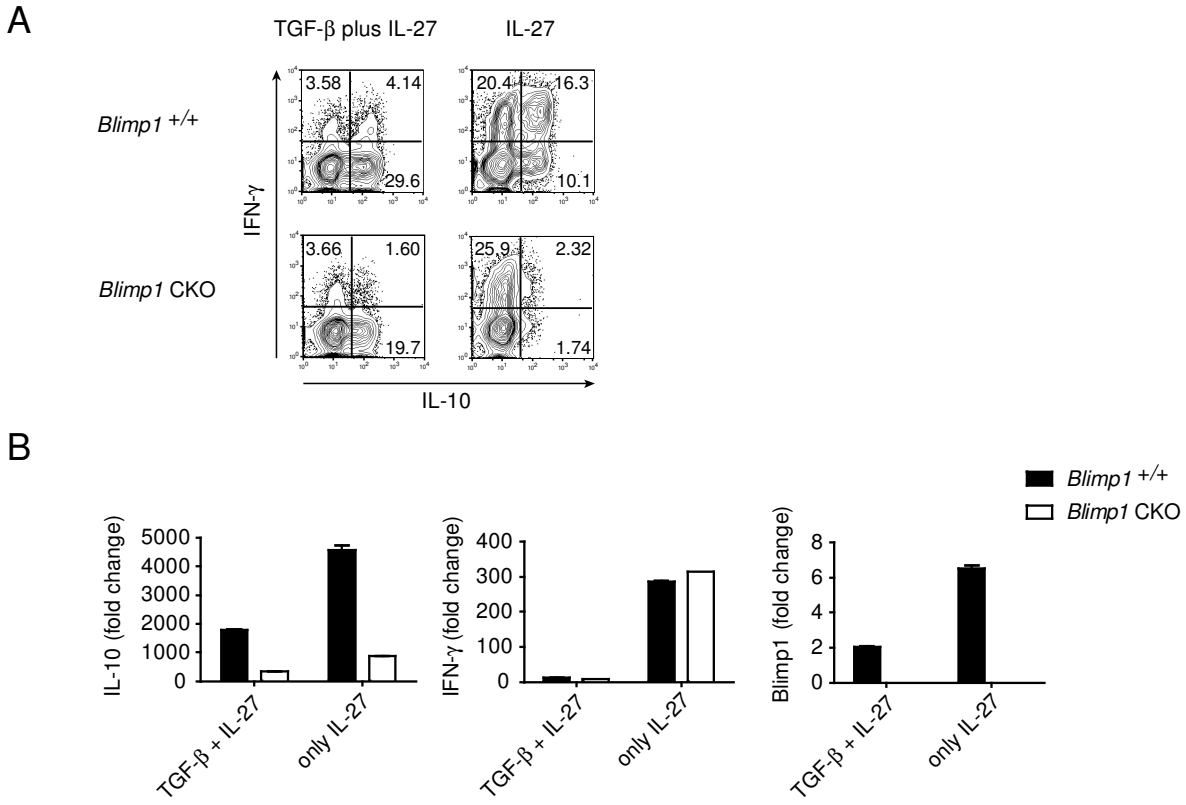

Figure 7: Blimp1 is required to induce IL-10 in Tr-1 T cells. Naïve T cells were purified from *Blimp1*$^{wt/wt}$ x *CD4*$^{Cre+}$ (control) and *Blimp1*$^{flox/flox}$ x *CD4*$^{Cre+}$ (*Blimp1* CKO) mice and were differentiated into Tr-1 cells for 72 hours and stained for intracellular cytokines (A). Frequencies of cytokines were indicated from the live CD4$^+$ T cells-Gate. (B) Differentiated T cells from both groups were analyzed for gene expression by quantitative RT-PCR on day 3.

In summary, these data indicate that (i) Blimp1 is required for IL-27 driven IL-10 production in the absence of TGF-β and (ii) increasing TGF-β concentrations skew the IL-10 driving pathway away from Blimp1 towards other transcription factors most likely including c-Maf and Ahr. We presume that Tr-1 cells feature two different pathways of IL-10 production to maintain the production of IL-10 in different ambient conditions.

4.1.3 Th17 and Tr-1 cells are equipped with IL-12R and IL-23R

Blimp1 was necessary and sufficient to drive IL-10 production in Tr-1 cells (differentiated *in vitro* starting from naive CD4$^+$ T cells). We wondered whether Blimp1 induction in already committed pro-inflammatory effector T helper cells would be a means to reprogram effector T cells into regulatory IL-10 producing T cells. I had noticed that besides IL-27, IL-12 also was associated

with Blimp1 expression (see analysis of Th1 cells). Indeed, we searched for potential modulators of Blimp1 expression that have an impact on already committed T cells and are available at the site of inflammation. Thus, we focused on cytokines of the IL-6 and IL-12 cytokine family, which meet these criteria. I took the approach to first analyze the expression profile of cytokine receptors of IL-6/IL-12 family cytokines on committed T cell subsets.

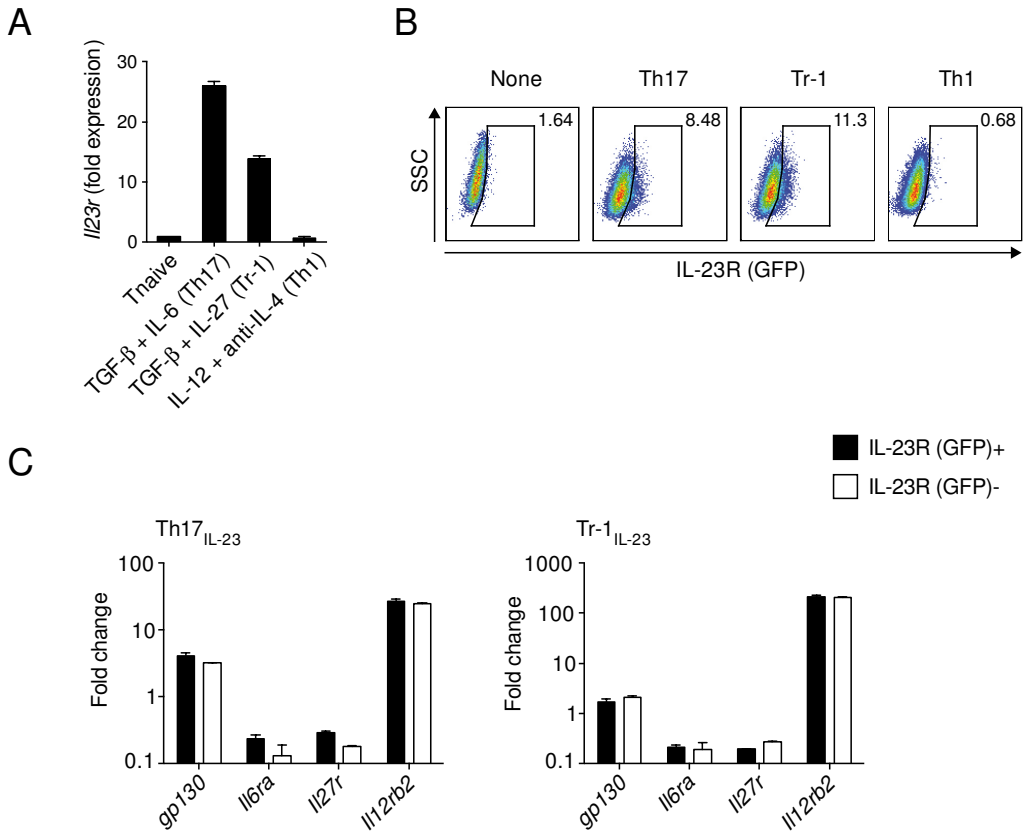

Figure 8: Differentiated Th17 and Tr-1 cells are responsive to both IL-12 and IL-23, but less to IL-6 or IL-27. (A) FACS sorted naïve T cells from wild type mice were differentiated into Th17, Tr-1 and Th1 cells. *Il23r* expression was analyzed by RT-PCR on day 3. (B) Cells from *Il23r* reporter mice (*Il23r*$^{gfp/+}$) were differentiated for 3 days into Th0 (called None), Th1, Th17 and Tr-1 T cells. The *Il23r* expression was detected based on eGFP signal by Flow cytometry. (C) Naïve T cells from *Il23r*$^{gfp/+}$ mice were differentiated into Th17 and Tr-1 T cells in the presence of IL-23 (added on day 2; 10 ng/ml). IL-23R^{+} and IL-23R^{-} T cells were sorted on day 5 of differentiation. Expression of indicated receptors was analyzed by quantitative RT-PCR and plotted as fold change of the expression level in naïve T cells. Representative data of three similar experiments.

Naïve T cells were differentiated for 3 days and the cytokine receptor repertoire including IL-6R (gp130/IL-6Rα) and IL-27R (gp130/IL-27R) as well as of IL-12R (IL-12Rβ1/IL-12Rβ2) and IL-23R (IL-12Rβ1/ IL-23R) was analyzed by quantitative RT-PCR analysis or flow cytometry (Figure 8). Th17 and Tr-1 cells from *Il23r* reporter mice (*Il23r*$^{gfp/+}$) showed a high expression of *Il23r* mRNA

(Figure 8A) and a higher frequency of the IL-23R$^+$ (GFP$^+$) cells (Figure 5B), in comparison to Th1 cells, which lacked the IL-23R (Figure 8A and Figure 8B).
In order to analyze the cytokine receptor repertoire of individual IL-23R$^+$ cells, we differentiated Th17 and Tr-1 cells from *Il23r* reporter mice and sorted IL-23R$^+$ and IL-23R$^-$ cells, based on their GFP expression. To enhance the generation of *bona fide* Th17 cells and to promote the transcriptional activity of *Il23r* correlating with IRES-driven GFP transcription, we added IL-23 to the cultures. Importantly, IL-23R$^+$ and IL-23R$^-$ cells expressed equal amounts of IL-12Rβ2 (Figure 8C). Neither Th17 nor Tr-1 cells differentially expressed IL-12Rβ2 as a function of IL-23R equipment. Thus, individual cells co-express IL-23R and IL-12Rβ2 and are thus able to sense both cytokines. In contrast, the receptor subunits IL-6Rα and IL-27R were down-regulated in committed Th17 and Tr-1 cells compared to naïve T cells (Figure 8C).

The mRNA of IL-6 and IL-27 receptors was down-regulated in Th17 cells and Tr-1 cells in comparison to naïve T cells. To test whether committed Th17 and Tr-1 cells still responded to IL-27 and IL-6, we evaluated the activation of Stat1 and Stat3 upon stimulation of Th17 and Tr-1 cells with IL-27 or IL-6, respectively. Both cytokines activate Stat3 and IL-27 acts additionally via Stat1 (El-behi et al., 2009; Yang et al., 2007). An activation of Stat molecules results in their phosphorylation with subsequent translocation into the nucleus and binding to specific regulatory gene regions. Thus, we detected Stat1 and Stat3 as well as pStat1 and pStat3 to analyze the ratio pStat/total Stat corresponding to their activation status after cytokine stimulation. To test the functional relevance of down modulation of receptor IL-6 and IL-27 subunits expression on the surface of Th17 and Tr-1 cells, differentiated Th17 and Tr-1 cells were shortly stimulated with IL-6 and IL-27, after 3 days of differentiation. Protein lysates were generated and the Stat molecules with or without phosphorylation were detected with antibody by Western Blot (Figure 9). In accordance with previous data (El-behi et al., 2009), we detected phosphorylation of Stat1 and Stat3 in Th17 and Tr-1 stimulated with IL-6 or IL-27 (Figure 9A and Figure 9B). In comparison to naïve T cells, Th17 and Tr-1 cells showed lower gain in Stat1 and Stat3 phosphorylation, but still clearly responded to IL-6 and IL-27. Thus, *in vitro* differentiated Th17 and Tr-1 cells were able to respond to IL-12 and IL-23, and also IL-6 and IL-27.

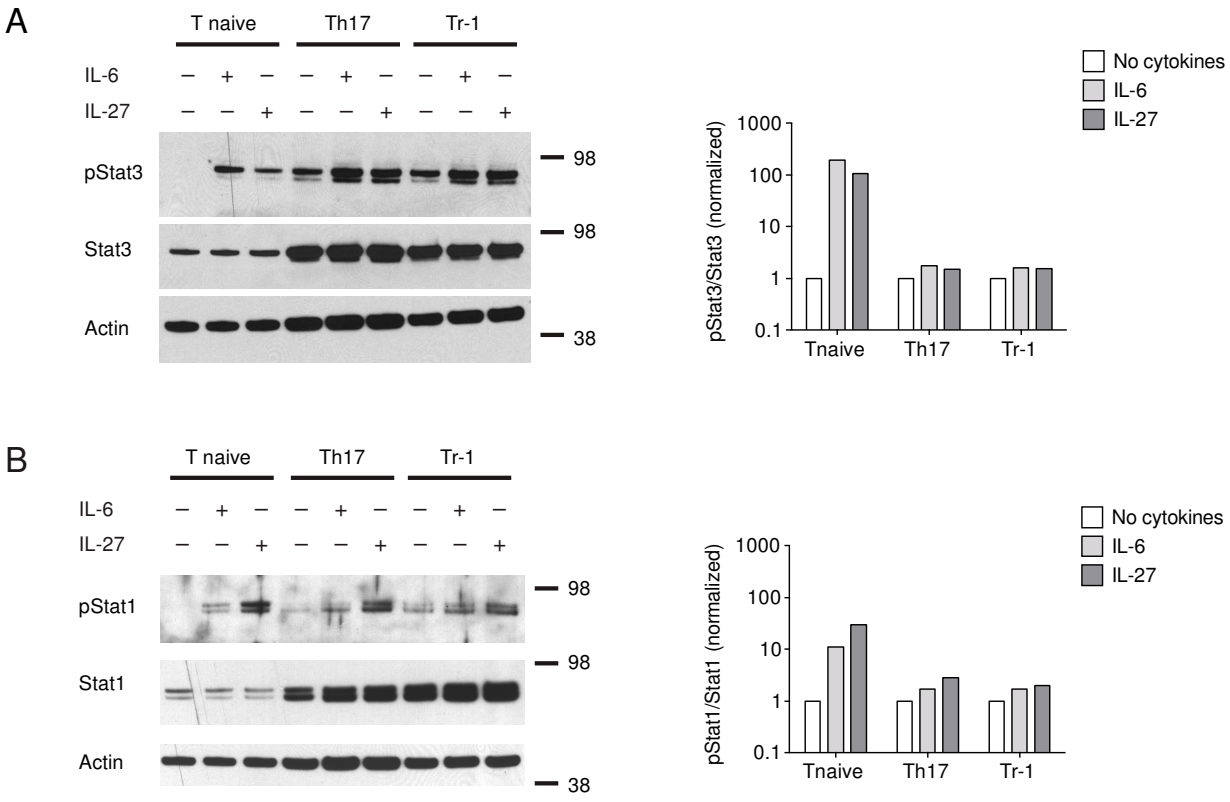

Figure 9: Pre-committed Th17 and Tr-1 cells show reduced responsiveness to IL6 and IL-27. Naïve T cells were differentiated into Th17 and Tr-1 T cells for 3 days. Cells were re-stimulated for 3 hours without exogenous cytokines, IL-6 (50 ng/ml), or IL-27 (100 ng/ml). Cell lysates were generated and Stat3, pStat3 (A) or Stat1 and pStat1 (B) were analyzed by Western Blot. Actin served as internal control. A densitometric analysis was performed using Image J software and the data are representative of two independent experiments.

4.2 IL-12 induces Blimp1 expression in pre-committed Th cell subsets

4.2.1 IL-12 enhances Blimp1 expression in Tr-1 cells

Committed Tr-1 and Th17 cells were responsive to the stimulation with IL-12 cytokine family members IL-12, IL-23, IL-6, and IL-27. I have shown before that IL-6 was not able to induce Blimp1 (see Figure 5) while IL-27 was a strong inducer of Blimp1 in naive T cells. Here, we focused on IL-12 and IL-23 regarding their capacity to induce Blimp1 in pre-committed T cell species. I investigated the effect of IL-12 and IL-23 on the Blimp1 expression as well as the IL-10 and IFN-γ production in pre-committed Tr-1 cells. Different knockout mice (*Il12rb2*$^{-/-}$ and

Il23r$^{-/-}$*)* were used in order to control genetically for the presence of only one signaling pathway, either IL-12 or IL-23. Stimulation with IL-12 in *Il12rb2*$^{-/-}$ mice and stimulation with IL-23 stimulation in *Il23r*$^{-/-}$ mice served as negative controls. Tr-1 cells from *Il12rb2*$^{-/-}$ and *Il23r*$^{-/-}$ mice were differentiated for 3 days, rested in the presence of IL-2 for 6 days and were then polyclonally re-stimulated with IL-12 or IL-23 for 3 days. With this experimental setup, we generated committed Tr-1 cells, which were re-activated in presence of IL-12 or IL-23.
T cells from *Il23r*$^{-/-}$ mice, which can only sense IL-12 signals, expressed a high amount of *Blimp1* mRNA after re-stimulation with IL-12, in comparison to non-responsive *Il12rb2*$^{-/-}$ cells. In contrast, IL-23 stimulation of *Il12rb2*$^{-/-}$ cells, did not induce Blimp1 expression (Figure 10A). This observation was validated with Tr-1 cells from wild type mice, which showed a significantly increased Blimp1 expression after stimulation with IL-12 (Figure 10B) but not with IL-23. A significantly higher expression of IL-10, IFN-γ, and T-bet was associated with Blimp1 induction. In contrast, IL-23 induced *Rorc* and *Il17* mRNA in pre-committed Tr-1 cells (Figure 10B). To validate the impact of IL-12 on the protein level, we analyzed the cytokine production after stimulation by flow cytometry. IL-12 stimulation resulted in a massively induced frequency of IL-10^{+} and IL-10^{+}IFN-γ^{+} T cells in the Tr-1 culture. In contrast, IL-23 stimulation resulted in a slight increase in IL-17 producing cells within the pre-committed Tr-1 cell culture (Figure 10C).

To sum up, these data show that stimulation of pre-committed Tr-1 cells with IL-12 increased Blimp1 expression resulting in the maintenance and further accumulation of IFN-γ^{+}IL-10^{+} producing cells. Thus, the stimulation of Tr-1 cells with IL-12 resulted in a support of anti-inflammatory properties in these cells. In contrast, the response of Tr-1 cells to IL-23 stimulation was characterized by de novo induction of pro-inflammatory conditions in these cells including secretion of IL-17.

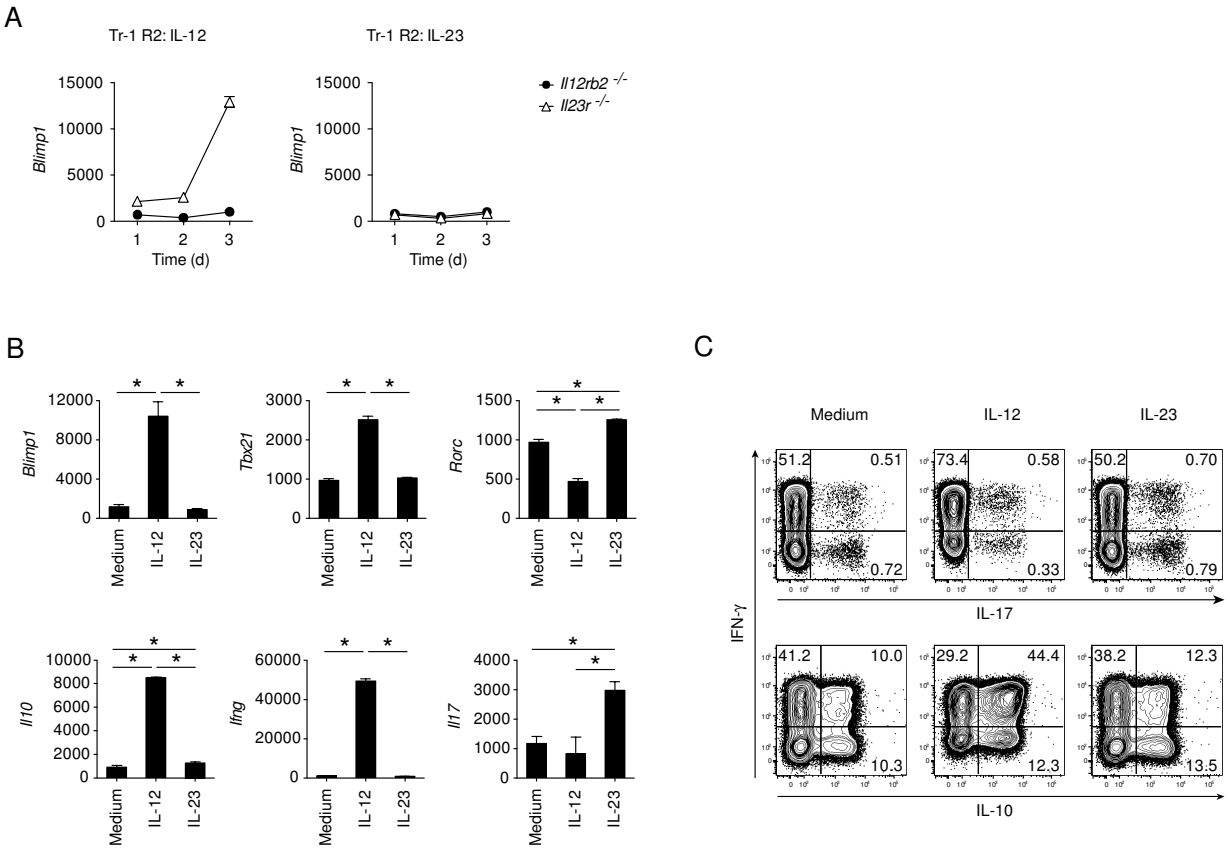

Figure 10: IL-12 induced Blimp1 expression is associated with increased IL-10 and IFN-γ expression in Tr-1 cells. (A) Naïve T cells from *Il12rb2*$^{-/-}$ and *Il23r*$^{-/-}$ mice were differentiated into Tr-1 cells for 3 days. After a resting phase of 6 days (2 ng/ml IL-2), the cells were polyclonally re-stimulated for 3 days (R2) with IL-12 (10 ng/ml) or with IL-23 (10 ng/ml). The RT-PCR analysis of Blimp1 expression after 3 days of re-stimulation is illustrated. (B) WT Tr-1 cells were differentiated for 3 day, rested and re-stimulated. Expression levels were detected by RT-PCR and are depicted as fold change compared to naïve T cells. (A, B) Representative data from three similar experiments. (C) T naïve cells from TCR transgenic SMARTA mice were differentiated antigen-specifically into Tr-1 in R1. Cells were re-stimulated antigen-specifically in presence of IL-12 or IL-23 or without cytokines. The cells were differentiated and stimulated with cognate peptide in presence of antigen presenting cells (APC). Two independent experiments.

4.2.2 The IL-12 induced IL-10/IFN-γ co-expression is dependent on Blimp1

To formally prove that the IL-12 maintained and further induced IL-10 production in pre-committed Tr-1 cells, I used T cell conditional Blimp1 knockout mice. To evaluate the influence of Blimp1 on the production of IFN-γ and IL-10 after IL-12 stimulation, Tr-1 cells were differentiated from wild type and Blimp1 CKO mice, rested and re-stimulated polyclonally without exogenous cytokines or in the presence of IL-12 (Figure 11). After the first round, the IL-10

production in Blimp1 CKO T cells was significantly reduced, but not completely absent, confirming data illustrated in Figure 7. After the second stimulation in the presence of IL-12, Blimp1 CKO T cells failed to generate IFN-γ$^+$IL-10$^+$ double producing or IL-10$^+$ single producing cells (Figure 11A). A significant reduction of *Il10* mRNA from Blimp1 CKO Tr-1 cells was detected by quantitative RT-PCR analysis (Figure 11B), which confirmed the hypothesis that IL-12 driven IL-10 production in Tr-1 cells is dependent on the expression of Blimp1.

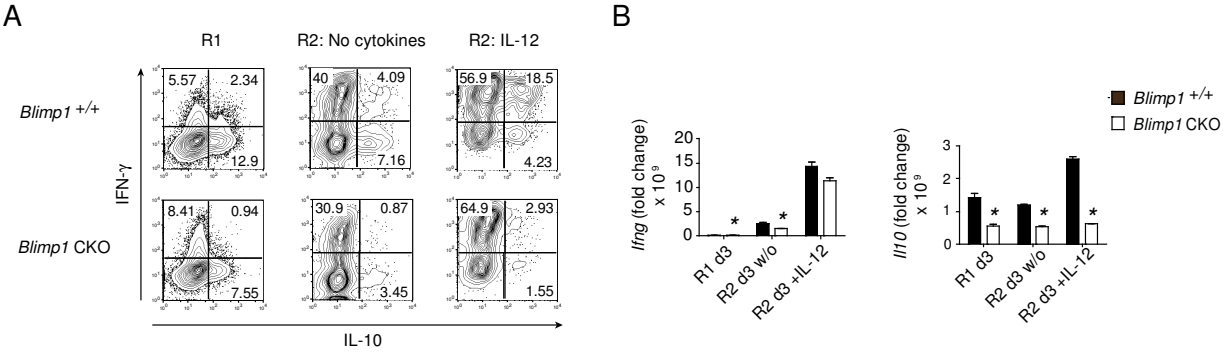

Figure 11: The IL-12 driven induction of IFN-γ$^+$IL-10$^+$ double producers is Blimp1 dependent. Cytokine production in Tr-1 cells which were differentiated from *Blimp1$^{wt/wt}$* x *CD4^{Cre+}* (*Blimp1$^{+/+}$*) and *Blimp1$^{flox/flox}$* x *CD4^{Cre+}* (*Blimp1* CKO) mice. T cells were differentiated into Tr-1 cells in the first round of stimulation (R1, for 3 days), rested for 6 days in presence of IL-2 (2 ng/ml) followed by a second round of stimulation (R2) in presence of IL-12 or no exogenous cytokines for 3 days. Representative for three similar experiments, intracellular cytokines were measured by flow cytometry (A). IL-10 and IFN-γ expression was detected by quantitative RT-PCR analysis (B).

IL-12 stimulation enhanced the frequency of both IL-10$^+$ single producers and IL-10$^+$IFN-γ$^+$ double producers in pre-committed Tr-1 cells. Therefore, we wanted to investigate which Tr-1 subset (based on the secretion of IL-10 and/or IFN-γ) expressed the largest amounts of Blimp1 in the starting Tr-1 cultures and in the second stimulation round cultures after IL-12 stimulation. Thus, we generated Tr-1 cells (R1) as well as re-stimulated Tr-1 cells (R2) and purified cells according to their status of IL-10 and IFN-γ secretion. The RNA of every sorted population was isolated, transcribed and used as template for quantitative RT-PCR analysis of transcription factors and cytokines. The highest Blimp1 expression was detected in IFN-γ$^+$IL-10$^+$ double positive cells in the first and second round. After IL-12 stimulation, the IL-10$^+$ single producing cells expressed equal amount of Blimp1 as double producing cells (Figure 12).

In summary, these data suggest that IL-12 induces a Blimp1 dependent transcriptional program in pre-committed Tr-1 T cells that results in the maintenance of IL-10 production and further induction of IFN-γ$^+$IL-10$^+$ double producing cells.

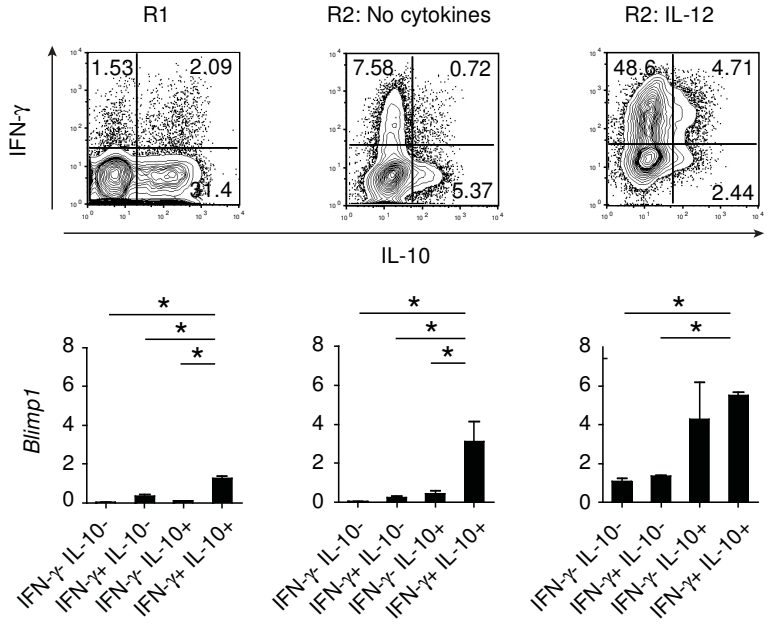

Figure 12: Blimp1 is expressed in IL-10 single and IFN-γ IL-10 double expressing Tr-1. Naïve T cells were differentiated into Tr-1 cells for 3 days (R1), rested and re-stimulated without exogenous cytokines or with IL-12 (R2). On day 3 of R1 and R2, T cells were sorted by FACS according to their production of IL-10 or/and IFN-γ. The RNA was isolated from all sorted fractions followed by detection of *Blimp1* expression by quantitative RT-PCR analysis.

4.2.3 IL-12 induces re-programming of Th17 cells

Pre-committed Tr-1 cells responded to IL-12 with enhanced expression of Blimp1 and a secretion of IL-10 and IFN-γ. The results suggested that (i) the IL-12 induced IL-10 expression was Blimp1 dependent and (ii) that IL-23 counteracted the down-modulatory effects of IL-12 in this context by inducing pro-inflammatory cytokines like IL-17. Not only pre-committed Tr-1 cells are responsive to IL-12 and IL-23, Th17 also possess the receptor equipment for these IL-12 family cytokines (Figure 8). We hypothesized that Blimp1 is a molecular modulator of balancing between IL-12 (anti-inflammatory) and IL-23 (pro-inflammatory) signals in T helper cell subsets, which might constitute an important determinant of their plasticity during inflammation. In order to determine whether stimulation with IL-12 and IL-23 has a functional relevance on pre-committed effector Th17 cells, we used again *Il12rb2*$^{-/-}$ and *Il23r*$^{-/-}$ cells. Naïve T cells from *Il12rb2*$^{-/-}$ and *Il23r*$^{-/-}$ mice were differentiated into Th17, rested with IL-2 for 6 days and polyclonally re-stimulated in the absence of exogenous cytokines or in the presence of IL-12 or IL-23. Th17 cells from *Il23r*$^{-/-}$ mice responded to IL-12 stimulation with increased Blimp1 mRNA. No changes in the Blimp1 expression were detectable after IL-23 stimulation of *Il12rb2*$^{-/-}$ in

comparison to the control conditions (Figure 13A). The expression level of Blimp1 in Th17 cells after IL-12 stimulation was clearly lower than in Tr-1 cells after the same stimulation (Figure 10A), but a distinct induction of Blimp1 expression in pro-inflammatory Th17 cells due to IL-12 stimulation was clearly detectable. To correlate the increasing Blimp1 expression after IL-12 stimulation to the cytokine profile of Th17 cells, naïve T cells were differentiated antigen-specifically, rested and peptide-specifically re-stimulated without cytokines, with IL-12 or IL-23. After stimulation in the presence of IL-12, Th17 cells produced higher levels of IL-10 and IFN-γ and the frequencies of IFN-γ^+IL-10^+ double producing T cells were increased in second round Th17 cultures. In comparison, in the presence of IL-23 the 'bona fide' Th17 phenotype was stabilized by preservation of IL-17 production in second round Th17 cultures (Figure 13B).

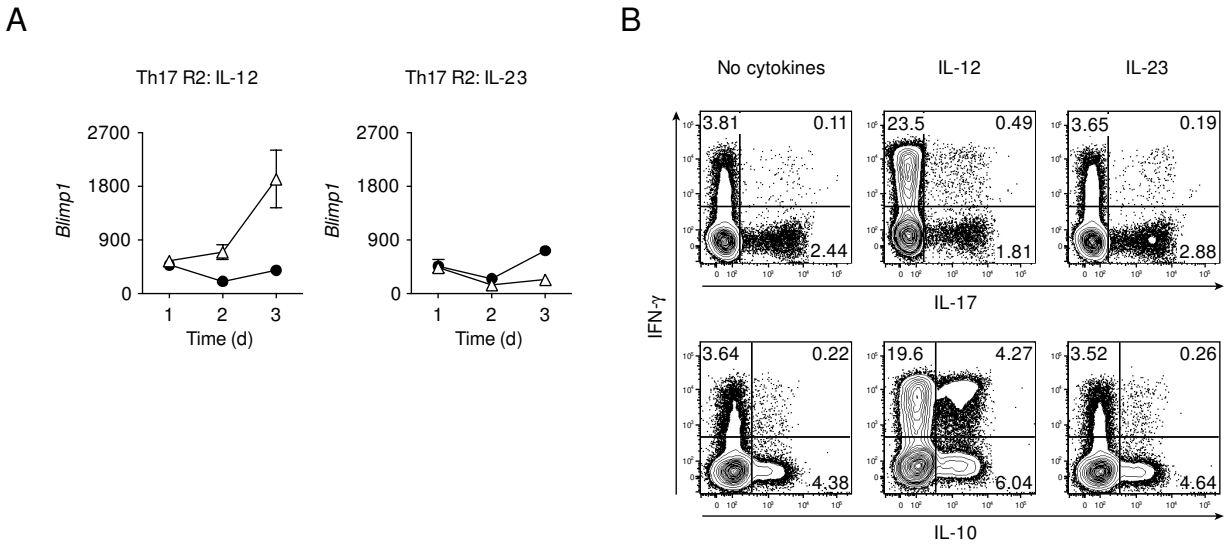

Figure 13: IL-12 induces a reprogramming of Th17 cells into T cells expressing Blimp1 and IL10/IFN-γ. (A) Naïve T cells from *Il12rb2*$^{-/-}$ and *Il23r*$^{-/-}$ mice were differentiated into Th17 T cells for 3 days, rested for 6 days and re-stimulated with IL-12 or IL-23. The expression of *Blimp1* mRNA during round 2 of re-stimulation was analyzed by RT-PCR. Representative data from three similar experiments. (B) T naïve cells from TCR transgenic SMARTA mice were differentiated antigen-specifically into Th17, rested and in R2 re-stimulated antigen-specifically in absence of cytokines or in presence of IL-12 or IL-23 (10 ng/ml each). The cells were differentiated and stimulated with cognate peptide in presence of antigen presenting cells (APC). Two independent experiments.

To confirm that IL-12 stimulation induced a true re-reprogramming of bona-fide Th17 cells into IL-10 producing cells, true IL-17 producers were separated from partially or not committed T cells by cytokine capture assay (Miltenyi). Naïve T cells were polyclonally differentiated into Th17 cells for 3 days. After initial differentiation, IL-17^+ as well as IL-17^- T cells were isolated and rested for 6 days in the presence of IL-2. Both T cell fractions were polyclonally re-stimulated

without exogenous cytokines or in the presence of IL-12 or IL-23. Quantitative RT-PCR analysis revealed that IL-17$^+$ and IL-17$^-$ T cells reacted to IL-12 stimulation with enhanced Blimp1 and T-bet expression. In contrast, IL-23 stimulation promoted Ror-γt expression coinciding with the stabilization of the Th17 phenotype (Figure 14A). A higher Blimp1 expression after IL-12 stimulation was also detected by Western Blot analysis (Figure 14B) in both IL-17$^-$ and IL-17$^+$ T cells. The increased Blimp1 expression was associated with a significantly higher secretion of IL-10 and IFN-γ. The Ror-γt expression induced by IL-23 was linked with IL-17 expression (Figure 14C), which confirmed the RT-PCR analysis.

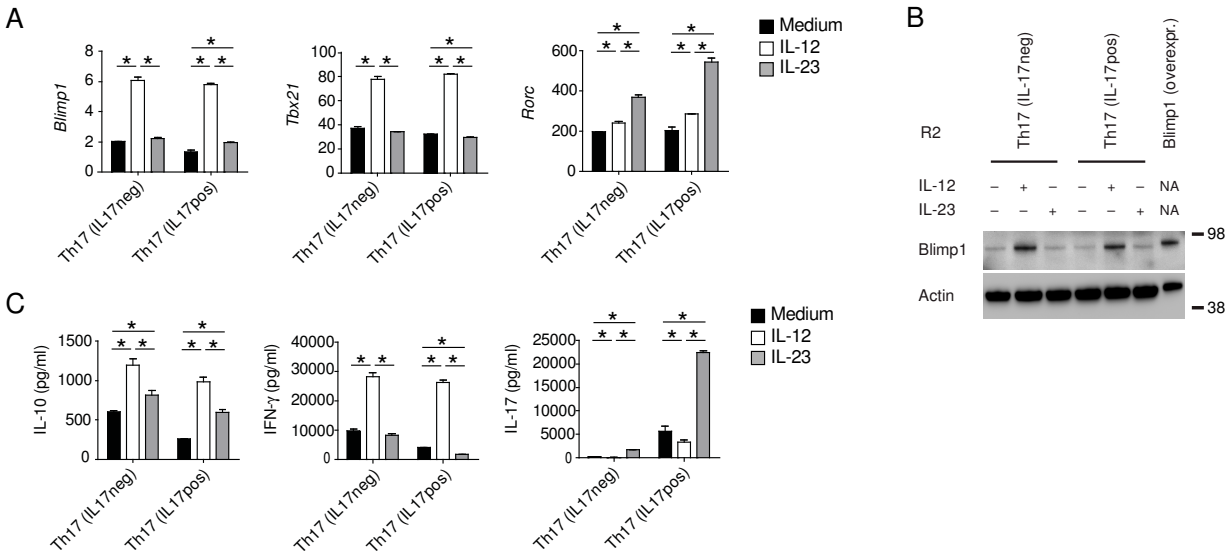

Figure 14: Bona-fide Th17 cells convert into anti-inflammatory IL-10 producing T cells upon IL-12 stimulation. T naïve cells from wild type mice were differentiated with polyclonal stimulus into Th17 cells for 3 days. IL-17 producing cells and IL-17-negativ cells (~90% purity) were separated using IL-17 secretion assay, rested for 6 days and re-stimulated without exogenous cytokines or in presence of IL-12 or IL-23. (A) Expression of *Blimp1*, *T-bet* and *Rorc* was detected by RT-PCR analysis from IL-17 positive and negative cells out of the Th17 differentiation culture on day 3 of re-stimulation. (B) Cell lysates were generated after polyclonal re-stimulation (R2) with indicated conditions. Depicted was the expression of Blimp1 in both IL-17$^+$ and IL-17$^-$ fractions of the initial Th17 cultures by Western Blot. Actin served as loading control and overexpressed Blimp1 in retrovirally transduced Th0 cells as positive control. (C) The cytokine production of IL-10, IFN-γ and IL-17 analyzed by ELISA after re-stimulation.

In conclusion, it is to say that pre-committed Th17 respond to IL-23 signals by stabilization of the pathogenic Th17 phenotype which is characterized by high Ror-γt expression and secretion of IL-17. However, IL-12 signals induce a Tr-1-like phenotype characterized by IL-10 and IFN-γ production in a Blimp1 dependent manner. Thus, IL-12 might re-program Th17 cells into a T cell subset with anti-inflammatory properties.

4.2.4 Stat4 is activated upon IL-12R signaling and binds directly to the Blimp1 locus

Our results suggested that Blimp1 could be a molecular switch regulating the cell-intrinsic down-modulation of pro-inflammatory properties in pre-committed effector T cells and IL-12 appeared to be a strong inducer of Blimp1 in pre-committed Th17 cells. Next, we wanted to investigate in more detail the signaling pathway of Blimp1 expression during T cell differentiation and in pre-committed Tr-1 and Th17 cells. The differentiation cytokines of Tr-1 and Th17 cells, IL-27 and IL-6, signal both via Stat3. On the one hand, IL-6 alone did not induce Blimp1 (Figure 5). On the other hand, IL-21 has been reported to induce IL-10 expression in $CD4^+$ and $CD8^+$ T cells in a Stat3 dependent manner (Spolski et al., 2009). In order to analyze whether Stat3 signals have an impact on Blimp1 expression during differentiation of IL-10 producing Tr-1 cells, we differentiated Tr-1 cells, and as control Th1 and Th17 cells, from wild type and Stat3 T cell conditional knock-out mice. In fact, the extent of Blimp1 induction differed according to the differentiation regime as shown above (see Figure 5). However, deficiency of Stat3 did not immediately affect the induction of Blimp1 under Tr-1 differentiation conditions (Figure 15). In contrast to Blimp1 expression, c-Maf expression in Th17 and Tr-1 cells was Stat3 dependent (Figure 15).

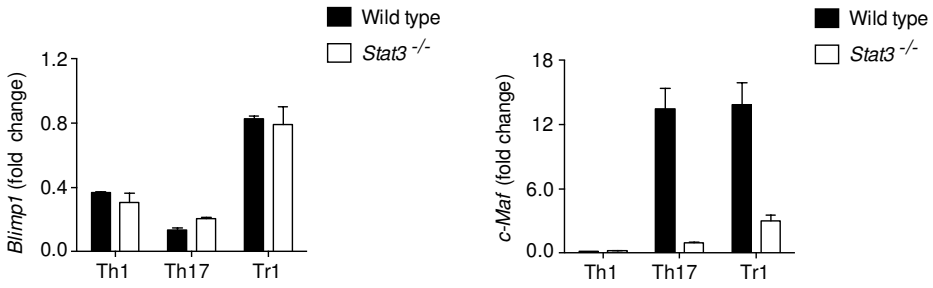

Figure 15: Stat3 is not a determinant of Blimp1 induction in Tr-1 cells. Naïve T cells from wild type or T cell conditional Stat3 deficient mice (*Stat3*$^{-/-}$) were differentiated into Th1, Th17 or Tr-1 cells for 2 days. The relative expression of *Blimp1* and *c-Maf* was measured by RT-PCR in relation to b-actin. T naïve cells served as reference and representative diagram from three similar and independent experiments.

Moreover, IL-12 does not activate Stat3 but is an activator of Stat4. Thus, I wanted to test whether Blimp1 induction in Tr-1 cells was dependent on Stat4 activation. Almost 20 years ago, Kaplan et al. demonstrated that Stat4 is essential for mediating responses to IL-12 in lymphocytes (Kaplan et al., 1996). Thus, we assessed the contribution of Stat4 during the Blimp1 dependent IL-10 induction by IL-12 in the second round cultures. Naïve T cells from Wild

type (Balbc) and Stat4$^{-/-}$ mice were polyclonally differentiated for 3 days into Tr-1 cells, rested for 6 days in presence of IL-2 and were re-stimulated without cytokines or with IL-12.

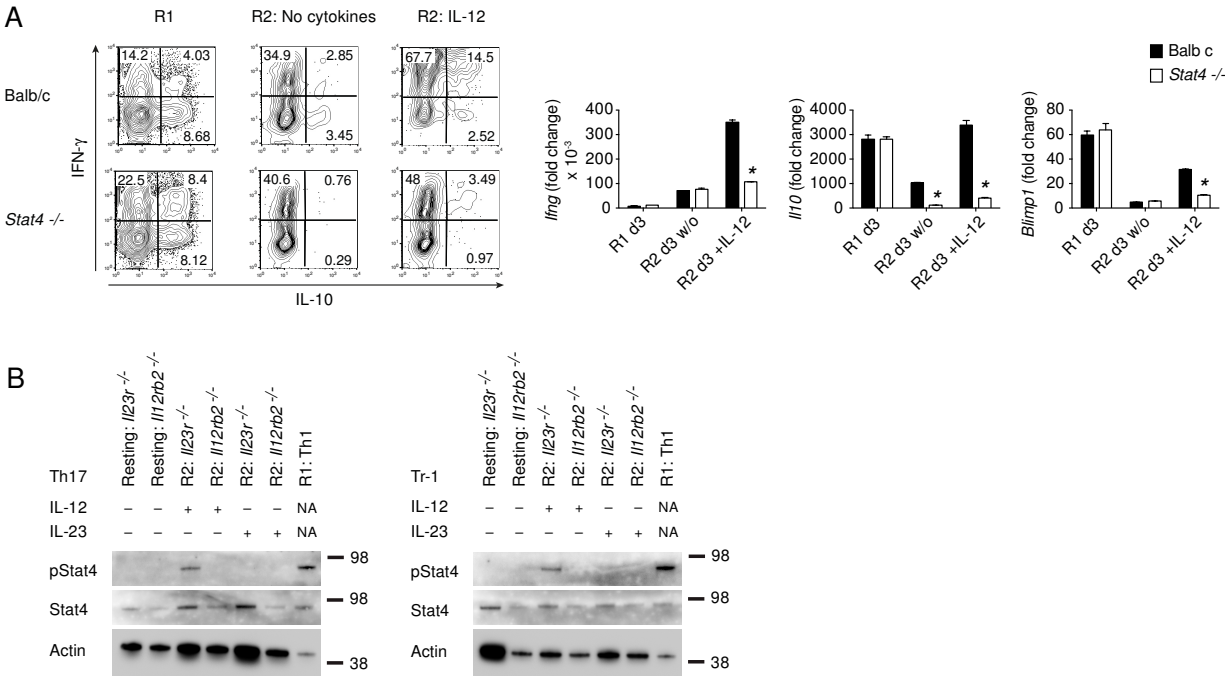

Figure 16: IL-12 driven induction of IL10/IFN-γ producing cells from pre-committed Tr-1 cells is dependent on Stat4 activation. (A) T naïve cells from wild type (Balb/c) or Stat4 deficient mice (*Stat4*$^{-/-}$) were differentiated into Tr-1 cells for 3 days (R1). After a resting phase of 6 days, Tr-1 cells were polyclonally re-stimulated without exogenous cytokines or with IL-12. The cytokine production on day 3 of R1 and day 3 of R2 was detected using flow cytometry and mRNA expression of indicated genes was analyzed by quantitative RT-PCR. (B) T naïve cells from *Il12rb2*$^{-/-}$ and *Il23r*$^{-/-}$ mice were differentiated into Th17 and Tr-1 cells for 3 days, rested for 6 days and re-stimulated as indicated. Cell Lysates were generated from cells before and after a short re-stimulation in presence of IL-12 or IL-23 for 2 hours. The lysates were analyzed by Western Blot for expression of Stat4 and the phosphorylated Stat4 protein. Actin served as loading control and Th1 T cells, differentiated from wild type mice for 3 days, as positive control. The diagrams are representative data of three similar experiments.

After the first round of differentiation, control cells and Stat4 deficient T cells produced similar amounts of cytokines. In contrast, after stimulation with IL-12, Stat4$^{-/-}$ Tr-1 cells failed to develop into IFN-γ^{+}IL-10^{+} double producers and showed significantly lower expression of IFN-γ, IL-10 and Blimp1 (Figure 16A). The data demonstrated that Stat4 was involved in the Blimp1-dependent induction of anti-inflammatory cytokines by IL-12 stimulation. To proof the direct involvement of Stat4 during signal transduction of IL-12, and to exclude that IL-23 could activate Stat4 we detected phosphorylated Stat4 (pStat4), by Western Blot. Using Th17 and Tr-1 cells

from *Il12rb2*$^{-/-}$ and *Il23r*$^{-/-}$ mice, phosphorylation of Stat4 was only detected after IL-12 stimulation in Th17 and Tr-1 cells (Figure 16B). Th1 cells served as positive control.

These data demonstrated that IL-12 induced Stat4 activation might be important for inducing Blimp1 expression in Th17 and Tr-1 cells. To define whether Stat4 bound directly to the Blimp1 genomic region, a chromatin immunoprecipitation (ChIP) was performed. We stimulated Th1, Th17 and Tr-1 cells in a second round with IL-12 to enhance the binding of transcription factors to regulatory gene sequences. This binding of transcription factors to the DNA were cross linked to generated a stable complex, which can be separated by immunoprecipitation (IP) with an antibody against the antigen of interest, in this case the transcription factor Stat4. After IP, the crosslinks between protein and DNA were removed and the released gene sequences, which were putative targets of the transcription factor after stimulation, can be detected by PCR methods. Primers were designed to amplified sequences of target gens (in our case Blimp1), which contained probable binding sequences of Stat4. The whole Blimp1 gene sequence is not completely decoded, results is putative nomination of transcription factor binding sites or regulatory sequences, like promoter regions. Two putative binding sites of Stat4 were predicted within the Blimp1 locus using the software VISTA, searching for the Stat4 consensus sequence described by Wei et al. (Wei et al., 2010) (genome.lbl.gov/vista/ index.shtml). Primer pairs to amplify region A in the putative promoter region and region B in intron 3 of the *Blimp1* locus were generated. Primer pairs to amplify control regions derived from IL-4 and T-bet genes were also generated and served as negative and positive control, respectively. In contrast to the descripted binding of Stat4 to T-bet locus, amplification of IL-4 sequence acted as negative control, because no binding was expected.

To analyze the binding of Stat4 on the Blimp1 locus after IL-12 stimulation, we differentiated Th1, Th17 and Tr-1 cells for 3 days and re-stimulated them in the second round with IL-12. Afterwards, samples were fixed, precipitated with anti-Stat4 antibody or irrelevant IgG, and the pull down of Blimp1 genomic regions was detected by PCR with Blimp1 primer pair A or Blimp1 primer pair B. The data demonstrated that Stat4 bound to both predicted regions, the putative promoter region and in intron 3 of the *Blimp1* locus, in Th1, Th17 and Tr-1 cells (Figure 17A). Our results were verified by data from another method, which were generated with kind support of our cooperation partner Warren J. Leonard (Laboratory of Molecular Immunology and Immunology Center, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, USA). The data from the ChIP-sequencing (chromatin immunoprecipitation sequencing) demonstrated the same binding region of Stat4 within the putative promoter region and in intron 3 of the Blimp1 genomic sequence (Figure 17B).

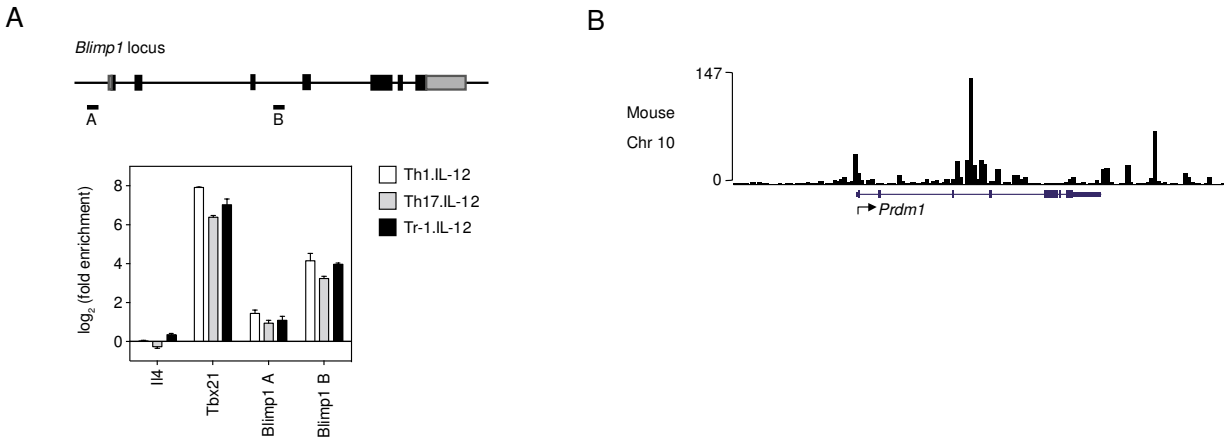

Figure 17: Stat4 binds directly to the *Blimp1* locus. (A) Naïve T cells from wild type mice were differentiated into Th1, Th17 or Tr-1 cells for 3 days, rested for 6 days and re-stimulated for 2 hours in presence of IL-12. To detect whether Stat4 binds on the Blimp1 locus, chromatin was precipitated with anti-Stat4 antibody or irrelevant IgG antibody and amplified by PCR with primers for putative Stat4 binding sites (Wei et al., 2010) in Blimp1 genomic regions. The upper part of the picture shows the Blimp1 locus with putative promoter region, introns and exons. Marked with A and B are Blimp 1 primer pairs. Amplification of Tbx21 promoter and Il4 intron sequences served as positive and negative control respectively. Signals were detected by qPCR with SYBR Green and depicted as fold enrichment in relation to signals from control IgG precipitates. Data include three independent experiments (B) Stat4 ChIP sequencing tracks for activated murine Th1 cells were illustrated. Gene orientation is from 5´ to 3´ end.

To sum up, IL-12 receptor signals activate Stat4. Phospho-Stat4 translocates into the nucleus and binds to the putative promoter regions or to the intron 3 of the *Blimp1* locus, which likely leads to direct transactivation of the *Blimp1* gene. The results are one indication that Stat4 plays important role in the activation of the Blimp1 and that Stat4 potentially act as gene regulation molecule on the Blimp1 gen.

4.3 The balance between IL-12R and IL-23R signals *in vivo*

4.3.1 Blimp1 is essential for developing Tr-1 like cells *in vivo*

Our results from the *in vitro* experiments suggested that Blimp1 is a transcription factor that had the capacity to integrate pro- and anti-inflammatory cytokine cues in pre-committed T helper cell subsets. To test the hypothesis that Blimp1 mediates the balance between IL-12 and IL-23 signals *in vivo* during autoimmune inflammation like EAE, control mice or T cell conditional

Blimp1 deficient mice (called *Blimp1* CKO) were immunized with MOG_{35-55} peptide in complete Freund's adjuvant (CFA) followed by monitoring of the EAE severity (Figure 18A).

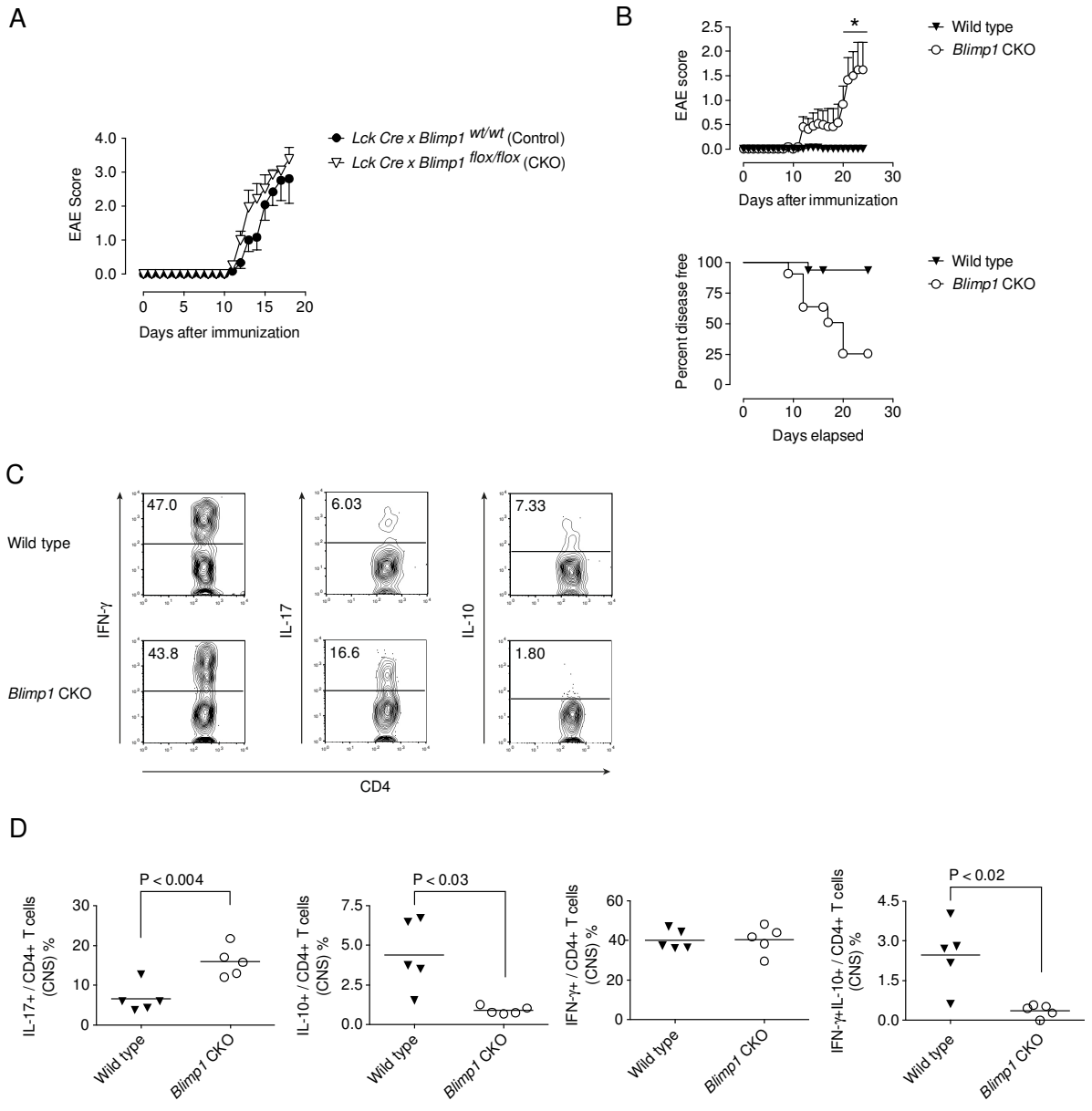

Figure 18: Blimp1 is essential for the development of IL-10 producers *in vivo*. (A) *RAG1*$^{-/-}$ mice were lethally irradiated, reconstituted with bone marrow of control or T cell conditional *Blimp1* KO mice and immunized with MOG_{35-55} in CFA plus 200 ng PTx on day 0 and 2 (standard EAE protocol). Mean EAE score of 6 mice per group are shown. Depicted are representative data for three similar experiments. (B) Wild type or T cell conditional *Blimp1* deficient (*Blimp1* CKO) mice were immunized with MOG_{35-55} in CFA plus 50 ng PTx on day 0 and 2 (suboptimum EAE protocol). EAE score and disease incidence of three independent experiments (n=11) are shown. (C and D) On day 20 (suboptimum EAE protocol), mononuclear cells were isolated from the CNS and cytokine profiles were evaluated by flow cytometry. Two independent experiments were performed.

With the standard EAE protocol, containing 200 ng PTx per mouse on day 0 and 2 after immunization, both groups developed severe EAE symptoms. No differences could be detected between both groups, because even the wild type group reached a ceiling effect due to massive EAE symptoms. To unmask potential differences in the susceptibility to EAE, we used a suboptimum EAE protocol with reduced PTx concentrations (50 ng instead of 200 ng PTx). Wild type mice and *Blimp1* CKO mice were immunized and the EAE score was assessed (Figure 18B). Clinical signs of EAE were only induced in Blimp1 CKO mice, whereas the wild type mice developed only marginal signs of EAE. Thus, Blimp1 CKO mice appeared more susceptible to EAE than their wild type littermates. The analysis of CNS infiltrates showed that Blimp1 CKO mice failed to generate IL-10 positive T cells and double producing IL-10/IFN-γ T cells at the site of inflammation (Figure 18C). In addition, wild type $CD4^+$ T cells in the CNS showed a significantly reduced frequency of $IL\text{-}17^+$ T cells in comparison to *Blimp1* CKO T cells (Figure 18D).

In summary, Blimp1 is required for the development and generation of IL-10 producing T cells in the inflammatory tissue the CNS. Blimp1 deficient $CD4^+$ cells failed to express anti-inflammatory cytokines, like IL-10. In addition, the frequency of pro-inflammatory cytokines like IL-17 is increased in T cell conditional Blimp1 KO mice and promotes severe CNS inflammation.

4.3.2 IL-12R signals induce the IL-10 production in effector T cells *in vivo*

To evaluate whether the shift of effector T cells from IL-17/IFN-γ to Tr-1 like T cells was due to IL-12R signaling *in vivo*, we transferred committed Th17 or Tr-1 T cells from wild type and IL-12Rβ2 deficient mice (controls) into immunized *CD45.1* mice. For transfer, I used 2D2 T cells that bear a transgenic (MOG)-specific TCR. The majority of $CD4^+$ splenocytes express the transgenic TCR (as defined by Valpha3.2 and Vbeta11 expression), which allow the isolation or transfer of high amount of antigen-specific T cells. The aim was to investigate the impact of IL-12R signals on pre-committed T cells in inflammatory conditions by analyzing their cytokine profile. Based on the congenic marker of the transferred T cells, donor T cells were re-sorted from the CNS and analyzed by RT-PCR. Th17 (Figure 19A) and Tr-1 (Figure 19B) *Il12rb2*$^{-/-}$ cells expressed significantly less *IFNg* and *Il10* mRNA in comparison to the wild type control T cells. The frequency of IL-10 single producing and IL-10/IFN-γ double producing T cells was also reduced in absence of IL-12 signaling in both transferred Tr-1 and Th17 cells. The data

demonstrated that IL-12 signaling was required for generation of IL-10 and IL-10/IFN-γ producing T cells in an inflammatory milieu *in vivo*. Both pre-committed Th17 and Tr-1 cells needed to sense IL-12 signals to induce this anti-inflammatory cytokine profile *in vivo*.

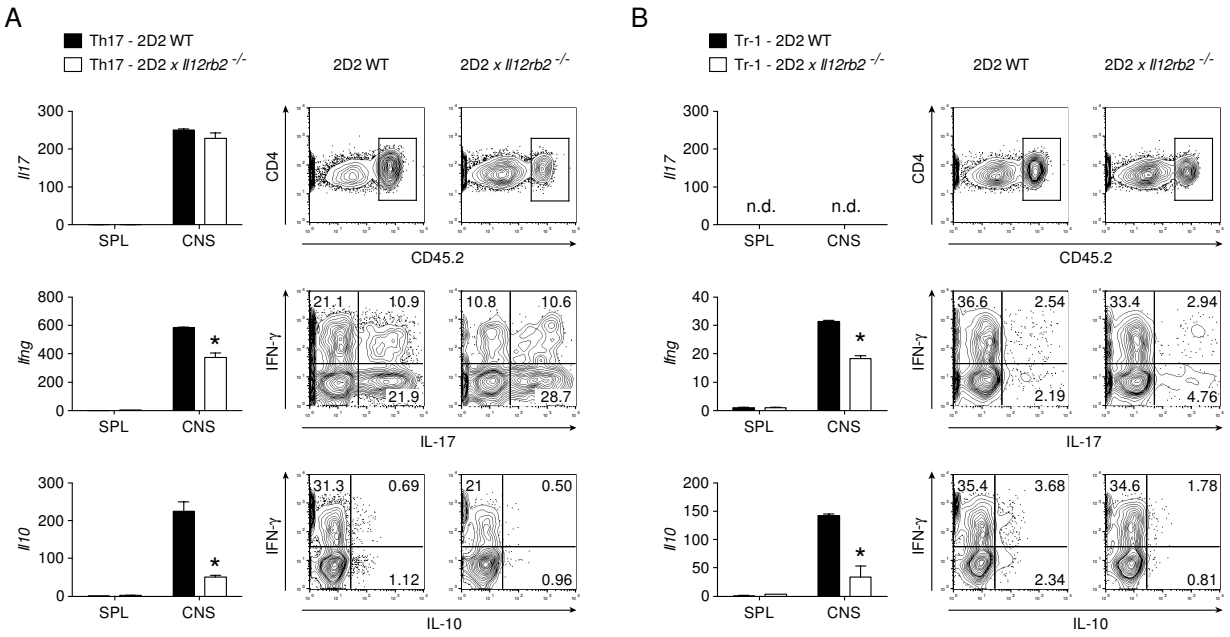

Figure 19: IL-12R signaling is required for IL-10 and IFN-γ expression *in vivo*. Naïve T cells from wild type *2D2* or *Il12rb2*$^{-/-}$ *x 2D2* mice (CD45.2) were polyclonally differentiated into Th17 (A) and Tr-1 (B) cells for 3 days. Congenic CD45.1 mice were immunized with MOG_{35-55} in CFA in combination with PTx. 10 million pre-committed T cells were intravenously injected into CD45.1 mice on day 6 after immunization and were re-sorted from the CNS at the peak of disease (d17). The mRNA level of cytokines was analyzed by quantitative RT-PCR and intracellular cytokine staining was evaluated by Flow cytometry. The data are representative of two independent experiments each containing at least three mice per group.

In order to further corroborate this finding, we generated mixed bone marrow chimeras (mBMC). Here, wild type cells and *Il12rb2* KO cells were mixed and used to reconstitute irradiated Rag1 KO host mice. After complete reconstitution of the T cell pool, mice were immunized with MOG_{35-55} in CFA. At the peak of CNS autoimmune disease, d16, T cells were isolated, based on the congenic marker, from the spleen and analyzed for the expression of *Blimp1*, *Il10*, and *Ifng* expression by quantitative PCR. *Il12rb2*$^{-/-}$ splenic T cells showed decreased expression of IL-10, IFN-γ and Blimp1 as compared to their IL-12R sufficient counterparts (Figure 20). Thus, the results revealed that IL-12 contributed to the expression of Blimp1, IL-10 and IFN-γ in $CD4^+$ T cells *in vivo*.

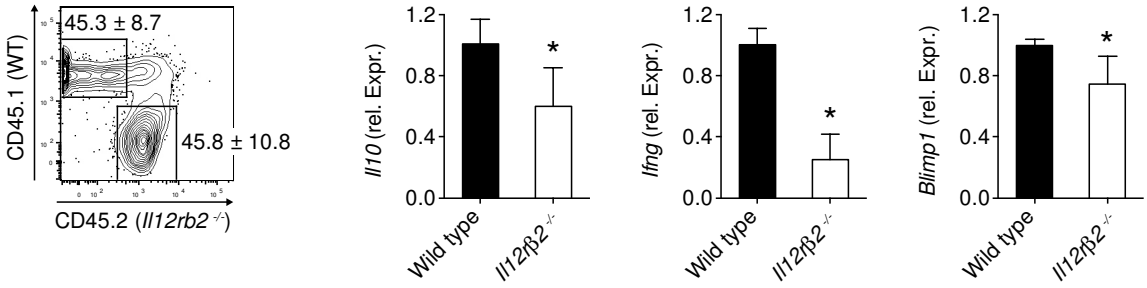

Figure 20: Without IL-12R signaling, CD4$^+$ T cells reduce express *Blimp1 in vivo*. Bone marrow cells from wild type CD45.1 and IL-12rb2 deficient mice (CD45.2) were injected into irradiated *Rag1*$^{-/-}$ mice to generate mBMC. After 5 weeks of cell transfer, EAE was induced by injection of MOG_{35-55} in CFA with PTx on day 0 and 2. At the peak of EAE (d17), engrafted CD4$^+$ Donor T cells were isolated from the spleen and sorted according to their congenic marker. The expression of *Il10*, *Ifng* and *Blimp1* was analyzed by quantitative RT-PCR. Data were representative for two similar independent experiments.

4.3.3 Genetic ablation of the Tr-1 cytokines IL-10 and IFN-γ abolishes the EAE resistance of *Il23r* deficient mice

The results indicated that IL-12 contributed to the development of IL-10/ IFN-γ producing T cells in the CNS in a Blimp1 dependent manner. Accordingly, IL-12 skewed pathogenic Th17 T cells into IL-10 producers *in vivo*. Thus, I hypothesized that the IL-12/Blimp1/IL-10 axis might counteract the effects of IL-23 in T cells *in vivo* and it was possible that the resistance of Il23r$^{-/-}$ mice to EAE might be connected to exaggerated IL-12 driven Tr-1 cell functions. To evaluate the functional relevance of IL-10/IFN-γ expression in IL-23R deficient mice, a new mouse model was generated. *IL23r*$^{-/-}$ mice were crossed with IL-10 and IFN-γ deficient mice to generate triple deficient mice (TKO: *Il23r*$^{-/-}$ *x Il10*$^{-/-}$ *x Ifng*$^{-/-}$).

In comparison to *Il23r*$^{-/-}$ mice, which are resistant, triple deficient mice (TKO) developed severe EAE symptoms without remission as observed for wild type *Il23r*$^{+/-}$ mice. Control double deficient mice (*Il23r*$^{-/-}$ x *Ifng*$^{-/-}$ and *Il23r*$^{-/-}$ x *Il10*$^{-/-}$) developed only negligible symptoms in comparison to triple knock-out mice (Figure 21). These data indicated that the ablation of both cytokines, IL-10 and IFN-γ, rendered IL-23R deficient mice susceptible to EAE. The CNS infiltrating CD4$^+$ T cells from all groups were analyzed by flow cytometry (Figure 22). Infiltrates in triple deficient mice contained reduced amounts of IL-17 producing T cells and unchanged amounts of GM-CSF producers compared with wild type cells. Possibly, GM-CSF a potent pro-inflammatory cytokine (Codarri et al., 2011; El-Behi et al., 2011) was the major pro-inflammatory T cell effector cytokine in this scenario.

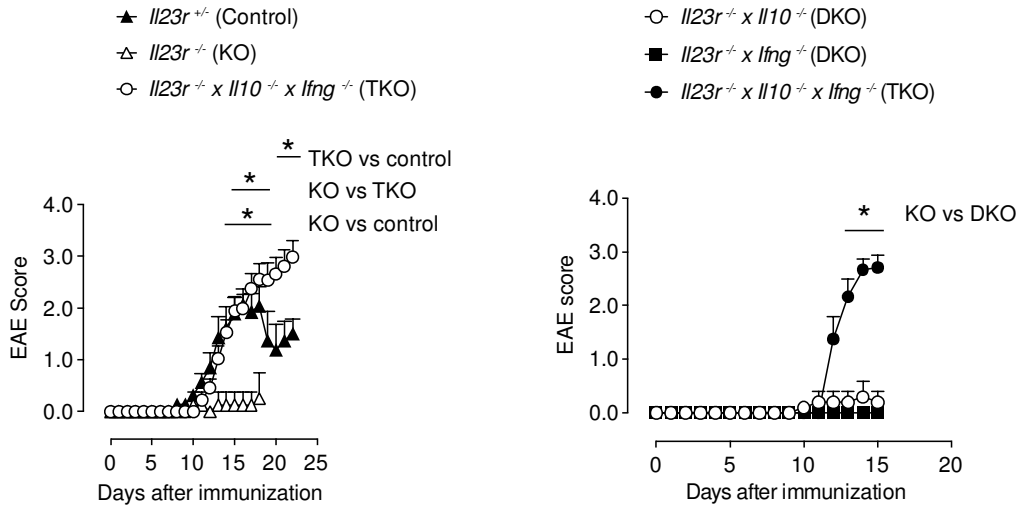

Figure 21: Depletion of the hallmark cytokines of Tr-1 IL-10 and IFN-γ leads to severe autoimmune CNS inflammation. Wild type (*Il23r*$^{+/-}$), *Il23r* deficient mice (*Il23r*$^{-/-}$), double knock-out (*Il23r*$^{-/-}$ *x Il10*$^{-/-}$*; Il23r*$^{-/-}$ *x Ifng*$^{-/-}$) as well as triple knock-out mice (*Il23r*$^{-/-}$ *x Il10*$^{-/-}$ *x Ifng*$^{-/-}$) were immunized with MOG_{35-55} in CFA. Depicted are the mean clinical scores with SEM.

Taken together, the absence of the Tr-1 hallmark cytokines (IL-10 and IFN-γ) rescues the EAE phenotype of *Il23r*$^{-/-}$ mice, which are describe as strictly resistant to EAE induction. Thus, IL-10 and IFN-γ are necessary to suppress immunopathology of CNS inflammation. Finally, IL-12 was an important mediator to regulate this Blimp1 dependent development of anti-inflammatory IL-10 producers. In conclusion, suppressing the “down-modulatory” IL-12/Blimp1/IL-10 axis might be an equally important function of IL-23 as the induction of pro-inflammatory properties in $CD4^+$ T cells.

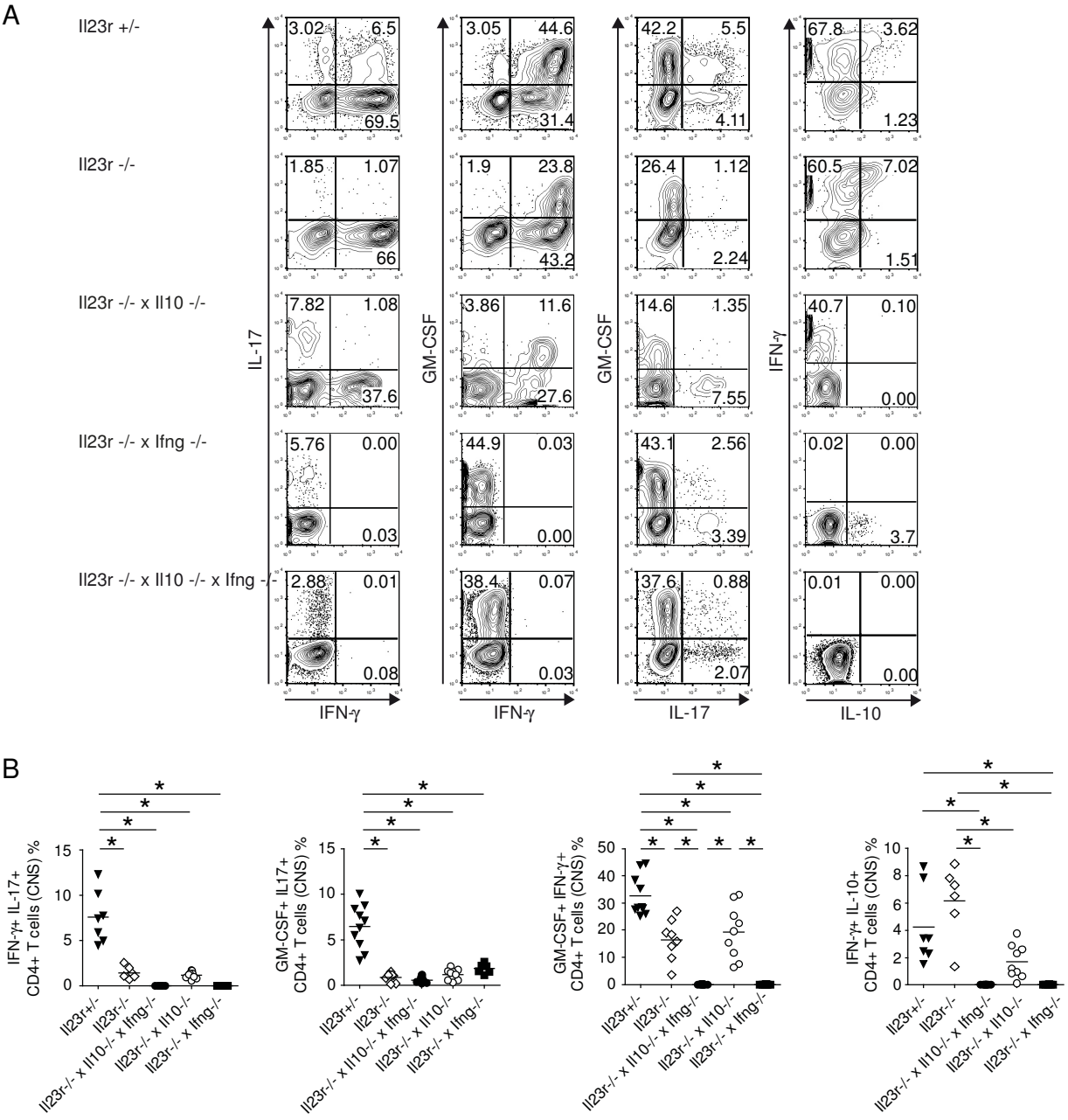

Figure 22: Depletion of characteristic Tr-1 cytokines IL-10 and IFN-γ leads to a GM-CSF cytokine phenotype. Wild type (*Il23r*$^{+/-}$), *Il23r*$^{-}$ deficient mice (*Il23r*$^{-/-}$), double knock-out (*Il23r*$^{-/-}$ *x Il10*$^{-/-}$*; Il23r*$^{-/-}$ *x Ifng*$^{-/-}$) as well as triple knock-out mice (*Il23r*$^{-/-}$ *x Il10*$^{-/-}$ *x Ifng*$^{-/-}$) were immunized with MOG_{35-55} in CFA. (A) At the peak of disease (d18 to d20) the cytokine production of CNS infiltrates were analyzed by flow cytometry. Representative cytograms of two independent experiments are displayed. (B) Fraction of IL-17^{+}IFN-γ^{+}, GM-CSF^{+}IL-17^{+}, GM-CSF^{+}IFN-γ^{+} and IFN-γ^{+}IL-10^{+} double producing CD4^{+} T cells of the CNS are shown. ANOVA with Turkey´s multiple comparison test were used.

5 Discussion and outlook

5.1 Different transcriptional expressions in Tr-1 vs. Th17 cells

The aim of the present work was to evaluate the biological function of IL-12 cytokine family members, mainly IL-23, for the modulation of pathogenic T cell responses in organ specific autoimmunity. Multiple Sclerosis and corresponding animal models are T cell mediated diseases, in which auto-reactive effector T cells induce partly irreversible tissue damage in the target organ CNS. Cytokines of the IL-12 cytokine family are described to effect the generation of T cell lineages, and thus the development of pathogenic T helper cells. However, these cytokines also modulate the plasticity of T cells during inflammatory conditions. In this study we focused on the evaluation of T cell-intrinsic functions of IL-12 cytokines family members to modify the transcriptional expression in pro-inflammatory and anti-inflammatory T cell subsets. The *in vitro* generation of anti-inflammatory IL-10 producing Tr-1 cells is dependent on TGF-β and IL-27 (Awasthi et al., 2007; Fitzgerald et al., 2007; Stumhofer et al., 2007). TGF-β, a pleiotropic cytokine, promotes in combination with IL-6 the generation of IL-17 producing Th17 cells (Bettelli et al., 2006; Mangan et al., 2006; Veldhoen et al., 2006). Both, IL-6 and IL-27 act via Stat3 signals (Batten et al., 2008; Stumhofer et al., 2007; Xu et al., 2009). This demonstrates that similar signaling pathways induce the generation of fundamentally different T cell subsets. Transcription factors are key modulators for T cell differentiation and control the development and plasticity of T cells. For that reason we compared *in vitro* differentiated anti-inflammatory Tr-1 cells and pro-inflammatory Th17 cells in a screening assay to identify transcription factor candidates which were differentially expressed and potentially modulated by IL-12 cytokine family members. We observed that 4 transcription factors (*Irf1*, *Irf8*, *Tbx21* and *Prdm1*) are at least 3-fold higher expressed in Tr-1 cells compared to Th17 cells (Figure 4).

Irf8 has a critical role in silencing Th17 cell differentiation. The group of Xiong reported that a conditional knockout of the *Irf8* gene in T cells results in enhanced Th17 cell generation while exhibiting no significant effect on Tregs, Th1 cells or Th2 cells, as measured by unchanged frequencies of IFN-γ^+, IL-4^+, IL-10^+ and Foxp3 positive cells (Ouyang et al., 2011). They concluded that T cell conditional Irf8 deficiency resulted in a more severe inflammatory response in an experimental model of colitis, and that Irf8 has a suppressive role in the control of Th17

differentiation. In addition, Irf8 has profound functions in APCs of the myeloid lineage. Irf8, expressed in APCs, induces a cytokine milieu that favors induction and maintenance of Th1 and Th17 cells, via stimulating IL-12 and IL-23 production. On the other hand, Irf8 inhibits IL-27 during EAE. Furthermore, global Irf8$^{-/-}$ mice are resistant to EAE induction (Yoshida et al., 2014). Our microarray data revealed that Irf8 is highly expressed in Tr-1 cells. It is likely that Irf8 has different functions in T cells vs. APCs. Concerning its role in T cells, our results are in line with the conclusion of Ouyang et al. that Irf8 has T cell intrinsic inhibitory functions in the generation of Th17 cells.

Irf1 has an essential function regulating the expression of *Il12rb1*. Kano et al. reported that in the absence of Irf1, naïve CD4$^+$ T cells did not differentiate into IFN-γ producing Th1 cells due to defective IL-12 signaling (Kano et al., 2008). However, Irf1-mediated induction of IL-12Rβ1 was dispensable for IL23 induced Th17 cell differentiation. Over-expression of the transcription factor Tbx21 (T-bet) which is essential for Th1 development by inducing IFN-γ (Szabo et al., 2000), did not normalize IFN-γ production in *Irf1*$^{-/-}$ CD4$^+$ T cells. This demonstrates that both, functional IL-12 signaling and T-bet expression, seem to be required for full Th1 differentiation (Kano et al., 2008). Our microarray data associate Irf1 and Tbx21 also with the Tr-1 transcriptional program and both transcription factors might have similar targets in Tr-1 cells as in Th1 cells. In fact, Tr-1 cells also express IFN-γ besides their signature cytokine IL-10.

Finally, we identified Blimp1 to be differentially expressed in Tr-1 cells vs. Th17 cells. Since Blimp1 has been associated with IL-10 production in other cell types before (Cui et al., 2011; Iwasaki et al., 2013; Lin et al., 2013; Sun et al., 2011), our microarray data provided a strong hint that IL-10 production in CD4$^+$ T cells might also be controlled by Blimp1.

5.2 The role of Blimp1 during differentiation of T helper cells

The microarray analyzes indicated that Blimp1 is differentially expressed in T helper subsets (Figure 4) which is in accordance with other reports (Salehi et al., 2012). Tr-1 and Th1 cells express large amounts of Blimp1 while Th17 cells fail to express Blimp1. While expression of Blimp1 in CD4$^+$ T cells correlates with their ability to produce IL-10, other pathways of IL-10 induction might be operational and the availability of TGF-β determines the transcriptional pathway of IL-10 induction. Without TGF-β, the IL-10 expression is Blimp1-dependent. A report from the group of Martins is in line with our results because they show that with increasing TGF-β concentrations the IL-10 inducing effect of Blimp1 wanes (Salehi et al., 2012). Indeed we found that with TGF-β the production of IL-10 in Tr-1 cells is Blimp1-independent and correlates

with expression of c-Maf and Ahr (Figure 6 and Figure 7). Apetoh and colleagues demonstrated that Ahr interacts with c-Maf to promote Tr-1 differentiation induced by IL-27 and TGF-β (Apetoh et al., 2010). They show that the presence of TGF-β is essential for the Ahr expression and that the Ahr expression increases during differentiation of Tr-1 cells with IL-27 and TGF-β. Our data are in accordance with these findings.

Fitzgerald et al. observed an induction of IL-10 in IFN-γ^+T-bet$^+$Foxp3$^-$ cells by IL-27. They also show that IL-10 mediates the suppressive effect of IL-27 on encephalitogenic T cells in adoptive transfer EAE (Fitzgerald et al., 2007). Our results corroborate these data and give hints that the IL-27 induced IL-10 production in these IFN-γ^+T-bet$^+$Foxp3$^-$ cells (probably Tr-1 cells) is Blimp1 dependent. In fact, we show that in absence of TGF-β the production of IL-10 is ensured by Blimp1. Thus, we propose that different transcriptional pathways guarantee the IL-10 expression by Tr-1 cells depending on the presence of TGF-β.

5.3 Impact of Blimp1 on pre-committed T helper cells

When Tr-1 cells and Th17 cells are differentiated *in vitro* starting from naive (CD62L high) CD4$^+$ T cells, Blimp1 is strongly expressed in Tr-1 cells but not Th17 cells. In this project, I also evaluated the impact of Blimp1 expression in committed (no longer naive) T cells. The receptor equipment of committed Th17 and Tr-1 cells permits differentiated T cells to sense signals from both IL-23 and IL-12 (Figure 8). Stimulation of committed Tr-1 cells with IL-12 results in enhanced IL-10 and IFN-γ production which is Blimp1-dependent. In contrast, stimulation with IL-23 induces IL-17 in Tr-1 cells. Interestingly, the Blimp1-dependent induction of an anti-inflammatory phenotype (i.e. the induction of IL-10 together with IFN-γ) is not limited to specific T cell subsets. It rather is also operational in IL23R positive Th17 cells which are also equipped to receive IL-12R signals. Indeed, in this project, I showed that pre-committed Th17 cells adapt an anti-inflammatory phenotype upon sensing of IL-12. As a result of IL-12 stimulation, the IL-17 production is reduced while the production of IL-10 and IFN-γ is increased. In this context signals from IL-12R counteract IL-23R signals, which stabilize the pathogenic Th17 phenotype. We present a mechanism to re-program committed pathogenic T cells into anti-inflammatory IL-10 producing cells. IL-12 cytokine family members play a central role in modulation of transcriptional pathways, which are essential for T cell plasticity. Our *in vitro* data exhibit that distinct subsets of committed T cells, i.e. Tr-1 cells and Th17 cells, can in principle be modulated into contrary acting cells. Salehi et al. demonstrated in a current report that IL-12 stimulation leads to the induction of Blimp1 (Salehi et al., 2012). In 2008 Lexberg et al. reported that *in vitro*

generated Th17 cells can convert into Th1 cells (Lexberg et al., 2008) although this had been a matter of debate until fate tracking experiments showed that IL-17 producing T cells really convert into IFN-γ producers *in vivo* (Hirota et al., 2011; Kurschus et al., 2010). In a follow-up study Lexberg and colleagues found that *in vivo* generated Th17 cells were relatively stable and failed to express IL-12Rβ2, which means that Th17 cells are not responsive to IL-12. Thus, a simultaneous stimulation with IFN-γ and IL-12 of *ex vivo* isolated Th17 induced first the expression of IL-12Rβ2 and results later in a stable induction of T-bet and IFN-γ production. These *ex vivo* isolated Th17 cells co-expressed Th1 and Th17 specific transcription factors and cytokines which lead to their description as Th1/Th17 cells (Lexberg et al., 2010).

Kurschus and colleagues used an IL-17 fate mapping mouse strain (IL-17F Cre x R26-YFP) and reported that *in vivo* generated Th17 cells expressed IL-17 and IFN-γ. They concluded that *in vivo* generated Th17 cells do not represent a terminally differentiated cell population and that Th17 cells are able to alter their cytokine secretion profile (Kurschus et al., 2010). Gitta Stockinger's group confirmed these findings and further suggested that IL-23 was necessary for double expression of IL-17A and IFN-γ in historic IL-17 single producers because T-bet failed to be up-regulated in previous IL-17 producers in IL-23p19 deficient mice (Hirota et al., 2011). Similar observations were reported by Casey Weaver's group (Lee et al., 2009). They used an IL-17F reporter mouse and reported that not IL-23 but TGF-β was essential for the maintenance of IL-17 expression while committed Th17 cells responded to IL-23 stimulation (in the absence of TGF-β) with an extinction of IL-17F and a promotion of IFN-γ production (Lee et al., 2009). In addition, Lee and colleagues tested the response of Th17 cells to IL-12 and found that the transition of Th17 cell into a Th1 like phenotype was Stat4 and T-bet dependent (Lee et al., 2009). A more recent report suggested that – in line with our observation – Th17 cells were able to respond to IL-12 *in vivo* and that IL-12 induced an IL-17/IFN-γ double producing T cell population through cooperation of T-bet with Runx-1 or Runx-3 (Wang et al., 2014). In this study, IL-10 production has not been assessed. In our experimental systems, Th17 cells responded to IL-12 by adapting an IFN-γ/IL-10 secreting phenotype thus adopting the cytokine profile of Tr-1 cells. Therefore, we propose that IL-12 might contribute to re-program pathogenic Th17 cells into an effector T cell subset that serves regulatory functions.

5.4 Involvement of Stat molecules in the regulation of Blimp1 expression

Since IL-12 and IL-27 induced Blimp1 in naive T cells and in pre-committed Th17 cells and Tr-1 cells, the role of the Stat proteins downstream of the IL-12 receptor (Stat4) and IL-27 receptor (among others Stat3) needed to be tested for their capacity to directly transactivate Blimp1 expression. Notably, IL-6, which also signals through Stat3, cannot induce Blimp1 expression while IL-27 induces it (Figure 5). In order to test the requirement for Stat3 in IL-12 and IL-27 induced Blimp1 induction, Th1 cells (IL-12 plus anti-IL-4), Tr-1 cells (TGF-β plus IL-27) and Th17 cells (TGF-β plus IL-6) were differentiated from naive T cells derived from wild type mice or from mice that lacked Stat3 in their T cells (CD4 Cre x $Stat3^{flox/flox}$). Our results show that the Blimp1 expression, which is highly induced in Th1 and Tr-1, is Stat3 independent in this cells (Figure 15). Fornek and colleagues revealed that IL-21 induced the differentiation of B cells into plasma cells in a Stat3 and Blimp1 dependent manner (Fornek et al., 2006). Kwon et al. reinforced these data with the observation of a functional cooperation between Stat3 and IRF4 in IL-21-induced Blimp1 expression and discussed the same findings in T cells (Kwon et al., 2009). In $CD4^+$ T cells, Stumhofer et al. reported that IL-27 induce the secretion of IL-10 in Th1 cells in a Stat1 and Stat3-dependent fashion. On the other hand, they reported that the effect of IL-27 induced IL-10 production in Th1 cells was Stat4 and T-bet-independent (Stumhofer et al., 2007). In the present study I confirmed that Blimp1 expression during differentiation of Tr-1 cells with IL-27 was Stat4 independent (Figure 16). More recently, it was found out that IL-27 induced IL-10 production in $CD4^+$ T cells by the joint action of Egr-2 and Blimp1 (Iwasaki et al., 2013). Iwasaki and colleagues reported that IL-27 mediated induction of Blimp1, which was important for IL-10 production, was impaired in the absence of Egr-2. Egr-2 deficient $CD4^+$ T cells showed enhanced IFN-γ and IL-17 production in response to IL-27 stimulation. Thus, they summarized that Egr-2 was crucial in maintaining the balance between regulatory and inflammatory cytokines (Iwasaki et al., 2013). But both reports from Stumhofer et al. and Iwasaki et al. only illustrate that Stat1 and Stat3 is required for the expression of Egr2 or for the IL-10 production, but not directly for Blimp1 expression. Egr-2 expression was Stat3 dependent and it is possible that under specific conditions Stat3 contributes to full induction of Blimp1. However, in our experiments we showed that the IL-12/Stat4 axis is equally able to induce Blimp1 and eventually Blimp1 dependent transcriptional modules in committed T helper cell subsets. The group of O'Garra reported that *in vitro* differentiated Th1 cells required IL-12-induced Stat4 signaling for the IL-10 production and Stat4 sustained the phosphorylation of Erk1 and Erk2 (Saraiva et al., 2009). In this study, we found that IL-12 induced IL-10 in committed Tr-1 and Th17 cells via Blimp1.

Furthermore, our ChIP experiments demonstrated direct binding of Stat4 in the Blimp1 locus. Thus, by identifying Blimp1 as a putative target of Stat4, we found a new pathway for the modulation of the cytokine phenotype of T helper cell species, which might be one molecular explanation for their plasticity during inflammatory responses.

Apart from Stat3 and Stat4, other Stat molecules might be involved in the regulation of Blimp1 expression. For example, Stat5 regulates Blimp1 expression in Tfh cells. Blimp1 inhibits Tfh development by negatively regulating Bcl6, the master transcription factor of Tfh cells (Nurieva et al., 2012). The group of Wang demonstrated that Stat5 effectively inhibited Tfh differentiation by suppressing the expression of Tfh associated factors like CXCR5, c-Maf and Bcl6. On the other hand, Blimp1 overexpression inhibits Tfh gene expression in Stat5 deficient T cells, suggesting that the Stat5 function regulates Blimp1 expression (Nurieva et al., 2012).
In summary, the regulation of Blimp1 is highly context dependent. For example in Treg cells, Irf4 directly interacts with Blimp1 in order to induce proficient IL-10 production in Tregs (Cretney et al., 2011). By identifying the IL-12/Stat4/Blimp1 axis, we characterized a novel pathway in committed T helper cell species that leads to the production of IL-10 and might provide a means to restrain immunopathology in highly inflammatory milieus in a T cell intrinsic manner.

5.5 Opposing effects of IL-12 cytokine family member signals during autoimmune CNS inflammation *in vivo*

In contrast to IL-12 (and IL-27), IL-23 did not promote the up-regulation of Blimp1. Instead, the pathogenic (pro-inflammatory) T helper cell phenotype was stabilized by IL-23R signals – a process that was independent of Blimp1 (Bettelli et al., 2007; Martins and Calame, 2008). We performed *in vivo* experiments to test how the IL-23/Ror-γt/IL-17 axis might be balanced against the IL-12/Blimp1/IL-10 axis in T cell mediated autoimmunity.
Our conditional T cell specific Blimp1 knock-out experiments illustrate the important role of Blimp1 expression for the induction of IL-10 producing T cells which have suppressive functions to ameliorate EAE (Figure 18). In line with this function of Blimp1 in CNS autoimmunity, Blimp1 deficient mice develop severe colitis and harbor an increased number of peripheral effector and memory T cells (Kallies et al., 2006; Martins et al., 2006). Both studies document an increased IFN-γ but reduced IL-10 production in Blimp1 deficient $CD4^+$ T cells. Interestingly, there is an ongoing discussion whether Blimp1 directly represses *Ifng* or whether the effects of Blimp1 on the production of IFN-γ by T cells are just indirect. One explanation how Blimp1 might inhibit

IFN-γ production was proposed by Cimmino et al. They described that Blimp1 binds directly to the regulatory regions of *Ifng*, *Tbx21* and *Bcl6* genes (Cimmino et al., 2008). In our experiments we did not detect an increased IFN-γ production as a result of Blimp1 deficiency in T cells, but rather a slight reduction. This might have to do with the cytokine milieu in the EAE model where abundant IL-12 drives maximum IFN-γ production by various pathways. As it was also reported by others (Cretney et al., 2011; Kallies et al., 2006; Martins et al., 2006), we found a strongly reduced IL-10 production in T cells in the absence of Blimp1. More recent studies analyzed the relationship between Blimp1 and IL-10 in autoimmune diabetes (NOD mice) or in inflammatory responses in the colon. Lin et al. were able to that in Blimp1 deficient mice Th17 cells were significantly increased and that *Blimp1* CKO $CD4^+$ T cells highly expressed IL-23R. They concluded that Blimp1 negatively regulated Th17 differentiation via modulation of *Rora* and *Rorc* and discussed that Blimp1 attenuated Th1 responses via the suppression of T-bet (Lin et al., 2013). The group of Martins confirmed these data and showed that Blimp1 deficient $CD4^+$ effector T cells harbored increased amounts of *Il23r* and *Rorc* mRNA which they explained through direct repression of these gens by Blimp1 (Salehi et al., 2012). Thus, it was proposed that Blimp1 somehow destabilized the pathogenic Th17 phenotype. Here, our results demonstrate the essential role of Blimp1 for the re-programming of pathogenic effector T cells including Th17 cells into anti-inflammatory IL-10 producing T cells (Figure 18). We illustrate that IL-12R signals induce Blimp1 expression and promote the generation of anti-inflammatory IL-10 producers during inflammatory conditions.

Based on our findings we propose that Blimp1 might be a transcriptional modulator in T cells that integrates IL-12 and IL-27 signals on the one hand and IL-23 signals on the other hand. IL-12 and IL-27 induced anti-inflammatory properties in committed T cells via induction of Blimp1. An exaggerated function of the IL-12/Blimp1 pathway might thus contribute to conferring resistance to T cell mediated immunopathology and it was conceivable that in IL-23R deficient mice, the IL-12/Blimp1 pathway was in fact unopposed and excessively active resulting in resistance to EAE of *Il23r*$^{-/-}$ mice. This hypothesis was tested by “disrupting” the IL-12/Blimp1 pathway in *Il23r*$^{-/-}$ mice by ablating its effector molecules, i.e. IL-10 and IFN-γ. We found that *Il23r*$^{-/-}$ *x Il10*$^{-/-}$ *x Ifng*$^{-/-}$ triple KO mice developed severe EAE. Notably, bona fide Th17 cells were detected in the inflamed CNS of these mice suggesting that IL-23R was dispensable for the generation of pathogenic Th17 cells but was indispensable for controlling the IL-12/Blimp1/IL-10 pathway. Intracellular staining of infiltrating T cells isolated from the CNS of triple knockout mice indicated that GM-CSF could be the relevant effector cytokine which induced CNS inflammation (Figure 22). McQualter et al. demonstrated that GM-CSF has a non-redundant role during autoimmune

inflammation because *GM-CSF*$^{-/-}$ mice are resistant to EAE induction (McQualter et al., 2001). A more detailed correlation between GM-CSF and EAE induction was reported by Becher's group ten years later. Cordarri et al. suggested that GM-CSF production was driven by IL-23 and Ror-γt while IL-27, IL-12 and IFN-γ inhibited its expression (Codarri et al., 2011). In addition, Rostami's group described a positive feedback loop whereby GM-CSF secreted by Th17 cells stimulated the production of IL-23 by APCs which in turn induced further GM-CSF (El-Behi et al., 2011). In fact, GM-CSF deficient T cells failed to adoptively transfer EAE. GM-CSF might be essential in inhibiting apoptosis and promoting IL-17 production as well as the survival of autoreactive CD4$^+$ T cells (Sonderegger et al., 2008). All in all, GM-CSF has been considered as non-redundant encephalitogenic cytokine of pathogenic T helper cells. This spurred the discussion that Th17 cells with low GM-CSF production, for example upon differentiation in the presence of TGF-β and IL-6 (and in the absence of IL-23), might constitute a different flavor of Th17 cells which would not be pathogenic (Ghoreschi et al., 2010; Lee et al., 2012).
In the present study we detected GM-CSF producing T cells in the CNS of *Il23r*$^{-/-}$ mice and the frequency of GM-CSF$^+$ CD4$^+$ T cells in the inflamed CNS of *Il23r*$^{-/-}$ *x Il10*$^{-/-}$ *x Ifng*$^{-/-}$ triple KO mice was comparable to wild type mice. These data refute the idea that GM-CSF production in T cells is strictly dependent on IL-23R signals. Furthermore, our observations also speak against the concept that the strict coupling of IL-23 to downstream induction of GM-CSF determines the pathogenic role of IL-23. It seems that medium amounts of Ror-γt expression are sufficient to drive GM-CSF in T cells in the absence of IL-23R signals.

5.6 Conclusion and outlook

The aim of the study was to investigate the biological function of IL-12 cytokine family members for the generation and maintenance of pathogenic T cells. We found that IL-12 induces Blimp1 expression in committed T cells, resulting in an anti-inflammatory Tr-1 like phenotype. We described the essential requirement of IL-12R signals and Blimp1 expression specifically in T cells for the generation of IL-10 producing T cells. IL-23 counteracts the up-regulation of Blimp1, but stabilizes the pro-inflammatory Th17 phenotype. IL-12 cytokine family members act opposing but both operate intrinsically to modulate the expression of transcription factors and cytokines. The observation that IL-12 can re-program Th17 cells results in the hypothesis that the abolition of IL-23R signals in *Il23r*$^{-/-}$ mice unmasks IL-12R signals, and thereby promotes the "exaggerated" re-programming of effector T cells into Tr-1 like suppressive T cells. With these

results we provide new aspects to the biological function of IL-23 and an explanation for the resistance of *Il23r*$^{-/-}$ mice to EAE.

However, some questions remain to be addressed in further studies. With the description of IL-12 induced re-programming of committed Th17 *in vivo* we provide further evidence for the plasticity of T helper cells. The strict classification of T helper lineages needs to be re-thought. For example, we propose that Tr-1 cells may not constitute a distinct lineage of T helper cells but rather might be a developmental endpoint of T helper differentiation in inflammatory conditions. Certain extrinsic and intrinsic factors like cytokines and transcription factors mediate and regulate IL-10 production in T helper cells dependent on the ambient cytokine milieu. We describe a new transcriptional pathway beside c-Maf and Ahr, whereby IL-12 drives Stat4- and Blimp1-dependent IL-10 production in Th17 cells converting them to Tr-1 like cells. Another affirming point against the lineage theory is the fact that no specific biomarker for Tr-1 cells was detected so far. Only the co-expression of surface markers CD49b and Lag-3 have been discussed to allow for identification of human and mouse Tr-1 cells (Gagliani et al., 2013). However, the expression of either CD49b or Lag-3 is not specific for Tr-1 cells and Th17 cells "converted" into Tr-1 cells also start to express Lag-3. Currently, Tr-1 cells are described by their cytokine profile and strong immune-suppressive activity (Apetoh et al., 2010; Awasthi et al., 2007; Fitzgerald et al., 2007; Pot et al., 2009; Stumhofer et al., 2007). Unless a "master transcription factor" for IL-10 producing cells is identified, it will be difficult to speak of Tr-1 cells as a "lineage" of their own.

6 References

Abdi, K. 2002. IL-12: the role of p40 versus p75. *Scand J Immunol* 56:1-11.

Abe, R. 2005. [Regulation of immune response by T cell co-signaling.]. *Nihon Rinsho Meneki Gakkai Kaishi* 28:21-32.

Afkarian, M., J.R. Sedy, J. Yang, N.G. Jacobson, N. Cereb, S.Y. Yang, T.L. Murphy, and K.M. Murphy. 2002. T-bet is a STAT1-induced regulator of IL-12R expression in naive CD4+ T cells. *Nat Immunol* 3:549-557.

Amor, S., N. Groome, C. Linington, M.M. Morris, K. Dornmair, M.V. Gardinier, J.M. Matthieu, and D. Baker. 1994. Identification of epitopes of myelin oligodendrocyte glycoprotein for the induction of experimental allergic encephalomyelitis in SJL and Biozzi AB/H mice. *J Immunol* 153:4349-4356.

Anderson, A.C., L.B. Nicholson, K.L. Legge, V. Turchin, H. Zaghouani, and V.K. Kuchroo. 2000. High frequency of autoreactive myelin proteolipid protein-specific T cells in the periphery of naive mice: mechanisms of selection of the self-reactive repertoire. *J Exp Med* 191:761-770.

Andersson, J., D.Q. Tran, M. Pesu, T.S. Davidson, H. Ramsey, J.J. O'Shea, and E.M. Shevach. 2008. CD4+ FoxP3+ regulatory T cells confer infectious tolerance in a TGF-beta-dependent manner. *J Exp Med* 205:1975-1981.

Angelin-Duclos, C., G. Cattoretti, K.I. Lin, and K. Calame. 2000. Commitment of B lymphocytes to a plasma cell fate is associated with Blimp-1 expression in vivo. *J Immunol* 165:5462-5471.

Apetoh, L., F.J. Quintana, C. Pot, N. Joller, S. Xiao, D. Kumar, E.J. Burns, D.H. Sherr, H.L. Weiner, and V.K. Kuchroo. 2010. The aryl hydrocarbon receptor interacts with c-Maf to promote the differentiation of type 1 regulatory T cells induced by IL-27. *Nat Immunol* 11:854-861.

Apostolou, I., and H. von Boehmer. 2004. In vivo instruction of suppressor commitment in naive T cells. *J Exp Med* 199:1401-1408.

Asseman, C., and M. von Herrath. 2002. About CD4pos CD25pos regulatory cells. *Autoimmun Rev* 1:190-197.

Astier, A.L., G. Meiffren, S. Freeman, and D.A. Hafler. 2006. Alterations in CD46-mediated Tr1 regulatory T cells in patients with multiple sclerosis. *J Clin Invest* 116:3252-3257.

Awasthi, A., Y. Carrier, J.P. Peron, E. Bettelli, M. Kamanaka, R.A. Flavell, V.K. Kuchroo, M. Oukka, and H.L. Weiner. 2007. A dominant function for interleukin 27 in generating interleukin 10-producing anti-inflammatory T cells. *Nat Immunol* 8:1380-1389.

Awasthi, A., L. Riol-Blanco, A. Jager, T. Korn, C. Pot, G. Galileos, E. Bettelli, V.K. Kuchroo, and M. Oukka. 2009. Cutting edge: IL-23 receptor gfp reporter mice reveal distinct populations of IL-17-producing cells. *J Immunol* 182:5904-5908.

Bacchetta, R., M. Bigler, J.L. Touraine, R. Parkman, P.A. Tovo, J. Abrams, R. de Waal Malefyt, J.E. de Vries, and M.G. Roncarolo. 1994. High levels of interleukin 10 production in vivo are associated with tolerance in SCID patients transplanted with HLA mismatched hematopoietic stem cells. *J Exp Med* 179:493-502.

Battaglia, M., S. Gregori, R. Bacchetta, and M.G. Roncarolo. 2006. Tr1 cells: from discovery to their clinical application. *Semin Immunol* 18:120-127.

Batten, M., N.M. Kljavin, J. Li, M.J. Walter, F.J. de Sauvage, and N. Ghilardi. 2008. Cutting edge: IL-27 is a potent inducer of IL-10 but not FoxP3 in murine T cells. *J Immunol* 180:2752-2756.

Batten, M., J. Li, S. Yi, N.M. Kljavin, D.M. Danilenko, S. Lucas, J. Lee, F.J. de Sauvage, and N. Ghilardi. 2006. Interleukin 27 limits autoimmune encephalomyelitis by suppressing the development of interleukin 17-producing T cells. *Nat Immunol* 7:929-936.

Bauquet, A.T., H. Jin, A.M. Paterson, M. Mitsdoerffer, I.C. Ho, A.H. Sharpe, and V.K. Kuchroo. 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* 10:167-175.

Becher, B., I. Bechmann, and M. Greter. 2006. Antigen presentation in autoimmunity and CNS inflammation: how T lymphocytes recognize the brain. *J Mol Med (Berl)* 84:532-543.

Becher, B., B.G. Durell, and R.J. Noelle. 2002. Experimental autoimmune encephalitis and inflammation in the absence of interleukin-12. *J Clin Invest* 110:493-497.

Ben-Nun, A., H. Wekerle, and I.R. Cohen. 1981. The rapid isolation of clonable antigen-specific T lymphocyte lines capable of mediating autoimmune encephalomyelitis. *Eur J Immunol* 11:195-199.

Bennett, C.L., J. Christie, F. Ramsdell, M.E. Brunkow, P.J. Ferguson, L. Whitesell, T.E. Kelly, F.T. Saulsbury, P.F. Chance, and H.D. Ochs. 2001. The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. *Nat Genet* 27:20-21.

Bettelli, E., Y. Carrier, W. Gao, T. Korn, T.B. Strom, M. Oukka, H.L. Weiner, and V.K. Kuchroo. 2006. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature* 441:235-238.

Bettelli, E., T. Korn, and V.K. Kuchroo. 2007. Th17: the third member of the effector T cell trilogy. *Curr Opin Immunol* 19:652-657.

Bettelli, E., M. Pagany, H.L. Weiner, C. Linington, R.A. Sobel, and V.K. Kuchroo. 2003. Myelin oligodendrocyte glycoprotein-specific T cell receptor transgenic mice develop spontaneous autoimmune optic neuritis. *J Exp Med* 197:1073-1081.

Bettelli, E., B. Sullivan, S.J. Szabo, R.A. Sobel, L.H. Glimcher, and V.K. Kuchroo. 2004. Loss of T-bet, but not STAT1, prevents the development of experimental autoimmune encephalomyelitis. *J Exp Med* 200:79-87.

Brunkow, M.E., E.W. Jeffery, K.A. Hjerrild, B. Paeper, L.B. Clark, S.A. Yasayko, J.E. Wilkinson, D. Galas, S.F. Ziegler, and F. Ramsdell. 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.

Buc, M. 2013. Role of regulatory T cells in pathogenesis and biological therapy of multiple sclerosis. *Mediators Inflamm* 2013:963748.

Chang, H.D., C. Helbig, L. Tykocinski, S. Kreher, J. Koeck, U. Niesner, and A. Radbruch. 2007. Expression of IL-10 in Th memory lymphocytes is conditional on IL-12 or IL-4, unless the IL-10 gene is imprinted by GATA-3. *Eur J Immunol* 37:807-817.

Chen, W., W. Jin, N. Hardegen, K.J. Lei, L. Li, N. Marinos, G. McGrady, and S.M. Wahl. 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* 198:1875-1886.

Chen, X., R.T. Winkler-Pickett, N.H. Carbonetti, J.R. Ortaldo, J.J. Oppenheim, and O.M. Howard. 2006. Pertussis toxin as an adjuvant suppresses the number and function of CD4+CD25+ T regulatory cells. *Eur J Immunol* 36:671-680.

Cho, M.L., J.W. Kang, Y.M. Moon, H.J. Nam, J.Y. Jhun, S.B. Heo, H.T. Jin, S.Y. Min, J.H. Ju, K.S. Park, Y.G. Cho, C.H. Yoon, S.H. Park, Y.C. Sung, and H.Y. Kim. 2006. STAT3 and NF-kappaB signal pathway is required for IL-23-mediated IL-17 production in spontaneous arthritis animal model IL-1 receptor antagonist-deficient mice. *J Immunol* 176:5652-5661.

Chu, C.Q., S. Wittmer, and D.K. Dalton. 2000. Failure to suppress the expansion of the activated CD4 T cell population in interferon gamma-deficient mice leads to exacerbation of experimental autoimmune encephalomyelitis. *J Exp Med* 192:123-128.

Chung, Y., S. Tanaka, F. Chu, R.I. Nurieva, G.J. Martinez, S. Rawal, Y.H. Wang, H. Lim, J.M. Reynolds, X.H. Zhou, H.M. Fan, Z.M. Liu, S.S. Neelapu, and C. Dong. 2011. Follicular regulatory T cells expressing Foxp3 and Bcl-6 suppress germinal center reactions. *Nat Med* 17:983-988.

Cimmino, L., G.A. Martins, J. Liao, E. Magnusdottir, G. Grunig, R.K. Perez, and K.L. Calame. 2008. Blimp-1 attenuates Th1 differentiation by repression of ifng, tbx21, and bcl6 gene expression. *J Immunol* 181:2338-2347.

Ciofani, M., A. Madar, C. Galan, M. Sellars, K. Mace, F. Pauli, A. Agarwal, W. Huang, C.N. Parkurst, M. Muratet, K.M. Newberry, S. Meadows, A. Greenfield, Y. Yang, P. Jain, F.K. Kirigin, C. Birchmeier, E.F. Wagner, K.M. Murphy, R.M. Myers, R. Bonneau, and D.R. Littman. 2012. A validated regulatory network for Th17 cell specification. *Cell* 151:289-303.

Codarri, L., G. Gyulveszi, V. Tosevski, L. Hesske, A. Fontana, L. Magnenat, T. Suter, and B. Becher. 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* 12:560-567.

Cottrez, F., S.D. Hurst, R.L. Coffman, and H. Groux. 2000. T regulatory cells 1 inhibit a Th2-specific response in vivo. *J Immunol* 165:4848-4853.

Cretney, E., A. Xin, W. Shi, M. Minnich, F. Masson, M. Miasari, G.T. Belz, G.K. Smyth, M. Busslinger, S.L. Nutt, and A. Kallies. 2011. The transcription factors Blimp-1 and IRF4 jointly control the differentiation and function of effector regulatory T cells. *Nat Immunol* 12:304-311.

Crotty, S. 2011. Follicular helper CD4 T cells (TFH). *Annu Rev Immunol* 29:621-663.

Cua, D.J., J. Sherlock, Y. Chen, C.A. Murphy, B. Joyce, B. Seymour, L. Lucian, W. To, S. Kwan, T. Churakova, S. Zurawski, M. Wiekowski, S.A. Lira, D. Gorman, R.A. Kastelein, and J.D. Sedgwick. 2003. Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain. *Nature* 421:744-748.

Cui, W., Y. Liu, J.S. Weinstein, J. Craft, and S.M. Kaech. 2011. An interleukin-21-interleukin-10-STAT3 pathway is critical for functional maturation of memory CD8+ T cells. *Immunity* 35:792-805.

de la Rosa, M., S. Rutz, H. Dorninger, and A. Scheffold. 2004. Interleukin-2 is essential for CD4+CD25+ regulatory T cell function. *Eur J Immunol* 34:2480-2488.

Disanto, G., J.M. Morahan, and S.V. Ramagopalan. 2012. Multiple sclerosis: risk factors and their interactions. *CNS Neurol Disord Drug Targets* 11:545-555.

Diveu, C., M.J. McGeachy, K. Boniface, J.S. Stumhofer, M. Sathe, B. Joyce-Shaikh, Y. Chen, C.M. Tato, T.K. McClanahan, R. de Waal Malefyt, C.A. Hunter, D.J. Cua, and R.A. Kastelein. 2009. IL-27 blocks RORc expression to inhibit lineage commitment of Th17 cells. *J Immunol* 182:5748-5756.

Domingues, H.S., M. Mues, H. Lassmann, H. Wekerle, and G. Krishnamoorthy. 2010. Functional and pathogenic differences of Th1 and Th17 cells in experimental autoimmune encephalomyelitis. *PLoS One* 5:e15531.

Ehrenstein, M.R., J.G. Evans, A. Singh, S. Moore, G. Warnes, D.A. Isenberg, and C. Mauri. 2004. Compromised function of regulatory T cells in rheumatoid arthritis and reversal by anti-TNFalpha therapy. *J Exp Med* 200:277-285.

El-Behi, M., B. Ciric, H. Dai, Y. Yan, M. Cullimore, F. Safavi, G.X. Zhang, B.N. Dittel, and A. Rostami. 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* 12:568-575.

El-behi, M., B. Ciric, S. Yu, G.X. Zhang, D.C. Fitzgerald, and A. Rostami. 2009. Differential effect of IL-27 on developing versus committed Th17 cells. *J Immunol* 183:4957-4967.

Ferber, I.A., S. Brocke, C. Taylor-Edwards, W. Ridgway, C. Dinisco, L. Steinman, D. Dalton, and C.G. Fathman. 1996. Mice with a disrupted IFN-gamma gene are susceptible to the induction of experimental autoimmune encephalomyelitis (EAE). *J Immunol* 156:5-7.

Fitzgerald, D.C., G.X. Zhang, M. El-Behi, Z. Fonseca-Kelly, H. Li, S. Yu, C.J. Saris, B. Gran, B. Ciric, and A. Rostami. 2007. Suppression of autoimmune inflammation of the central nervous system by interleukin 10 secreted by interleukin 27-stimulated T cells. *Nat Immunol* 8:1372-1379.

Fletcher, J.M., S.J. Lalor, C.M. Sweeney, N. Tubridy, and K.H. Mills. 2010. T cells in multiple sclerosis and experimental autoimmune encephalomyelitis. *Clin Exp Immunol* 162:1-11.

Fontenot, J.D., M.A. Gavin, and A.Y. Rudensky. 2003. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat Immunol* 4:330-336.

Fontenot, J.D., J.P. Rasmussen, M.A. Gavin, and A.Y. Rudensky. 2005a. A function for interleukin 2 in Foxp3-expressing regulatory T cells. *Nat Immunol* 6:1142-1151.

Fontenot, J.D., J.P. Rasmussen, L.M. Williams, J.L. Dooley, A.G. Farr, and A.Y. Rudensky. 2005b. Regulatory T cell lineage specification by the forkhead transcription factor foxp3. *Immunity* 22:329-341.

Fornek, J.L., L.T. Tygrett, T.J. Waldschmidt, V. Poli, R.C. Rickert, and G.S. Kansas. 2006. Critical role for Stat3 in T-dependent terminal differentiation of IgG B cells. *Blood* 107:1085-1091.

Fritz, R.B., C.H. Chou, and D.E. McFarlin. 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* 130:191-194.

Gagliani, N., C.F. Magnani, S. Huber, M.E. Gianolini, M. Pala, P. Licona-Limon, B. Guo, D.R. Herbert, A. Bulfone, F. Trentini, C. Di Serio, R. Bacchetta, M. Andreani, L. Brockmann, S. Gregori, R.A. Flavell, and M.G. Roncarolo. 2013. Coexpression of CD49b and LAG-3 identifies human and mouse T regulatory type 1 cells. *Nat Med* 19:739-746.

Gavin, M.A., J.P. Rasmussen, J.D. Fontenot, V. Vasta, V.C. Manganiello, J.A. Beavo, and A.Y. Rudensky. 2007. Foxp3-dependent programme of regulatory T-cell differentiation. *Nature* 445:771-775.

Ghoreschi, K., A. Laurence, X.P. Yang, C.M. Tato, M.J. McGeachy, J.E. Konkel, H.L. Ramos, L. Wei, T.S. Davidson, N. Bouladoux, J.R. Grainger, Q. Chen, Y. Kanno, W.T. Watford, H.W. Sun, G. Eberl, E.M. Shevach, Y. Belkaid, D.J. Cua, W. Chen, and J.J. O'Shea. 2010. Generation of pathogenic T(H)17 cells in the absence of TGF-beta signalling. *Nature* 467:967-971.

Godfrey, V.L., J.E. Wilkinson, and L.B. Russell. 1991. X-linked lymphoreticular disease in the scurfy (sf) mutant mouse. *Am J Pathol* 138:1379-1387.

Gol-Ara, M., F. Jadidi-Niaragh, R. Sadria, G. Azizi, and A. Mirshafiey. 2012. The role of different subsets of regulatory T cells in immunopathogenesis of rheumatoid arthritis. *Arthritis* 2012:805875.

Gold, R., C. Linington, and H. Lassmann. 2006. Understanding pathogenesis and therapy of multiple sclerosis via animal models: 70 years of merits and culprits in experimental autoimmune encephalomyelitis research. *Brain* 129:1953-1971.

Gran, B., G.X. Zhang, S. Yu, J. Li, X.H. Chen, E.S. Ventura, M. Kamoun, and A. Rostami. 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* 169:7104-7110.

Groux, H. 2003. Type 1 T-regulatory cells: their role in the control of immune responses. *Transplantation* 75:8S-12S.

Groux, H., A. O'Garra, M. Bigler, M. Rouleau, S. Antonenko, J.E. de Vries, and M.G. Roncarolo. 1997. A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature* 389:737-742.

Hall, A.O., J.S. Silver, and C.A. Hunter. 2012. The immunobiology of IL-27. *Adv Immunol* 115:1-44.

Harris, T.J., J.F. Grosso, H.R. Yen, H. Xin, M. Kortylewski, E. Albesiano, E.L. Hipkiss, D. Getnet, M.V. Goldberg, C.H. Maris, F. Housseau, H. Yu, D.M. Pardoll, and C.G. Drake. 2007.

Cutting edge: An in vivo requirement for STAT3 signaling in TH17 development and TH17-dependent autoimmunity. *J Immunol* 179:4313-4317.

Hegazy, A.N., M. Peine, C. Helmstetter, I. Panse, A. Frohlich, A. Bergthaler, L. Flatz, D.D. Pinschewer, A. Radbruch, and M. Lohning. 2010. Interferons direct Th2 cell reprogramming to generate a stable GATA-3(+)T-bet(+) cell subset with combined Th2 and Th1 cell functions. *Immunity* 32:116-128.

Hibbert, L., S. Pflanz, R. De Waal Malefyt, and R.A. Kastelein. 2003. IL-27 and IFN-alpha signal via Stat1 and Stat3 and induce T-Bet and IL-12Rbeta2 in naive T cells. *J Interferon Cytokine Res* 23:513-522.

Hirahara, K., A. Poholek, G. Vahedi, A. Laurence, Y. Kanno, J.D. Milner, and J.J. O'Shea. 2013. Mechanisms underlying helper T-cell plasticity: implications for immune-mediated disease. *J Allergy Clin Immunol* 131:1276-1287.

Hirota, K., J.H. Duarte, M. Veldhoen, E. Hornsby, Y. Li, D.J. Cua, H. Ahlfors, C. Wilhelm, M. Tolaini, U. Menzel, A. Garefalaki, A.J. Potocnik, and B. Stockinger. 2011. Fate mapping of IL-17-producing T cells in inflammatory responses. *Nat Immunol* 12:255-263.

Ho, I.C., and L.H. Glimcher. 2002. Transcription: tantalizing times for T cells. *Cell* 109 Suppl:S109-120.

Ho, I.C., M.R. Hodge, J.W. Rooney, and L.H. Glimcher. 1996. The proto-oncogene c-maf is responsible for tissue-specific expression of interleukin-4. *Cell* 85:973-983.

Hofstetter, H.H., S.M. Ibrahim, D. Koczan, N. Kruse, A. Weishaupt, K.V. Toyka, and R. Gold. 2005. Therapeutic efficacy of IL-17 neutralization in murine experimental autoimmune encephalomyelitis. *Cell Immunol* 237:123-130.

Holda, J.H., D. Silberg, and R.H. Swanborg. 1983. Autoimmune effector cells. IV. Induction of experimental allergic encephalomyelitis in Lewis rats without adjuvant. *J Immunol* 130:732-734.

Hori, S. 2010. Developmental plasticity of Foxp3+ regulatory T cells. *Curr Opin Immunol* 22:575-582.

Hori, S. 2011. Regulatory T cell plasticity: beyond the controversies. *Trends Immunol* 32:295-300.

Hori, S., T. Nomura, and S. Sakaguchi. 2003. Control of regulatory T cell development by the transcription factor Foxp3. *Science* 299:1057-1061.

Hou, W., Y. Wu, S. Sun, M. Shi, Y. Sun, C. Yang, G. Pei, Y. Gu, C. Zhong, and B. Sun. 2003. Pertussis toxin enhances Th1 responses by stimulation of dendritic cells. *J Immunol* 170:1728-1736.

Huber, M., S. Heink, A. Pagenstecher, K. Reinhard, J. Ritter, A. Visekruna, A. Guralnik, N. Bollig, K. Jeltsch, C. Heinemann, E. Wittmann, T. Buch, O. Prazeres da Costa, A. Brustle, D. Brenner, T.W. Mak, H.W. Mittrucker, B. Tackenberg, T. Kamradt, and M. Lohoff. 2013. IL-17A secretion by CD8+ T cells supports Th17-mediated autoimmune encephalomyelitis. *J Clin Invest* 123:247-260.

Hunter, C.A. 2005. New IL-12-family members: IL-23 and IL-27, cytokines with divergent functions. *Nat Rev Immunol* 5:521-531.

Hunter, C.A., and R. Kastelein. 2012. Interleukin-27: balancing protective and pathological immunity. *Immunity* 37:960-969.

Ivanov, II, B.S. McKenzie, L. Zhou, C.E. Tadokoro, A. Lepelley, J.J. Lafaille, D.J. Cua, and D.R. Littman. 2006. The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells. *Cell* 126:1121-1133.

Iwasaki, Y., K. Fujio, T. Okamura, A. Yanai, S. Sumitomo, H. Shoda, T. Tamura, H. Yoshida, P. Charnay, and K. Yamamoto. 2013. Egr-2 transcription factor is required for Blimp-1-mediated IL-10 production in IL-27-stimulated CD4+ T cells. *Eur J Immunol* 43:1063-1073.

Kallies, A., J. Hasbold, D.M. Tarlinton, W. Dietrich, L.M. Corcoran, P.D. Hodgkin, and S.L. Nutt. 2004. Plasma cell ontogeny defined by quantitative changes in blimp-1 expression. *J Exp Med* 200:967-977.

Kallies, A., E.D. Hawkins, G.T. Belz, D. Metcalf, M. Hommel, L.M. Corcoran, P.D. Hodgkin, and S.L. Nutt. 2006. Transcriptional repressor Blimp-1 is essential for T cell homeostasis and self-tolerance. *Nat Immunol* 7:466-474.

Kallies, A., and S.L. Nutt. 2007. Terminal differentiation of lymphocytes depends on Blimp-1. *Curr Opin Immunol* 19:156-162.

Kano, S., K. Sato, Y. Morishita, S. Vollstedt, S. Kim, K. Bishop, K. Honda, M. Kubo, and T. Taniguchi. 2008. The contribution of transcription factor IRF1 to the interferon-gamma-interleukin 12 signaling axis and TH1 versus TH-17 differentiation of CD4+ T cells. *Nat Immunol* 9:34-41.

Kaplan, M.H., Y.L. Sun, T. Hoey, and M.J. Grusby. 1996. Impaired IL-12 responses and enhanced development of Th2 cells in Stat4-deficient mice. *Nature* 382:174-177.

Kasprowicz, D.J., N. Droin, D.M. Soper, F. Ramsdell, D.R. Green, and S.F. Ziegler. 2005. Dynamic regulation of FoxP3 expression controls the balance between CD4+ T cell activation and cell death. *Eur J Immunol* 35:3424-3432.

Kastelein, R.A., C.A. Hunter, and D.J. Cua. 2007. Discovery and biology of IL-23 and IL-27: related but functionally distinct regulators of inflammation. *Annu Rev Immunol* 25:221-242.

Kay, A.B., S. Ying, V. Varney, M. Gaga, S.R. Durham, R. Moqbel, A.J. Wardlaw, and Q. Hamid. 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* 173:775-778.

Kennedy, M.K., D.S. Torrance, K.S. Picha, and K.M. Mohler. 1992. Analysis of cytokine mRNA expression in the central nervous system of mice with experimental autoimmune encephalomyelitis reveals that IL-10 mRNA expression correlates with recovery. *J Immunol* 149:2496-2505.

Kerfoot, S.M., E.M. Long, M.J. Hickey, G. Andonegui, B.M. Lapointe, R.C. Zanardo, C. Bonder, W.G. James, S.M. Robbins, and P. Kubes. 2004. TLR4 contributes to disease-inducing mechanisms resulting in central nervous system autoimmune disease. *J Immunol* 173:7070-7077.

Kim, J.M., J.P. Rasmussen, and A.Y. Rudensky. 2007. Regulatory T cells prevent catastrophic autoimmunity throughout the lifespan of mice. *Nat Immunol* 8:191-197.

Kobelt, G., L. Jonsson, F. Henriksson, S. Fredrikson, and B. Jonsson. 2000. Cost-utility analysis of interferon beta-1b in secondary progressive multiple sclerosis. *Int J Technol Assess Health Care* 16:768-780.

Koch, M.A., K.R. Thomas, N.R. Perdue, K.S. Smigiel, S. Srivastava, and D.J. Campbell. 2012. T-bet(+) Treg cells undergo abortive Th1 cell differentiation due to impaired expression of IL-12 receptor beta2. *Immunity* 37:501-510.

Koch, M.A., G. Tucker-Heard, N.R. Perdue, J.R. Killebrew, K.B. Urdahl, and D.J. Campbell. 2009. The transcription factor T-bet controls regulatory T cell homeostasis and function during type 1 inflammation. *Nat Immunol* 10:595-602.

Kohm, A.P., P.A. Carpentier, H.A. Anger, and S.D. Miller. 2002. Cutting edge: CD4+CD25+ regulatory T cells suppress antigen-specific autoreactive immune responses and central nervous system inflammation during active experimental autoimmune encephalomyelitis. *J Immunol* 169:4712-4716.

Komiyama, Y., S. Nakae, T. Matsuki, A. Nambu, H. Ishigame, S. Kakuta, K. Sudo, and Y. Iwakura. 2006. IL-17 plays an important role in the development of experimental autoimmune encephalomyelitis. *J Immunol* 177:566-573.

Korn, T. 2008. Pathophysiology of multiple sclerosis. *J Neurol* 255 Suppl 6:2-6.

Korn, T., A.C. Anderson, E. Bettelli, and M. Oukka. 2007a. The dynamics of effector T cells and Foxp3+ regulatory T cells in the promotion and regulation of autoimmune encephalomyelitis. *J Neuroimmunol* 191:51-60.

Korn, T., E. Bettelli, W. Gao, A. Awasthi, A. Jager, T.B. Strom, M. Oukka, and V.K. Kuchroo. 2007b. IL-21 initiates an alternative pathway to induce proinflammatory T(H)17 cells. *Nature* 448:484-487.

Korn, T., M. Mitsdoerffer, and V.K. Kuchroo. 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., J. Reddy, W. Gao, E. Bettelli, A. Awasthi, T.R. Petersen, B.T. Backstrom, R.A. Sobel, K.W. Wucherpfennig, T.B. Strom, M. Oukka, and V.K. Kuchroo. 2007c. Myelin-specific regulatory T cells accumulate in the CNS but fail to control autoimmune inflammation. *Nat Med* 13:423-431.

Kuchroo, V.K., A.C. Anderson, H. Waldner, M. Munder, E. Bettelli, and L.B. Nicholson. 2002. T cell response in experimental autoimmune encephalomyelitis (EAE): role of self and cross-reactive antigens in shaping, tuning, and regulating the autopathogenic T cell repertoire. *Annu Rev Immunol* 20:101-123.

Kuchroo, V.K., R.A. Sobel, T. Yamamura, E. Greenfield, M.E. Dorf, and M.B. Lees. 1991. Induction of experimental allergic encephalomyelitis by myelin proteolipid-protein-specific T cell clones and synthetic peptides. *Pathobiology* 59:305-312.

Kurata, H., H.J. Lee, A. O'Garra, and N. Arai. 1999. Ectopic expression of activated Stat6 induces the expression of Th2-specific cytokines and transcription factors in developing Th1 cells. *Immunity* 11:677-688.

Kurschus, F.C., A.L. Croxford, A.P. Heinen, S. Wortge, D. Ielo, and A. Waisman. 2010. Genetic proof for the transient nature of the Th17 phenotype. *Eur J Immunol* 40:3336-3346.

Kusam, S., L.M. Toney, H. Sato, and A.L. Dent. 2003. Inhibition of Th2 differentiation and GATA-3 expression by BCL-6. *J Immunol* 170:2435-2441.

Kwon, H., D. Thierry-Mieg, J. Thierry-Mieg, H.P. Kim, J. Oh, C. Tunyaplin, S. Carotta, C.E. Donovan, M.L. Goldman, P. Tailor, K. Ozato, D.E. Levy, S.L. Nutt, K. Calame, and W.J. Leonard. 2009. Analysis of interleukin-21-induced Prdm1 gene regulation reveals functional cooperation of STAT3 and IRF4 transcription factors. *Immunity* 31:941-952.

Lahl, K., C. Loddenkemper, C. Drouin, J. Freyer, J. Arnason, G. Eberl, A. Hamann, H. Wagner, J. Huehn, and T. Sparwasser. 2007. Selective depletion of Foxp3+ regulatory T cells induces a scurfy-like disease. *J Exp Med* 204:57-63.

Langrish, C.L., Y. Chen, W.M. Blumenschein, J. Mattson, B. Basham, J.D. Sedgwick, T. McClanahan, R.A. Kastelein, and D.J. Cua. 2005. IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. *J Exp Med* 201:233-240.

Lee, Y., A. Awasthi, N. Yosef, F.J. Quintana, S. Xiao, A. Peters, C. Wu, M. Kleinewietfeld, S. Kunder, D.A. Hafler, R.A. Sobel, A. Regev, and V.K. Kuchroo. 2012. Induction and molecular signature of pathogenic TH17 cells. *Nat Immunol* 13:991-999.

Lee, Y.K., H. Turner, C.L. Maynard, J.R. Oliver, D. Chen, C.O. Elson, and C.T. Weaver. 2009. Late developmental plasticity in the T helper 17 lineage. *Immunity* 30:92-107.

Leonard, J.P., K.E. Waldburger, and S.J. Goldman. 1995. Prevention of experimental autoimmune encephalomyelitis by antibodies against interleukin 12. *J Exp Med* 181:381-386.

Lexberg, M.H., A. Taubner, I. Albrecht, I. Lepenies, A. Richter, T. Kamradt, A. Radbruch, and H.D. Chang. 2010. IFN-gamma and IL-12 synergize to convert in vivo generated Th17 into Th1/Th17 cells. *Eur J Immunol* 40:3017-3027.

Lexberg, M.H., A. Taubner, A. Forster, I. Albrecht, A. Richter, T. Kamradt, A. Radbruch, and H.D. Chang. 2008. Th memory for interleukin-17 expression is stable in vivo. *Eur J Immunol* 38:2654-2664.

Liang, S.C., X.Y. Tan, D.P. Luxenberg, R. Karim, K. Dunussi-Joannopoulos, M. Collins, and L.A. Fouser. 2006. Interleukin (IL)-22 and IL-17 are coexpressed by Th17 cells and cooperatively enhance expression of antimicrobial peptides. *J Exp Med* 203:2271-2279.

Lin, M.H., F.C. Chou, L.T. Yeh, S.H. Fu, H.Y. Chiou, K.I. Lin, D.M. Chang, and H.K. Sytwu. 2013. B lymphocyte-induced maturation protein 1 (BLIMP-1) attenuates autoimmune diabetes in NOD mice by suppressing Th1 and Th17 cells. *Diabetologia* 56:136-146.

Linington, C., T. Berger, L. Perry, S. Weerth, D. Hinze-Selch, Y. Zhang, H.C. Lu, H. Lassmann, and H. Wekerle. 1993. T cells specific for the myelin oligodendrocyte glycoprotein mediate an unusual autoimmune inflammatory response in the central nervous system. *Eur J Immunol* 23:1364-1372.

Liu, Y., P. Zhang, J. Li, A.B. Kulkarni, S. Perruche, and W. Chen. 2008. A critical function for TGF-beta signaling in the development of natural CD4+CD25+Foxp3+ regulatory T cells. *Nat Immunol* 9:632-640.

Lock, C., G. Hermans, R. Pedotti, A. Brendolan, E. Schadt, H. Garren, A. Langer-Gould, S. Strober, B. Cannella, J. Allard, P. Klonowski, A. Austin, N. Lad, N. Kaminski, S.J. Galli, J.R. Oksenberg, C.S. Raine, R. Heller, and L. Steinman. 2002. Gene-microarray analysis of multiple sclerosis lesions yields new targets validated in autoimmune encephalomyelitis. *Nat Med* 8:500-508.

Lubberts, E., M.I. Koenders, B. Oppers-Walgreen, L. van den Bersselaar, C.J. Coenen-de Roo, L.A. Joosten, and W.B. van den Berg. 2004. Treatment with a neutralizing anti-murine interleukin-17 antibody after the onset of collagen-induced arthritis reduces joint inflammation, cartilage destruction, and bone erosion. *Arthritis Rheum* 50:650-659.

Lucas, S., N. Ghilardi, J. Li, and F.J. de Sauvage. 2003. IL-27 regulates IL-12 responsiveness of naive CD4+ T cells through Stat1-dependent and -independent mechanisms. *Proc Natl Acad Sci U S A* 100:15047-15052.

Magnani, C.F., G. Alberigo, R. Bacchetta, G. Serafini, M. Andreani, M.G. Roncarolo, and S. Gregori. 2011. Killing of myeloid APCs via HLA class I, CD2 and CD226 defines a novel mechanism of suppression by human Tr1 cells. *Eur J Immunol* 41:1652-1662.

Mangan, P.R., L.E. Harrington, D.B. O'Quinn, W.S. Helms, D.C. Bullard, C.O. Elson, R.D. Hatton, S.M. Wahl, T.R. Schoeb, and C.T. Weaver. 2006. Transforming growth factor-beta induces development of the T(H)17 lineage. *Nature* 441:231-234.

Marie, J.C., J.J. Letterio, M. Gavin, and A.Y. Rudensky. 2005. TGF-beta1 maintains suppressor function and Foxp3 expression in CD4+CD25+ regulatory T cells. *J Exp Med* 201:1061-1067.

Martins, G., and K. Calame. 2008. Regulation and functions of Blimp-1 in T and B lymphocytes. *Annu Rev Immunol* 26:133-169.

Martins, G.A., L. Cimmino, M. Shapiro-Shelef, M. Szabolcs, A. Herron, E. Magnusdottir, and K. Calame. 2006. Transcriptional repressor Blimp-1 regulates T cell homeostasis and function. *Nat Immunol* 7:457-465.

Matusevicius, D., P. Kivisakk, B. He, N. Kostulas, V. Ozenci, S. Fredrikson, and H. Link. 1999. Interleukin-17 mRNA expression in blood and CSF mononuclear cells is augmented in multiple sclerosis. *Mult Scler* 5:101-104.

Maynard, C.L., L.E. Harrington, K.M. Janowski, J.R. Oliver, C.L. Zindl, A.Y. Rudensky, and C.T. Weaver. 2007. Regulatory T cells expressing interleukin 10 develop from Foxp3+ and Foxp3- precursor cells in the absence of interleukin 10. *Nat Immunol* 8:931-941.

McGeachy, M.J., and D.J. Cua. 2007. The link between IL-23 and Th17 cell-mediated immune pathologies. *Semin Immunol* 19:372-376.

McGeachy, M.J., L.A. Stephens, and S.M. Anderton. 2005. Natural recovery and protection from autoimmune encephalomyelitis: contribution of CD4+CD25+ regulatory cells within the central nervous system. *J Immunol* 175:3025-3032.

McQualter, J.L., R. Darwiche, C. Ewing, M. Onuki, T.W. Kay, J.A. Hamilton, H.H. Reid, and C.C. Bernard. 2001. Granulocyte macrophage colony-stimulating factor: a new putative therapeutic target in multiple sclerosis. *J Exp Med* 194:873-882.

Minagar, A. 2013. Current and future therapies for multiple sclerosis. *Scientifica (Cairo)* 2013:249101.

Moore, K.W., R. de Waal Malefyt, R.L. Coffman, and A. O'Garra. 2001. Interleukin-10 and the interleukin-10 receptor. *Annu Rev Immunol* 19:683-765.

Moore, K.W., P. Vieira, D.F. Fiorentino, M.L. Trounstine, T.A. Khan, and T.R. Mosmann. 1990. Homology of cytokine synthesis inhibitory factor (IL-10) to the Epstein-Barr virus gene BCRFI. *Science* 248:1230-1234.

Morinobu, A., M. Gadina, W. Strober, R. Visconti, A. Fornace, C. Montagna, G.M. Feldman, R. Nishikomori, and J.J. O'Shea. 2002. STAT4 serine phosphorylation is critical for IL-12-induced IFN-gamma production but not for cell proliferation. *Proc Natl Acad Sci U S A* 99:12281-12286.

Mosmann, T.R., H. Cherwinski, M.W. Bond, M.A. Giedlin, and R.L. Coffman. 1986. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J Immunol* 136:2348-2357.

Mosmann, T.R., and R.L. Coffman. 1989. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu Rev Immunol* 7:145-173.

Muller, M.S., L.F. Obel, H.S. Waagepetersen, A. Schousboe, and L.K. Bak. 2013. Complex actions of ionomycin in cultured cerebellar astrocytes affecting both calcium-induced calcium release and store-operated calcium entry. *Neurochem Res* 38:1260-1265.

Murphy, K.M., W. Ouyang, J.D. Farrar, J. Yang, S. Ranganath, H. Asnagli, M. Afkarian, and T.L. Murphy. 2000. Signaling and transcription in T helper development. *Annu Rev Immunol* 18:451-494.

Nakamura, K., A. Kitani, and W. Strober. 2001. Cell contact-dependent immunosuppression by CD4(+)CD25(+) regulatory T cells is mediated by cell surface-bound transforming growth factor beta. *J Exp Med* 194:629-644.

Nakayamada, S., Y. Kanno, H. Takahashi, D. Jankovic, K.T. Lu, T.A. Johnson, H.W. Sun, G. Vahedi, O. Hakim, R. Handon, P.L. Schwartzberg, G.L. Hager, and J.J. O'Shea. 2011. Early Th1 cell differentiation is marked by a Tfh cell-like transition. *Immunity* 35:919-931.

Nath, N., R. Prasad, S. Giri, A.K. Singh, and I. Singh. 2006. T-bet is essential for the progression of experimental autoimmune encephalomyelitis. *Immunology* 118:384-391.

Nurieva, R., X.O. Yang, G. Martinez, Y. Zhang, A.D. Panopoulos, L. Ma, K. Schluns, Q. Tian, S.S. Watowich, A.M. Jetten, and C. Dong. 2007. Essential autocrine regulation by IL-21 in the generation of inflammatory T cells. *Nature* 448:480-483.

Nurieva, R.I., A. Podd, Y. Chen, A.M. Alekseev, M. Yu, X. Qi, H. Huang, R. Wen, J. Wang, H.S. Li, S.S. Watowich, H. Qi, C. Dong, and D. Wang. 2012. STAT5 protein negatively regulates T follicular helper (Tfh) cell generation and function. *J Biol Chem* 287:11234-11239.

Oestreich, K.J., S.E. Mohn, and A.S. Weinmann. 2012. Molecular mechanisms that control the expression and activity of Bcl-6 in TH1 cells to regulate flexibility with a TFH-like gene profile. *Nat Immunol* 13:405-411.

Oh, S.A., and M.O. Li. 2013. TGF-beta: guardian of T cell function. *J Immunol* 191:3973-3979.

Oppmann, B., R. Lesley, B. Blom, J.C. Timans, Y. Xu, B. Hunte, F. Vega, N. Yu, J. Wang, K. Singh, F. Zonin, E. Vaisberg, T. Churakova, M. Liu, D. Gorman, J. Wagner, S. Zurawski, Y. Liu, J.S. Abrams, K.W. Moore, D. Rennick, R. de Waal-Malefyt, C. Hannum, J.F. Bazan, and R.A. Kastelein. 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* 13:715-725.

Ouyang, W., S.H. Ranganath, K. Weindel, D. Bhattacharya, T.L. Murphy, W.C. Sha, and K.M. Murphy. 1998. Inhibition of Th1 development mediated by GATA-3 through an IL-4-independent mechanism. *Immunity* 9:745-755.

Ouyang, X., R. Zhang, J. Yang, Q. Li, L. Qin, C. Zhu, J. Liu, H. Ning, M.S. Shin, M. Gupta, C.F. Qi, J.C. He, S.A. Lira, H.C. Morse, 3rd, K. Ozato, L. Mayer, and H. Xiong. 2011. Transcription factor IRF8 directs a silencing programme for TH17 cell differentiation. *Nat Commun* 2:314.

Parham, C., M. Chirica, J. Timans, E. Vaisberg, M. Travis, J. Cheung, S. Pflanz, R. Zhang, K.P. Singh, F. Vega, W. To, J. Wagner, A.M. O'Farrell, T. McClanahan, S. Zurawski, C. Hannum, D. Gorman, D.M. Rennick, R.A. Kastelein, R. de Waal Malefyt, and K.W. Moore. 2002. A receptor for the heterodimeric cytokine IL-23 is composed of IL-12Rbeta1 and a novel cytokine receptor subunit, IL-23R. *J Immunol* 168:5699-5708.

Park, W.R., M. Nakahira, N. Sugimoto, Y. Bian, Y. Yashiro-Ohtani, X.Y. Zhou, Y.F. Yang, T. Hamaoka, and H. Fujiwara. 2004. A mechanism underlying STAT4-mediated up-regulation of IFN-gamma induction inTCR-triggered T cells. *Int Immunol* 16:295-302.

Pestka, S., C.D. Krause, D. Sarkar, M.R. Walter, Y. Shi, and P.B. Fisher. 2004. Interleukin-10 and related cytokines and receptors. *Annu Rev Immunol* 22:929-979.

Petermann, F., and T. Korn. 2011. Cytokines and effector T cell subsets causing autoimmune CNS disease. *FEBS Lett* 585:3747-3757.

Pettinelli, C.B., and D.E. McFarlin. 1981. Adoptive transfer of experimental allergic encephalomyelitis in SJL/J mice after in vitro activation of lymph node cells by myelin basic protein: requirement for Lyt 1+ 2- T lymphocytes. *J Immunol* 127:1420-1423.

Pflanz, S., L. Hibbert, J. Mattson, R. Rosales, E. Vaisberg, J.F. Bazan, J.H. Phillips, T.K. McClanahan, R. de Waal Malefyt, and R.A. Kastelein. 2004. WSX-1 and glycoprotein 130 constitute a signal-transducing receptor for IL-27. *J Immunol* 172:2225-2231.

Pflanz, S., J.C. Timans, J. Cheung, R. Rosales, H. Kanzler, J. Gilbert, L. Hibbert, T. Churakova, M. Travis, E. Vaisberg, W.M. Blumenschein, J.D. Mattson, J.L. Wagner, W. To, S. Zurawski, T.K. McClanahan, D.M. Gorman, J.F. Bazan, R. de Waal Malefyt, D. Rennick, and R.A. Kastelein. 2002. IL-27, a heterodimeric cytokine composed of EBI3 and p28 protein, induces proliferation of naive CD4(+) T cells. *Immunity* 16:779-790.

Piccirillo, C.A., J.J. Letterio, A.M. Thornton, R.S. McHugh, M. Mamura, H. Mizuhara, and E.M. Shevach. 2002. CD4(+)CD25(+) regulatory T cells can mediate suppressor function in the absence of transforming growth factor beta1 production and responsiveness. *J Exp Med* 196:237-246.

Pot, C., L. Apetoh, A. Awasthi, and V.K. Kuchroo. 2010. Molecular pathways in the induction of interleukin-27-driven regulatory type 1 cells. *J Interferon Cytokine Res* 30:381-388.

Pot, C., H. Jin, A. Awasthi, S.M. Liu, C.Y. Lai, R. Madan, A.H. Sharpe, C.L. Karp, S.C. Miaw, I.C. Ho, and V.K. Kuchroo. 2009. Cutting edge: IL-27 induces the transcription factor c-Maf, cytokine IL-21, and the costimulatory receptor ICOS that coordinately act together to promote differentiation of IL-10-producing Tr1 cells. *J Immunol* 183:797-801.

Powell, B.R., N.R. Buist, and P. Stenzel. 1982. An X-linked syndrome of diarrhea, polyendocrinopathy, and fatal infection in infancy. *J Pediatr* 100:731-737.

Riemann, M., R. Endres, S. Liptay, K. Pfeffer, and R.M. Schmid. 2005. The IkappaB protein Bcl-3 negatively regulates transcription of the IL-10 gene in macrophages. *J Immunol* 175:3560-3568.

Robinson, D.S., Q. Hamid, S. Ying, A. Tsicopoulos, J. Barkans, A.M. Bentley, C. Corrigan, S.R. Durham, and A.B. Kay. 1992. Predominant TH2-like bronchoalveolar T-lymphocyte population in atopic asthma. *N Engl J Med* 326:298-304.

Rodgers, J.M., and S.D. Miller. 2012. Cytokine control of inflammation and repair in the pathology of multiple sclerosis. *Yale J Biol Med* 85:447-468.

Roncarolo, M.G., R. Bacchetta, C. Bordignon, S. Narula, and M.K. Levings. 2001. Type 1 T regulatory cells. *Immunol Rev* 182:68-79.

Roncarolo, M.G., S. Gregori, M. Battaglia, R. Bacchetta, K. Fleischhauer, and M.K. Levings. 2006. Interleukin-10-secreting type 1 regulatory T cells in rodents and humans. *Immunol Rev* 212:28-50.

Sadovnick, A.D., I.M. Yee, and G.C. Ebers. 2000. Factors influencing sib risks for multiple sclerosis. *Clin Genet* 58:431-435.

Sakaguchi, S. 2004. Naturally arising CD4+ regulatory t cells for immunologic self-tolerance and negative control of immune responses. *Annu Rev Immunol* 22:531-562.

Salehi, S., R. Bankoti, L. Benevides, J. Willen, M. Couse, J.S. Silva, D. Dhall, E. Meffre, S. Targan, and G.A. Martins. 2012. B lymphocyte-induced maturation protein-1 contributes to intestinal mucosa homeostasis by limiting the number of IL-17-producing CD4+ T cells. *J Immunol* 189:5682-5693.

Saraiva, M., J.R. Christensen, M. Veldhoen, T.L. Murphy, K.M. Murphy, and A. O'Garra. 2009. Interleukin-10 production by Th1 cells requires interleukin-12-induced STAT4 transcription factor and ERK MAP kinase activation by high antigen dose. *Immunity* 31:209-219.

Schoenhaut, D.S., A.O. Chua, A.G. Wolitzky, P.M. Quinn, C.M. Dwyer, W. McComas, P.C. Familletti, M.K. Gately, and U. Gubler. 1992. Cloning and expression of murine IL-12. *J Immunol* 148:3433-3440.

Schubert, L.A., E. Jeffery, Y. Zhang, F. Ramsdell, and S.F. Ziegler. 2001. Scurfin (FOXP3) acts as a repressor of transcription and regulates T cell activation. *J Biol Chem* 276:37672-37679.

Shapiro-Shelef, M., K.I. Lin, L.J. McHeyzer-Williams, J. Liao, M.G. McHeyzer-Williams, and K. Calame. 2003. Blimp-1 is required for the formation of immunoglobulin secreting plasma cells and pre-plasma memory B cells. *Immunity* 19:607-620.

Sher, A., D. Fiorentino, P. Caspar, E. Pearce, and T. Mosmann. 1991. Production of IL-10 by CD4+ T lymphocytes correlates with down-regulation of Th1 cytokine synthesis in helminth infection. *J Immunol* 147:2713-2716.

Shevach, E.M. 2009. Mechanisms of foxp3+ T regulatory cell-mediated suppression. *Immunity* 30:636-645.

Shoemaker, J., M. Saraiva, and A. O'Garra. 2006. GATA-3 directly remodels the IL-10 locus independently of IL-4 in CD4+ T cells. *J Immunol* 176:3470-3479.

Sonderegger, I., G. Iezzi, R. Maier, N. Schmitz, M. Kurrer, and M. Kopf. 2008. GM-CSF mediates autoimmunity by enhancing IL-6-dependent Th17 cell development and survival. *J Exp Med* 205:2281-2294.

Spolski, R., H.P. Kim, W. Zhu, D.E. Levy, and W.J. Leonard. 2009. IL-21 mediates suppressive effects via its induction of IL-10. *J Immunol* 182:2859-2867.

Starnes, T., M.J. Robertson, G. Sledge, S. Kelich, H. Nakshatri, H.E. Broxmeyer, and R. Hromas. 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* 167:4137-4140.

Stewart, J.P., and C.M. Rooney. 1992. The interleukin-10 homolog encoded by Epstein-Barr virus enhances the reactivation of virus-specific cytotoxic T cell and HLA-unrestricted killer cell responses. *Virology* 191:773-782.

Stoeckle, C., and E. Tolosa. 2010. Antigen processing and presentation in multiple sclerosis. *Results Probl Cell Differ* 51:149-172.

Stumhofer, J.S., J.S. Silver, A. Laurence, P.M. Porrett, T.H. Harris, L.A. Turka, M. Ernst, C.J. Saris, J.J. O'Shea, and C.A. Hunter. 2007. Interleukins 27 and 6 induce STAT3-mediated T cell production of interleukin 10. *Nat Immunol* 8:1363-1371.

Sullivan, B.M., O. Jobe, V. Lazarevic, K. Vasquez, R. Bronson, L.H. Glimcher, and I. Kramnik. 2005. Increased susceptibility of mice lacking T-bet to infection with Mycobacterium tuberculosis correlates with increased IL-10 and decreased IFN-gamma production. *J Immunol* 175:4593-4602.

Sun, J., H. Dodd, E.K. Moser, R. Sharma, and T.J. Braciale. 2011. CD4+ T cell help and innate-derived IL-27 induce Blimp-1-dependent IL-10 production by antiviral CTLs. *Nat Immunol* 12:327-334.

Szabo, S.J., S.T. Kim, G.L. Costa, X. Zhang, C.G. Fathman, and L.H. Glimcher. 2000. A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell* 100:655-669.

Tai, X., M. Cowan, L. Feigenbaum, and A. Singer. 2005. CD28 costimulation of developing thymocytes induces Foxp3 expression and regulatory T cell differentiation independently of interleukin 2. *Nat Immunol* 6:152-162.

Takeda, A., S. Hamano, A. Yamanaka, T. Hanada, T. Ishibashi, T.W. Mak, A. Yoshimura, and H. Yoshida. 2003. Cutting edge: role of IL-27/WSX-1 signaling for induction of T-bet through activation of STAT1 during initial Th1 commitment. *J Immunol* 170:4886-4890.

Tartakoff, A.M., and P. Vassalli. 1977. Plasma cell immunoglobulin secretion: arrest is accompanied by alterations of the golgi complex. *J Exp Med* 146:1332-1345.

Thakker, P., M.W. Leach, W. Kuang, S.E. Benoit, J.P. Leonard, and S. Marusic. 2007. IL-23 is critical in the induction but not in the effector phase of experimental autoimmune encephalomyelitis. *J Immunol* 178:2589-2598.

Tzartos, J.S., M.A. Friese, M.J. Craner, J. Palace, J. Newcombe, M.M. Esiri, and L. Fugger. 2008. Interleukin-17 production in central nervous system-infiltrating T cells and glial cells is associated with active disease in multiple sclerosis. *Am J Pathol* 172:146-155.

Veldhoen, M., R.J. Hocking, C.J. Atkins, R.M. Locksley, and B. Stockinger. 2006. TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. *Immunity* 24:179-189.

Veldhoen, M., C. Uyttenhove, J. van Snick, H. Helmby, A. Westendorf, J. Buer, B. Martin, C. Wilhelm, and B. Stockinger. 2008. Transforming growth factor-beta 'reprograms' the differentiation of T helper 2 cells and promotes an interleukin 9-producing subset. *Nat Immunol* 9:1341-1346.

Viglietta, V., C. Baecher-Allan, H.L. Weiner, and D.A. Hafler. 2004. Loss of functional suppression by CD4+CD25+ regulatory T cells in patients with multiple sclerosis. *J Exp Med* 199:971-979.

Villarino, A., L. Hibbert, L. Lieberman, E. Wilson, T. Mak, H. Yoshida, R.A. Kastelein, C. Saris, and C.A. Hunter. 2003. The IL-27R (WSX-1) is required to suppress T cell hyperactivity during infection. *Immunity* 19:645-655.

Wakatsuki, A., P. Borrow, K. Rigley, and P.C. Beverley. 2003. Cell-surface bound pertussis toxin induces polyclonal T cell responses with high levels of interferon-gamma in the absence of interleukin-12. *Eur J Immunol* 33:1859-1868.

Wang, Y., J. Godec, K. Ben-Aissa, K. Cui, K. Zhao, A.B. Pucsek, Y.K. Lee, C.T. Weaver, R. Yagi, and V. Lazarevic. 2014. The Transcription Factors T-bet and Runx Are Required for the Ontogeny of Pathogenic Interferon-gamma-Producing T Helper 17 Cells. *Immunity* 40:355-366.

Wei, L., G. Vahedi, H.W. Sun, W.T. Watford, H. Takatori, H.L. Ramos, H. Takahashi, J. Liang, G. Gutierrez-Cruz, C. Zang, W. Peng, J.J. O'Shea, and Y. Kanno. 2010. Discrete roles of STAT4 and STAT6 transcription factors in tuning epigenetic modifications and transcription during T helper cell differentiation. *Immunity* 32:840-851.

Wekerle, H., K. Kojima, J. Lannes-Vieira, H. Lassmann, and C. Linington. 1994. Animal models. *Ann Neurol* 36 Suppl:S47-53.

Wildin, R.S., F. Ramsdell, J. Peake, F. Faravelli, J.L. Casanova, N. Buist, E. Levy-Lahad, M. Mazzella, O. Goulet, L. Perroni, F.D. Bricarelli, G. Byrne, M. McEuen, S. Proll, M. Appleby, and M.E. Brunkow. 2001. X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy syndrome is the human equivalent of mouse scurfy. *Nat Genet* 27:18-20.

Willenborg, D.O., S. Fordham, C.C. Bernard, W.B. Cowden, and I.A. Ramshaw. 1996. IFN-gamma plays a critical down-regulatory role in the induction and effector phase of myelin

oligodendrocyte glycoprotein-induced autoimmune encephalomyelitis. *J Immunol* 157:3223-3227.

Wohlfert, E.A., J.R. Grainger, N. Bouladoux, J.E. Konkel, G. Oldenhove, C.H. Ribeiro, J.A. Hall, R. Yagi, S. Naik, R. Bhairavabhotla, W.E. Paul, R. Bosselut, G. Wei, K. Zhao, M. Oukka, J. Zhu, and Y. Belkaid. 2011. GATA3 controls Foxp3(+) regulatory T cell fate during inflammation in mice. *J Clin Invest* 121:4503-4515.

Wu, C., X. Wang, M. Gadina, J.J. O'Shea, D.H. Presky, and J. Magram. 2000. IL-12 receptor beta 2 (IL-12R beta 2)-deficient mice are defective in IL-12-mediated signaling despite the presence of high affinity IL-12 binding sites. *J Immunol* 165:6221-6228.

Xu, J., Y. Yang, G. Qiu, G. Lal, Z. Wu, D.E. Levy, J.C. Ochando, J.S. Bromberg, and Y. Ding. 2009. c-Maf regulates IL-10 expression during Th17 polarization. *J Immunol* 182:6226-6236.

Yamamoto, K., F. Shibata, O. Miura, R. Kamiyama, S. Hirosawa, and N. Miyasaka. 1999. Physical interaction between interleukin-12 receptor beta 2 subunit and Jak2 tyrosine kinase: Jak2 associates with cytoplasmic membrane-proximal region of interleukin-12 receptor beta 2 via amino-terminus. *Biochem Biophys Res Commun* 257:400-404.

Yang, X.O., A.D. Panopoulos, R. Nurieva, S.H. Chang, D. Wang, S.S. Watowich, and C. Dong. 2007. STAT3 regulates cytokine-mediated generation of inflammatory helper T cells. *J Biol Chem* 282:9358-9363.

Yang, X.O., B.P. Pappu, R. Nurieva, A. Akimzhanov, H.S. Kang, Y. Chung, L. Ma, B. Shah, A.D. Panopoulos, K.S. Schluns, S.S. Watowich, Q. Tian, A.M. Jetten, and C. Dong. 2008. T helper 17 lineage differentiation is programmed by orphan nuclear receptors ROR alpha and ROR gamma. *Immunity* 28:29-39.

Yoshida, S., and S. Plant. 1992. Mechanism of release of Ca2+ from intracellular stores in response to ionomycin in oocytes of the frog Xenopus laevis. *J Physiol* 458:307-318.

Yoshida, Y., R. Yoshimi, H. Yoshii, D. Kim, A. Dey, H. Xiong, J. Munasinghe, I. Yazawa, M.J. O'Donovan, O.A. Maximova, S. Sharma, J. Zhu, H. Wang, H.C. Morse, 3rd, and K. Ozato. 2014. The transcription factor IRF8 activates integrin-mediated TGF-beta signaling and promotes neuroinflammation. *Immunity* 40:187-198.

Yura, M., I. Takahashi, M. Serada, T. Koshio, K. Nakagami, Y. Yuki, and H. Kiyono. 2001. Role of MOG-stimulated Th1 type "light up" (GFP+) CD4+ T cells for the development of experimental autoimmune encephalomyelitis (EAE). *J Autoimmun* 17:17-25.

Zhang, X., D.N. Koldzic, L. Izikson, J. Reddy, R.F. Nazareno, S. Sakaguchi, V.K. Kuchroo, and H.L. Weiner. 2004. IL-10 is involved in the suppression of experimental autoimmune encephalomyelitis by CD25+CD4+ regulatory T cells. *Int Immunol* 16:249-256.

Zheng, W., and R.A. Flavell. 1997. The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells. *Cell* 89:587-596.

Zhou, L., Ivanov, II, R. Spolski, R. Min, K. Shenderov, T. Egawa, D.E. Levy, W.J. Leonard, and D.R. Littman. 2007. IL-6 programs T(H)-17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways. *Nat Immunol* 8:967-974.

Zou, W. 2006. Regulatory T cells, tumour immunity and immunotherapy. *Nat Rev Immunol* 6:295-307.

List of figures

Supplementary tables

Supplementary Table 1:. **Upregulated genes in Tr-1 cells vs. Th17 cells.** *In vitro* differentiated Tr-1 cells and Th17 cells were compared by microarray (n=5). List of genes with at least 3-fold upregulation ($p < 0.05$).

Input	Input Type	MGI Gene/Marker ID	Symbol	Name	Feature Type	Entrez Gene ID
10338959		No associated gene				
10341932		No associated gene				
10343452		No associated gene				
10349108	Affy 1.0 ST	MGI:109579	Serpinb5	serine (or cysteine) peptidase inhibitor, clade B, member 5	protein coding gene	20724
10349603	Affy 1.0 ST	MGI:96537	Il10	interleukin 10	protein coding gene	16153
10366586	Affy 1.0 ST	MGI:107656	Ifng	interferon gamma	protein coding gene	15978
10368970	Affy 1.0 ST	MGI:99655	Prdm1	PR domain containing 1, with ZNF domain	protein coding gene	12142
10372028	Affy 1.0 ST	MGI:1890127	Plxnc1	plexin C1	protein coding gene	54712
10376060	Affy 1.0 ST	MGI:96590	Irf1	interferon regulatory factor 1	protein coding gene	16362
10376324	Affy 1.0 ST	MGI:3649299	Gm12250	predicted gene 12250	pseudogene	631323
10379721	Affy 1.0 ST	MGI:98261	Ccl4	chemokine (C-C motif) ligand 4	protein coding gene	20303
10385518	Affy 1.0 ST	MGI:98734	Tgtp1	T cell specific GTPase 1	protein coding gene	21822
10385533	Affy 1.0 ST	MGI:98734	Tgtp1	T cell specific GTPase 1	protein coding gene	21822
10389231	Affy 1.0 ST	MGI:98260	Ccl3	chemokine (C-C motif) ligand 3	protein coding gene	20302
10390328	Affy 1.0 ST	MGI:1888984	Tbx21	T-box 21	protein coding gene	57765
10398039	Affy 1.0 ST	MGI:2182838	Serpina3f	serine (or cysteine) peptidase inhibitor, clade A, member 3F	protein coding gene	238393
10408935		No associated gene				
10413419	Affy 1.0 ST	MGI:1918954	Arhgef3	Rho guanine nucleotide exchange factor (GEF) 3	protein coding gene	71704
10420308	Affy 1.0 ST	MGI:109267	Gzmb	granzyme B	protein coding gene	14939
10428576	Affy 1.0 ST	MGI:1913730	Rpl15	ribosomal protein L15	protein coding gene	66480
10429573	Affy 1.0 ST	MGI:96882	Ly6c1	lymphocyte antigen 6 complex, locus C1	protein coding gene	17067
10435704	Affy 1.0 ST	MGI:101775	Cd80	CD80 antigen	protein coding gene	12519
10439895	Affy 1.0 ST	MGI:1313266	Alcam	activated leukocyte cell adhesion molecule	protein coding gene	11658
10441601	Affy 1.0 ST	MGI:3615484	Tagap	T cell activation Rho GTPase activating protein	protein coding gene	72536
10455954	Affy 1.0 ST	MGI:3644953	Gm4951	predicted gene 4951	protein coding gene	240327
10455961	Affy 1.0 ST	MGI:1926259	Iigp1	interferon inducible GTPase 1	protein coding gene	60440
10463070	Affy 1.0 ST	MGI:102805	Entpd1	ectonucleoside triphosphate diphosphohydrolase 1	protein coding gene	12495
10466127	Affy 1.0 ST	MGI:2147706	AW112010	expressed sequence AW112010	protein coding gene	107350
10475517	Affy 1.0 ST	MGI:3034182	AA467197	expressed sequence AA467197	protein coding gene	433470
10495186	Affy 1.0 ST	MGI:2139742	AI504432	expressed sequence AI504432	unclassified gene	229694
10496539	Affy 1.0 ST	MGI:2429943	Gbp5	guanylate binding protein 5	protein coding gene	229898
10496569	Affy 1.0 ST	MGI:2444421	Gbp7	guanylate binding protein 7	protein coding gene	229900
10511363	Affy 1.0 ST	MGI:104629	Penk	preproenkephalin	protein coding gene	18619
10512774	Affy 1.0 ST	MGI:1345966	Coro2a	coronin, actin binding protein 2A	protein coding gene	107684
10531724	Affy 1.0 ST	MGI:2445289	Plac8	placenta-specific 8	protein coding gene	231507
10531987	Affy 1.0 ST	MGI:97072	Gbp4	guanylate binding protein 4	protein coding gene	17472
10531994	Affy 1.0 ST	MGI:2140937	Gbp6	guanylate binding protein 6	protein coding gene	100702
10545135	Affy 1.0 ST	MGI:1270861	Il12rb2	interleukin 12 receptor, beta 2	protein coding gene	16162
10552406	Affy 1.0 ST	MGI:1931250	Nkg7	natural killer cell group 7 sequence	protein coding gene	72310
10576034	Affy 1.0 ST	MGI:96395	Irf8	interferon regulatory factor 8	protein coding gene	15900
10603151	Affy 1.0 ST	MGI:107672	Gpm6b	glycoprotein m6b	protein coding gene	14758
10608646		No associated gene				
10608681		No associated gene				

Supplementary Table 2. Up-regulated genes in Th17 cells vs. Tr-1 cells. *In vitro* differentiated Th17 cells and Tr-1 cells were compared by microarray (n=5). List of genes with at least 3-fold upregulation ($p < 0.05$).

Input	Input Type	MGI Gene/Marker ID	Symbol	Name	Feature Type	Entrez Gene ID
10338894		No associated gene				
10345032	Affy 1.0 ST	MGI:107364	Il17a	interleukin 17A	protein coding gene	16171
10345762	Affy 1.0 ST	MGI:96545	Il1r1	interleukin 1 receptor, type I	protein coding gene	16177
10347888	Affy 1.0 ST	MGI:1329031	Ccl20	chemokine (C-C motif) ligand 20	protein coding gene	20297
10353415	Affy 1.0 ST	MGI:2676631	Il17f	interleukin 17F	protein coding gene	257630
10362073	Affy 1.0 ST	MGI:1340062	Sgk1	serum/glucocorticoid regulated kinase 1	protein coding gene	20393
10367440	Affy 1.0 ST	MGI:102700	Itga7	integrin alpha 7	protein coding gene	16404
10405179	Affy 1.0 ST	MGI:1339365	S1pr3	sphingosine-1-phosphate receptor 3	protein coding gene	13610
10408557	Affy 1.0 ST	MGI:1913472	Serpinb1a	serine (or cysteine) peptidase inhibitor, clade B, member 1a	protein coding gene	66222
10409586	Affy 1.0 ST	MGI:96563	Il9	interleukin 9	protein coding gene	16198
10415392	Affy 1.0 ST	MGI:1309472	Ltb4r1	leukotriene B4 receptor 1	protein coding gene	16995
10422728	Affy 1.0 ST	MGI:109175	Dab2	disabled 2, mitogen-responsive phosphoprotein	protein coding gene	13132
10424119	Affy 1.0 ST	MGI:109185	Nov	nephroblastoma overexpressed gene	protein coding gene	18133
10436865	Affy 1.0 ST	MGI:107654	Ifngr2	interferon gamma receptor 2	protein coding gene	15980
10457168	Affy 1.0 ST	MGI:3039602	Cd226	CD226 antigen	protein coding gene	225825
10472893	Affy 1.0 ST	MGI:2443258	B230120H23Rik	RIKEN cDNA B230120H23 gene	protein coding gene	65964
10474419	Affy 1.0 ST	MGI:1891468	Lgr4	leucine-rich repeat-containing G protein-coupled receptor 4	protein coding gene	107515
10477717	Affy 1.0 ST	MGI:104596	Procr	protein C receptor, endothelial	protein coding gene	19124
10489204	Affy 1.0 ST	MGI:98731	Tgm2	transglutaminase 2, C polypeptide	protein coding gene	21817
10500656	Affy 1.0 ST	MGI:2685862	Cd101	CD101 antigen	protein coding gene	630146
10534667	Affy 1.0 ST	MGI:97608	Serpine1	serine (or cysteine) peptidase inhibitor, clade E, member 1	protein coding gene	18787
10544596	Affy 1.0 ST	MGI:1916348	Tmem176b	transmembrane protein 176B	protein coding gene	65963
10576639	Affy 1.0 ST	MGI:106206	Nrp1	neuropilin 1	protein coding gene	18186
10585794	Affy 1.0 ST	MGI:88582	Cyp11a1	cytochrome P450, family 11, subfamily a, polypeptide 1	protein coding gene	13070
10586700	Affy 1.0 ST	MGI:104661	Rora	RAR-related orphan receptor alpha	protein coding gene	19883
10598976	Affy 1.0 ST	MGI:98752	Timp1	tissue inhibitor of metalloproteinase 1	protein coding gene	21857

Attributions

The data (TGF-β titration experiments) shown in Figure 5 were generated by Franziska Petermann, Meike Mitsdörffer and Christopher Sie (own work group).

T cell differentiation and stimulation experiments depicted in Figure 10C and Figure 13B were performed by Sylvia Heink (own work group) in collaboration with cooperation partners in Australia (The Walter and Eliza Hall Institute of Medical Research, Parkville, Australia).

Secreation assay (Figure 14) and the ChIP assay (Figure 17A) were generated with support of Sylvia Heink (own work group).

The T cell differentiation with Stat3$^{-/-}$ mice depicted in Figure 15 was performed by Veit Rothammer (own work group).

The data (ChIP-sequencing) in Figure 17B were provided by our cooperation partner Warren J. Leonard.

The induction and monitoring of the EAE depicted in Figure 18A was performed by Sylvia Heink (own work group) in collaboration with cooperation partners in Australia (The Walter and Eliza Hall Institute of Medical Research, Parkville, Australia).

List of abbreviations

APC	Antigen presenting cell
APC (FACS)	Allophycocyanin
bp	Base pair
BSA	Bovine serum albumin
CD	Cluster of differentiation
CFA	Complete Freund’s adjuvant
CFSE	Carboxyfluorescein succinimidyl ester
CKO	Conditional kockout
CNS	Central nerve system
DC	Dendritic cell
ddH_2O	Double distilled water
DMEM	Dulbecco`s modified Eagle`s Medium
DMSO	Dimethyl sulfoxid
DNA	Deoxyribonucleic acid
DNase I	Deoxyribonuclease I
dNTP	Deoxyribonucleotide triphosphate
DTx	Diphtheria toxin
EAE	Experimental autoimmune encephalomyelitis
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence activated cell sorting
FCS	Fetal calf serum
FITC (FACS)	Fluorescein isothiocyanate
Foxp3	Forkhead box protein 3
FSC	Forward scatter
GM-CSF	Granulocyte-macrophage-colony stimmulating factor
GaH	Goat anti-hamster IgG antibody
GaM	Goat anti-mouse IgG antibody
GaR	Goat anti-rabbit IgG antibody
GaRAT	Goat anti-rat IgG antibody
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin

i.p.	Intraperitoneal
i.v.	Intravenous
kb	Kilobase
kDa	Kilodalton
KO	Knockout
m	Murine
mAb	Monoclonal antibody
MACS	Magnetic activated cell sorting
MHC	Major histocompatibility complex
MOG	Myelin oligodendrocyte glycoprotein
mRNA	Messenger ribonucleic acid
MS	Multiple sclerosis
n	Number of replicates
NaOH	Sodium hydroxide
NaCl	Sodium chloride
n.d.	Not detectable
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PcBl	Pacific Blue
PcO	Pacific Orange
PE	Phycoerythrin
PerCP (FACS)	Peridinin-Chlorophyll
p.i.	Post immunization
POD	Peroxidase
PTx	Pertussis toxin
RNA	Ribonucleic acid
rpm	rounds per minute
RT	room temperature
RT-PCR	Reverse transcription PCR
SA	Streptavidin
s.c.	Subcutaneous
SDS	Sodium dodecyl sulfate
SEM	Standard error of mean

SSC	Side scatter
STAT	Signal transducer and activator of transcription
Tab.	Table
T-bet	T-box expressed in T cells
TBS	Tris buffered saline
TCR	T cell receptor
TGF-β	Transforming growth factor beta
TNF-α	Tumor necrosis factor α
Treg	Regulatory T cell
Tris	Tris hydroxymethyl aminomethan
U	Units
vs.	*Versus*
v/v	Volume per volume
w/v	Weight per volume
w/w	Weight per weight
WT	Wildtyp

Publications

Heinemann C, Heink S, Petermann F, Vasanthakumar A, Rothhammer V, Doorduijn E, Mitsdörffer M, Sie C, Prazeres da Costa O, Buch T, Hemmer B, Oukka M, Kallies A, Korn T. 2014. IL-27 and IL-12 oppose pro-inflammatory IL-23 in $CD4^+$ T cells by inducing Blimp1. Nat Commun.

Huber M, Heink S, Pagenstecher A, Reinhard K, Ritter J, Visekruna A, Guralnik A, Bollig N, Jeltsch K, **Heinemann C**, Wittmann E, Buch T, Prazeres da Costa O, Brüstle A, Brenner D, Mak TW, Mittrücker HW, Tackenberg B, Kamradt T, Lohoff M. 2013. IL-17A secretion by $CD8^+$ T cells supports Th17-mediated autoimmune encephalomyelitis. J Clin Invest.

Berod L, **Heinemann C**, Heink S, Escher A, Stadelmann C, Drube S, Wetzker R, Norgauer J, Kamradt T. 2011. PI3Kγ deficiency delays the onset of experimental autoimmune encephalomyelitis and ameliorates its clinical outcome. Eur J Immunol.

Congress contributions

16th Meeting on T cells substes and functions, Marburg, 1.-2.7.2013

Talk: **Christina Heinemann**, Sylvia Heink, Franziska Petermann, Ajithkumar Vasanthakumar, Veit Rothhammer, Elien Doorduijn, Meike Mitsdoerffer, Christopher Sie, Olivia Prazeres da Costa, Thorsten Buch, Bernhard Hemmer, Mohamed Oukka, Peng Li, Warren J. Leonard, Axel Kallies, Thomas Korn; The balance of IL-12 and IL-23 signals controls Blimp1-dependent programming of CD4+ effector T cells into suppressive IL-10 producers in autoimmune inflammation; TU München

3. Wissenschaftssymposium „Neurowind e.V." in Motzen, 4.-6.11.2011

Talk: **Christina Heinemann**, Malte Claussen, Franziska Petermann, Sylvia Heink, Bernhard Hemmer, Mohamed Oukka, Thomas Korn;T helper cell intrinsic and extrinsic functions of IL-23 in autoimmune neuroinflammation; TU München

Berichtssymposium des Multipple Sklerose – Einzelantragsverfahrens der Geimeinnützigen Hertie-Stiftung (GHS) in Frankfurt, 11.10.2011

Talk: Capacity of regulatory T-lymphocytes to influence autoimmune attacks on the brain - examination of different EAE models; Christina Heinemann, Sebastian Berg, Tim Sparwasser, Christine Stadelmann, Thomas Korn, Thomas Kamradt, Sylvia Heink

14th German Meeting of T Cells: Subsets and functions, Marburg; 0.-21.06.2011

40. Jahrestagung der Deutschen Gesellschaft für Immunologie (DGfI), Leipzig, 22.-25.09.2010

Poster: Sebastian Berg, Sylvia Heink, **Christina Heinemann**, Tim Sparwasser, Chstine Stadelmann, Thomas Kamradt; Temporary selective depletion of regulatory T cells exacerbates conventional MOG_{35-55} peptide induced EAE only moderately; Friedrich-Schiller-Universität Jena

13th German Meeting of T Cells: Subsets and functions, Marburg, 30.6-1.7.2010

2nd European Congress of Immunology, Berlin, 13.-16.09.2009

Poster: Sebastian Berg, Sylvia Heink, **Christina Heinemann**, Tim Sparwasser, Chstine Stadelmann, Thomas Kamradt; Temporary selective depletion of regulatory T cells exacerbates conventional MOG_{35-55} peptide induced EAE only moderately; Friedrich-Schiller-Universität Jena

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	Betreuer: Prof. Dr. Benedikt Westermann

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Erklärung

Ich erkläre an Eides statt, dass ich die bei der promotionsführenden Einrichtung bzw. Fakultät für Medizin der TUM zur Promotionsprüfung vorgelegte Arbeit mit dem Titel: **IL-12 antagonizes IL-23 in autoimmune inflammation by Blimp1-dependent programming of CD4$^+$ effector T cells into suppressive IL-10 producers** in der Experimentellen Neurologie unter der Anleitung und Betreuung durch Herrn Prof. Dr. Thomas Korn ohne sonstige Hilfe erstellt und bei der Abfassung nur die gemäß § 6 Abs. 6 und 7 Satz 2 angegebenen Hilfsmittel benutzt habe.

(x) Ich habe keine Organisation eingeschaltet, die gegen Entgelt Betreuerinnen und Betreuer für die Anfertigung von Dissertationen sucht, oder die mir obliegenden Pflichten hinsichtlich der Prüfungsleistung für mich ganz oder teilweise erledigt.

(x) Ich habe die Dissertation in dieser oder ähnlicher Form in keinem anderen Prüfungsverfahren als Prüfungsleistung vorgelegt.

( ) Die vollständige Dissertation wurde in _____ veröffentlicht. Die promotionsführende Einrichtung _____ hat der Veröffentlichung zugestimmt.

(x) Ich habe den angestrebten Doktorgrad noch nicht erworben und bin nicht in einem früheren Promotionsverfahren für den angestrebten Doktorgrad endgültig gescheitert.

( ) Ich habe bereits am _____ bei der Fakultät für _____ der Hochschule _____ unter Vorlage einer Dissertation mit dem Thema _____ die Zulassung zur Promotion beantragt mit dem Ergebnis: _____.

Die öffentlich zugängliche Promotionsordnung der TUM ist mir gekannt, insbesondere habe ich die Bedeutung von § 28 (Nichtigkeit der Promotion) und § 29 (Entzug des Doktorgrades) zur Kenntnis genommen. Ich bin mir der Konsequenzen einer falschen Eidesstattlichen Erklärung bewusst.

Mit der Aufnahme meiner personenbezogenen Daten in die Alumni-Datei bei der TUM bin ich einverstanden.

München, der 13. Mai 2014 _____

Unterschrift

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