# Stability and plasticity of IL-17 expression in $T_H17$ cells

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# Summary

The cytokine IL-17, inter alia produced by $T_H17$ cells, plays an important role in autoimmune diseases, but also for the clearing of extracellular pathogens and for the recruitment of neutrophils during infection. Understanding the process, through which $T_H$ cells alter their cytokine-producing potential can provide interesting insights into effector cell commitment, gene regulation and provide us with important biological implications for designing therapeutic regimens. So far, very little is known about the stability and plasticity of the $T_H17$ phenotype.

In order to analyze the stability of IL-17 expression on the single cell level, in cooperation with Miltenyi Biotec, we have developed an IL-17 secretion assay, which allows us to isolate viable IL-17-producing $T_H$ cells. We show here that IL-$17^+$ $T_H$ cells generated *in vitro* with the canonical $T_H17$ differentiating signals TGFβ, IL-6 and IL-23 can readily be induced to express IFNγ with concomitant loss of IL-17 expression in response to IL-12. This conversion of $T_H17$ cells into $T_H1$ cells was T-bet-dependent. By contrast, IL-$17^+$ $T_H$ cells generated *in vivo* do neither respond to IL-12 with IFNγ expression nor to IL-4 with IL-4 expression. Transcriptome analysis comparing *in vitro* and *in vivo* generated $T_H17$ cells revealed that *in vivo* generated $T_H17$ cells do not express the β2 chain of the IL12R, rendering them refractory to IL-12 signaling. However, they express a functional IFNγR. As has been demonstrated for naïve $T_H$ cells and for $T_H2$ cells expression of the IL12Rβ2 chain could be induced by IFNγ restoring susceptibility to IL-12 signaling. Thus, we could demonstrate that in $T_H17$ cells generated *in vivo*, IFNγ expression can be induced by the synergistic action of IFNγ and IL-12. In such cells the master transcription factors RORγt and T-bet were co-expressed on the single cell level, as were the corresponding effector cytokines IL-17 and IFNγ. It remains to be clarified, however, whether such $T_H1/17$ cells represent a distinct stable lineage or a transitional state. We also analyzed the epigenetic imprinting of IL-$17^+$ $T_H$ cells and compared it to IFN$\gamma^+$ $T_H$ cells. We identified a region upstream of *il17* that is unmethylated in cells expressing IL-17 and methylated in IFN$\gamma^+$ $T_H$ cells. In addition, in CpG rich regions upstream of *rorγt* we identified an element, which was specifically methylated in IL-17-single-positive $T_H$ cells and may represent a putative silencer element.

In conclusion, the high plasticity of $T_H17$ cells could help the immune system to adapt and eliminate inflammation and infection. Thus, $T_H1/17$ cells could have a physical advantage through a combined effector repertoire of $T_H1$ and $T_H17$ cells on the single cell level, which would be worth considering in studies of autoimmune diseases or in optimization of vaccination strategies.

# Zusammenfassung

Das Zytokin IL-17, welches unter anderem von $T_H17$ Zellen produziert wird, spielt eine wichtige Rolle bei Autoimmunerkrankungen sowie bei der Eliminierung von extrazellulären Pathogenen und der Rekrutierung von Neutrophilen zum Entzündungsort. Die Kenntnis darüber, wie $T_H$ Zellen ihr Potential ändern ein bestimmtes Zytokin zu produzieren, eröffnet uns interessante Einblicke in die Steuerung von Effektor $T_H$ Zelldifferenzierung, was wiederum die Entwicklung von neuartigen therapeutischen Konzepten vorantreibt. Bisher war nicht viel über die Plastizität und Stabilität von $T_H17$ Zellen bekannt.

Um die Stabilität von $T_H17$ Zellen auf Einzel-Zell-Ebene zu untersuchen, entwickelten wir in Kooperation mit Miltenyi Biotec einen IL-17 Sekretionsassay, womit wir lebende IL-17-produzierende $T_H$ Zellen isolieren konnten. Wir zeigen hier, dass in $T_H17$ Zellen, die *in vitro* mit den bekannten $T_H17$ Differenzierungssignalen TGFβ, IL-6 und IL-23 generiert wurden, hinsichtlich der Produktion des Zytokins IL-17 instabil sind. So beginnen sie bei Stimulation mit IL-12 IFNγ zu produzieren, während gleichzeitig die IL-17 Expression verloren geht. Die Konversion von $T_H17$ zu $T_H1$ Zellen verlief T-bet-abhängig. *In vivo* generierte $T_H17$ Zellen verhalten sich allerdings stabil. So konnten diese weder durch IL-12 zur IFNγ Expression, noch durch IL-4 zur IL-4 Expression angeregt werden. Der Vergleich von *in vitro* vs *in vivo* generierten $T_H17$ Zellen in einer Transkriptomanalyse ergab, dass *in vivo* generierte $T_H17$ Zellen die β2 Kette des IL12R nicht exprimieren, was deren Nicht-reaktivität auf den IL-12 Stimulus erklärt. Allerdings exprimieren sie einen funktionellen IFNγR. Wie schon für naive und $T_H2$ Zellen gezeigt wurde, konnte auch hier IL12Rβ2 durch IFNγ Stimulus induziert werden. Wir zeigen daher, dass die IFNγ Expression in *in vivo* generierte $T_H17$ Zellen durch die synergistische Aktion von IFNγ und IL-12 induziert wird. Diese Zellen co-exprimieren die Haupttranskriptionsfaktoren T-bet und RORγt sowie die Zytokine IFNγ und IL-17. Inwieweit diese $T_H1/17$ Zellen eine stabile $T_H$ Zell-Population oder eine Übergangs-Population darstellen, muss weiter untersucht werden. Die epigenetische Prägung von IL-$17^+$ und IFN$\gamma^+$ $T_H$ Zellen wurde ebenfalls verglichen. Vorgelagerte Regionen von *il17* waren unmethyliert in IL-$17^+$ und methyliert in IFN$\gamma^+$ $T_H$ Zellen. Des weiteren haben wir in einer CpG-reichen Region die *rorc* vorgelagert ist, ein mögliches regulatorisches Element entdeckt, welches nur in IL-17-einzel-positiven $T_H$ Zellen methyliert war und ein putatives Silencer-Element darstellt.

$T_H17$ Zellen könnten durch ihre Plastizität dem Immunsystem helfen sich spezifischen Umständen anzupassen und dadurch Entzündungen und Infektionen effektiver eliminieren. Darüber hinaus könnten $T_H1/17$ Zellen durch ihr kombiniertes $T_H1$ und $T_H17$ Effektor-Repertoire einen Vorteil haben und sollten bei Autoimmunerkrankungen und bei Optimierung von Vakzinierungs-Konzepten berücksichtigt werden.

# 1 Table of Contents

# 2 Abbreviations

| | |
|---|---|
| AP-1 | activator protein 1 |
| APC | antigen-presenting cell |
| BBB | blood brain barrier |
| bp | base pair |
| BSA | bovine serum albumin |
| CCL | CC motif chemokine ligand |
| CCR | CC motif chemokine receptor |
| c-MAF | musculoaponeurotic fibrosarcoma oncogene homolog |
| CNS | conserved non-coding sequence |
| CpG | cytosine-phosphate-guanine |
| CTCF | CCCTC-binding factor |
| CTLA-8 | cytotoxic T-lymphocyte antigen-8 gene |
| CXCL | CXC motif chemokine ligand |
| DNA | desoxyribonucleic acid |
| EAE | experimental autoimmune encephalomyelitis |
| FACS | fluorescence-activated cell sorting |
| FITC | fluorescein isothiocyanat |
| G-CSF | granulocyte colony-stimulating factor |
| GM-CSF | granulocyte-macrophage colony-stimulating factor |
| GFP | green fluorescent protein |
| h | human |
| HDAC | histone deacetylase |
| Hlx | H2.0-like homeobox |
| HPRT | hypoxanthine guanine phosphoribosyl transferase |
| IFN | interferon |
| IL | interleukin |
| iTreg | inducible regulatory T cell |
| m | murine |

| | |
|---|---|
| MACS | magnetic cell sorting |
| MBD | methyl-CpG-binding domain protein |
| MHC | major histocompatibility complex |
| MMP | matrix metalloproteinases |
| MOG | myelin oligodendrocyte glycoprotein |
| MS | multiple sclerosis |
| NFAT | nuclear factor of activated T cells |
| NFκB | nuclear factor of κ light chain enhancer in B cells |
| NK | natural killer |
| NKT | natural killer T |
| OVA | ovalbumin |
| PBS | phosphate-buffered saline |
| PCR | polymerase chain reaction |
| PE | phycoerythrin |
| PMA | phorbol 12-myristate 13-acetate |
| r | recombinant |
| RA | rheumatoid arthritis |
| RNA | ribonucleic acid |
| ROR | retinoid-related orphan receptor |
| Runx3 | Runt-related transcription factor 3 |
| RT | room temperature |
| Stat | signal transducer of activated T cells |
| T-bet | T-box expressed in T cells |
| $T_{CM}$ | Central memory T |
| TCR | T cell receptor |
| $T_{EM}$ | Effector memory T |
| TGFβ1 | Transforming growth factor β1 |
| $T_H$ | T helper |
| TLR | toll like receptor |
| Treg | regulatory T cell |
| v/v | volume per volume |

| | |
|---|---|
| w/v | weight per volume |
| wt | wildtype |

# 3 Introduction

The main role of the immune system is to protect the body from infection. The system is divided into two major categories: the innate immune system and the adaptive immune system. Early protective immune mechanisms are mediated by the innate immune system and are followed by reactions of the adaptive immunity. Innate immunity to microbes stimulates adaptive immune responses and can influence the nature of the adaptive responses to make them optimally effective against different types of microbes.

The characteristics, which define the adaptive immunity, are the specificity for distinct molecules and an ability to “remember” and to respond more vigorously upon repeated exposures to the same microbe. Cells of the adaptive immune system, the T and B lymphocytes, bear variable antigen-recognition receptors on the cell surface encoded by somatically rearranged gene segments. Each T and B cell individually rearranges the genes that encode the variable part of the antigen receptor during the development in the thymus and bone marrow, respectively, creating an enormous diversity of different specificities. Since among these specificities some receptors potentially recognize self structures, it is necessary to eliminate self-reactive T and B cells from the repertoire before maturation of the cells. However, some autoreactive T and B cells escape elimination and are detectable in the blood of healthy individuals. Those potentially pathogenic cells are kept under control by a mechanism called peripheral tolerance. A delicate balance between inflammation and tolerance needs to be maintained as dysregulated immune reactions can lead to autoimmunity on one hand and allergy on the other. Aberrant $CD4^+$ T helper ($T_H$) type 1 ($T_H1$) and $T_H17$ cell responses play critical roles in organ-specific autoimmunity, whereas $T_H2$ cells have been implicated in the pathogenesis of asthma and allergy. $T_H$ cells have a central role in the immune system, coordinating both adaptive and innate responses. The function of $T_H$ cells is mainly mediated through the secretion of cytokines. The cytokines of the adaptive immunity are critical for the development of immune responses and for the activation of effector cells that serve to eliminate microbes and other antigens. The stability of cytokine production by a $T_H$ cell in a specific microenvironment can have a great impact on the development of an autoimmune disease. As observed in numerous diseases, excessive production or effects of cytokines can lead to pathologic consequences. For instance, Interleukin-17 (IL-17), a cytokine produced by $T_H17$ cells, has been shown to play a major part in autoimmune diseases, but also for the clearing

of extracellular pathogens and for the recruitment of neutrophils during infection. Therefore, a potential approach for modifying biologic responses associated with inflammatory diseases is the administration of cytokines or their inhibitors.

This work was focused on examining the plasticity and stability of IL-17 expression in $T_H17$ cells. Understanding the process through which $T_H$ cells alter their cytokine-producing potential will provide interesting insights into subset commitment and gene regulation. These findings may also allow the development of strategies to change $T_H$ function in autoimmunity and allergy.

## 3.1 $T_H$ cell memory

Upon exposure to an antigen, antigen specific T cells are activated, proliferate and differentiate into effector cells. The antigen specific effector cells act to clear the infection. While the majority of these cells die by apoptosis, some survive and as memory T cells provide a long-lasting protection upon re-exposure to the same antigen. These remaining pathogen-specific memory T cells are present at higher frequencies than the original naïve T cell, thereby increasing the probability that any re-infection with the same pathogen will be detected and cleared rapidly without having time to spread in the organism.

Memory cells can be distinguished from naïve cells based on the expression of surface molecules. After activation of T cells, the receptor for hyaluronate, CD44, is up-regulated. CD44 allows activated and memory T cells to enter inflamed peripheral sites. T cell activation further results in the down-regulation of CD62L and CCR7, molecules that are required to enter lymph nodes and to access the T cell area of the lymph node. Whereas the up-regulation of CD44 is probably a permanent change, some memory T cells re-express CD62L and CCR7, which is used to subdivide the memory T cells pool into two major populations (Sallusto et al., 1999). Central memory T ($T_{CM}$) cells are similar to naïve T cells in that they express CD62L and CCR7 and produce IL-2 following re-activation. Effector memory T ($T_{EM}$) cells rapidly produce effector cytokines (such as IL-4, IL-17 and IFNγ) upon activation by antigen. Due to the low expression of CD62L and CCR7 they are less likely to traffic through the lymph nodes and can be detected predominately in the peripheral organs, e.g. in cutaneous and intestinal tissue.

Another difference between memory and naïve T cells is that memory cells are able to mount cytokine responses faster than cells responding for the first time. Cytokine

production by T cells is transient and requires activation of the cell through the T cell receptor (TCR) recognizing its specific antigen. Upon primary activation it takes days until the cells express cytokine genes such as IL-4, IL-17, IFNγ, IL-2 and IL-10, although the kinetics of expression varies between the different cytokines. After secondary stimulation, cytokine expression is initiated within hours with similar and rapid kinetics for various cytokines. The ability of a re-activated $T_H$ cell to re-express the cytokines it was instructed to express in the absence of the instructing signals, is referred to as cytokine memory. Murine $T_H1$ and $T_H2$ cells generated *in vitro* show different degrees of stability of cytokine memory, depending on the duration of polarization. The memory for IL-4 is refractory to IL-12 after 1-2 weeks of $T_H2$ polarization, whereas the memory for IFNγ becomes refractory to IL-4 after 3-4 weeks of $T_H1$ polarization (Assenmacher et al., 1998; Murphy et al., 1996). Information about the memory for the pro-inflammatory cytokine IL-17 is so far very limited.

## 3.2 The role of cytokines in host defense

Activated $T_H$ cells proliferate and differentiate into effector cells whose functions are mediated largely by secreted cytokines. Their commitment depends on complex interactions with antigen-presenting cells, antigenic type and load, costimulatory molecules and cytokine signaling. Committed $CD4^+$ T cells may differentiate into $T_H1$, $T_H2$, $T_H17$ phenotypes, with distinct cytokine products and biological functions, or evolve into the inducible regulatory T cell (iTreg) lineage, with immunomodulatory functions. The cytokines present in the microenvironment of the cell during activation, determine the later phenotype of the cell.

Cytokines are polypeptides produced in response to microbes and other antigens and mediate many of the responses of innate and adaptive immunity. A complex network of cytokines exist which is crucial for the regulation of cellular events, thereby establishing a link between the innate and adaptive immune system. Most cytokines act in close proximity to where they are produced, either in an autocrine (effect on the same cell that secretes the cytokine) or in a paracrine (effect on a nearby cell) fashion. The actions of cytokines are very often pleiotropic, which refers to the ability of a cytokine to act on different cell types, allowing a cytokine to mediate diverse biological effects. This effect is further enhanced by the ability of cytokines to influence the synthesis and actions of other cytokines. Two cytokines may antagonize each other`s action, produce additive effects or synergistic effects.

A cytokine that has only recently been discovered to be of tremendous importance in the immune system is IL-17A. The IL-17A gene, originally called cytotoxic T-lymphocyte antigen-8 gene (CTLA-8), was first cloned from a murine cytotoxic T-lymphocyte hybridoma cDNA library (Rouvier et al., 1993). IL-17 is the founding member of the IL-17 family of cytokines, which includes IL-17A (also called IL-17), IL-17B, IL-17C, IL-17D, IL-17E (also called IL-25), and IL-17F (Aggarwal and Gurney, 2002; Kawaguchi et al., 2004). Among these cytokines, IL-17F has the highest amino acid homology to IL-17. The *il17f* gene is located close to the *il17a* gene in humans and mice. Both IL-17 and IL-17F induce the production of antimicrobial peptides (defensins and S100 proteins) (Kao et al., 2004; Liang et al., 2006), inflammatory cytokines (IL-6, granulocyte colony-stimulating factor (G-CSF)), and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Fossiez et al., 1996; Hymowitz et al., 2001; Kawaguchi et al., 2001; Numasaki et al., 2004); chemokines (CXCL1, CXCL5, IL-8, CCL2 and CCL7) (Kawaguchi et al., 2004; Kawaguchi et al., 2003; Kawaguchi et al., 2002; Yang et al., 2008a) and matrix metalloproteinases (MMP1, MMP3, MMP9 and MMP13) (Chabaud et al., 2000; Koenders et al., 2005; Park et al., 2005; Prause et al., 2004), from fibroblasts, endothelial cells and epithelial cells. Besides being produced by $T_H17$ cells, both IL-17 and IL-17F are also produced by a variety of cell types, including γδ T cells, natural killer (NK) cells, natural killer T (NKT) cells, neutrophils, CD8 cells and eosinophils (Ferretti et al., 2003; He et al., 2006; Liu et al., 2007; Lockhart et al., 2006; Molet et al., 2001; Starnes et al., 2001).

Numerous studies have investigated the role of IL-17 in immunity. Using a model of LPS-induced lung inflammation in mice it was shown that IL-17, produced mainly by $CD4^+$ cells, but also by neutrophils, played a major role in the mobilization of lung neutrophils following bacterial challenge (Ferretti et al., 2003). IL-17 overexpression in knee joints of mice induces joint inflammation, bone erosion and cartilage proteoglycan loss (Lubberts et al., 2001). Furthermore, numerous groups have shown that blockade or deficiency of IL-17 receptor (IL-17R) or neutralization of IL-17 with antibodies reduces the severity of murine collagen-induced arthritis and adjuvant-induced arthritis and has a significant protective effect against joint damage (Bush et al., 2002; Lubberts et al., 2004; Nakae et al., 2003). Due to these and many other studies, blocking IL-17 is considered a future therapy in rheumatoid arthritis and other autoimmune diseases.

## 3.3 $T_H$ lymphocyte differentiation

Naïve $CD4^+$ T cells, which mature in the thymus and have not yet encountered antigen, are the common precursors of $T_H$ cells. In response to an antigen naïve $CD4^+$ T cells may differentiate into subsets of effector cells that produce distinct sets of cytokines enable them to perform distinct effector functions. The adapted fate of the T cells is preserved in subsequent rounds of cell division. In this respect, $CD4^+$ T cell subsets are thought to be distinct lineages, as defined by the cytokines they produce. Classically divided into $T_H1$ and $T_H2$ cell (Mosmann et al., 1986; Mosmann and Coffman, 1989), a novel lineage of $CD4^+$ T cells was recently added, which is characterized by the production of IL-17 and named $T_H17$ (Harrington et al., 2005; Park et al., 2005). Regulatory $T_H$ cell subsets ($CD4^+CD25^+/Foxp3^+$) are essential for immune homeostasis by controlling activation, proliferation and functionality of the effector lineages. Regulatory T cells, which differentiate from naïve $CD4^+$ T cells are called inducible Tregs (iTreg) (Figure 1).

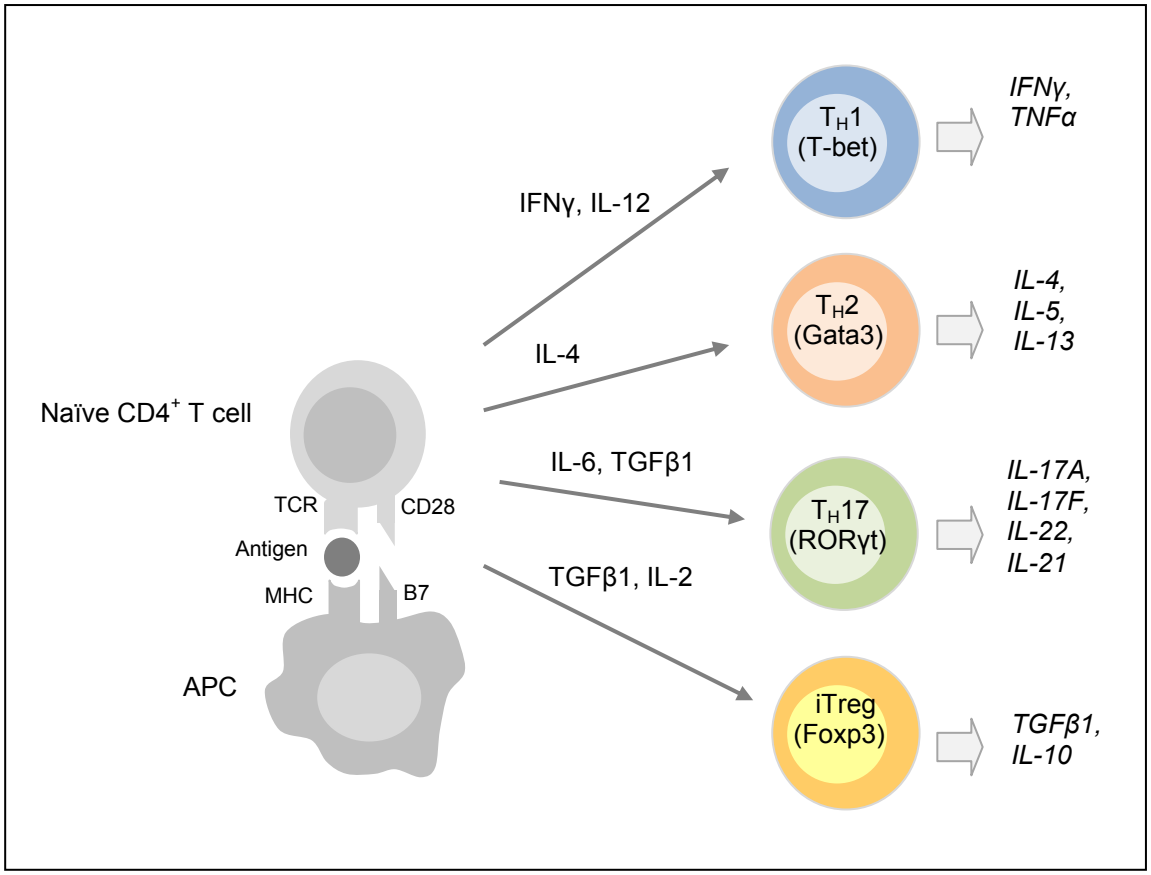


**Figure 1. The cytokine milieu determines $CD4^+$ T cell differentiation.** After recognizing an antigen presented by antigen-presenting cells (APC), naïve $T_H$ cells become activated and differentiate into effector ($T_H1$, $T_H2$, $T_H17$ cells) and regulatory (iTreg) subsets, depending on instructive signals. The master transcription factors of the associated $T_H$ cell subset are in parentheses. Cytokines, produced by these cells, are listed (*italic*).

### *3.3.1 $T_H$1 differentiation*

$T_H$1 cells are essential for clearing intracellular bacteria and viruses, and their signature cytokine is Interferon gamma (IFNγ). $T_H$1 differentiation (Figure 2) is induced by signals from antigen-presenting cells (APC): IL-12, which is mainly produced by monocytes and dendritic cells and IFNγ, which is secreted by already differentiated $T_H$1 cells and by NK and NKT cells. IFNγ activates signal transducer and activator of transcription 1 (Stat1) via the IFNγ receptor (IFNγR). Together with the TCR-induced transcription factors (NFAT, NFκB and AP-1), these signals activate the transcription factor Tbox21 (T-bet). T-bet is a member of the T-box family of transcription factors and is considered to be the master regulator of $T_H$1 differentiation. Subsequently, T-bet induces the production of IFNγ and the activation of the transcription factor H2.0-like homeobox (Hlx) and Runx3. TCR-signaling represses the up-regulation of the IL12Rβ2 subunit in an NFAT-dependent manner (Afkarian et al., 2002). The termination of TCR-signaling finally allows the up-regulation of IL12Rβ2. As a consequence, Stat4 activation through IL-12 signaling together with T-bet, Hlx and Runx3 activate the *ifnγ* locus and thereby positively enhance Stat1 signaling (Schulz et al., 2009). The ability of IFNγ to stimulate T-bet expression and the ability of T-bet to enhance IFNγ transcription sets up a positive feedback loop which drives differentiation of T cells toward the $T_H$1 phenotype. The stability of the phenotype is further enhanced by the cooperation of Runx3 with T-bet in silencing of the *il4* gene in $T_H$1 cells by binding to the *il4* silencer and by binding to the *ifnγ* promoter to further promote IFNγ production (Djuretic et al., 2007; Naoe et al., 2007).

### *3.3.2 $T_H$2 differentiation*

$T_H$2 cells, essential in eliminating extracellular pathogens, including helminths, express IL-4, IL-5, IL-10, IL-13, and IL-25. The differentiation of antigen-stimulated T cells to the $T_H$2 subset (Figure 2) is dependent on IL-4 provided by mast cells, basophils, NKT cells, eosinophils or previously differentiated $T_H$2 cells. IL-4 signaling through the IL-4 receptor, which is expressed on naïve $CD4^+$ T cells, activates the transcription factor Stat6, which together with TCR signals induces the expression of Gata-binding protein 3 (Gata3) (Zheng and Flavell, 1997). Gata3 is a transcription factor that acts as a master regulator of $T_H$2 differentiation, enhancing expression of IL-4, IL-5 and IL-13, which are located in the same genetic locus. Gata3 induces the transcription of the long form of viral musculoaponeurotic fibrosarcoma oncogene homolog (c-MAF), which additionally helps to activate *il4* transcription (Kurata et al., 1999; Ouyang et al., 2000). This activation results in a strong autocrine feedback loop that activates *il4*, *il5* and *il13*. Furthermore, IL-4 appears to repress IL-12 signaling through inhibition of IL12Rβ2

expression, thus antagonizing $T_H1$ differentiation and stabilizing the $T_H2$ phenotype (Szabo et al., 1997).

### *3.3.3 $T_H17$ differentiation*

The $T_H17$ subset, determinant in fighting Gram negative bacteria, fungi, and some protozoa, secretes IL-17, IL-21 and IL-22 with strong proinflammatory effects. Transforming growth factor β1 (also called TGFβ) provided by dendritic and epithelial cells, is required for the generation of $T_H17$ cells and also Treg cells, a process that is dependent on co-stimulatory signals and occurs in a concentration-dependent manner (Veldhoen et al., 2006). At the same time TGFβ inhibits $T_H1$ and $T_H2$ differentiation. If the cytokine milieu additionally provides IL-6, which is mainly produced by monocytes, differentiation into the $T_H17$ lineage is induced (Bettelli et al., 2006). The IL6R activates Stat3, which subsequently induces the master transcription factor of $T_H17$ differentiation, the retinoid-related orphan receptors, RORγt and RORα (Ivanov et al., 2006; Yang et al., 2008b). However, full induction of RORγt is only achieved in the presence of TGFβ (Zhou et al., 2008). IL-6 has been implicated in the induction of *il21* expression, which is induced in a Stat3-dependent manner. IL-21, together with TGFβ, also induces IL-17 production and expression of RORγt (Wei et al., 2007). It has been suggested that IL-21 serves as an autocrine factor secreted by $T_H17$ cells that promotes or sustains $T_H17$ lineage commitment. RORγt expression has no impact on IL-21 production, as RORγt KO mice express normal levels of IL-21 (Zhou et al., 2007). In addition, IL-23 plays a critical role establishing inflammatory immunity and enhancing IL-17 production *in vivo*. At lower concentrations, together with IL-6 or IL-21, TGFβ is required for the initial induction of IL23R on naïve $CD4^+$ T cells, which renders $T_H17$ cells responsive to IL-23 and therefore promotes their maturation. IL-23 further up-regulates IL23R expression, by this imposing another positive feedback loop (Zhou et al., 2008).

Conversely, the $T_H1$ and $T_H2$ cytokines IFNγ and IL-4 both inhibit the induction of IL-17 (Harrington et al., 2005). Additionally, IL-27, a member of the heterodimeric IL-12 cytokine family, promotes $T_H1$ cell differentiation by inducing T-bet and IL12Rβ2 (Lucas et al., 2003; Takeda et al., 2003) and concurrently inhibits $T_H17$ cell differentiation in a Stat1-dependent manner (Batten et al., 2006; Diveu et al., 2009; Stumhofer et al., 2007). The genetic loss of T-bet strongly favors IL-17 expression in $CD4^+$ T cells.

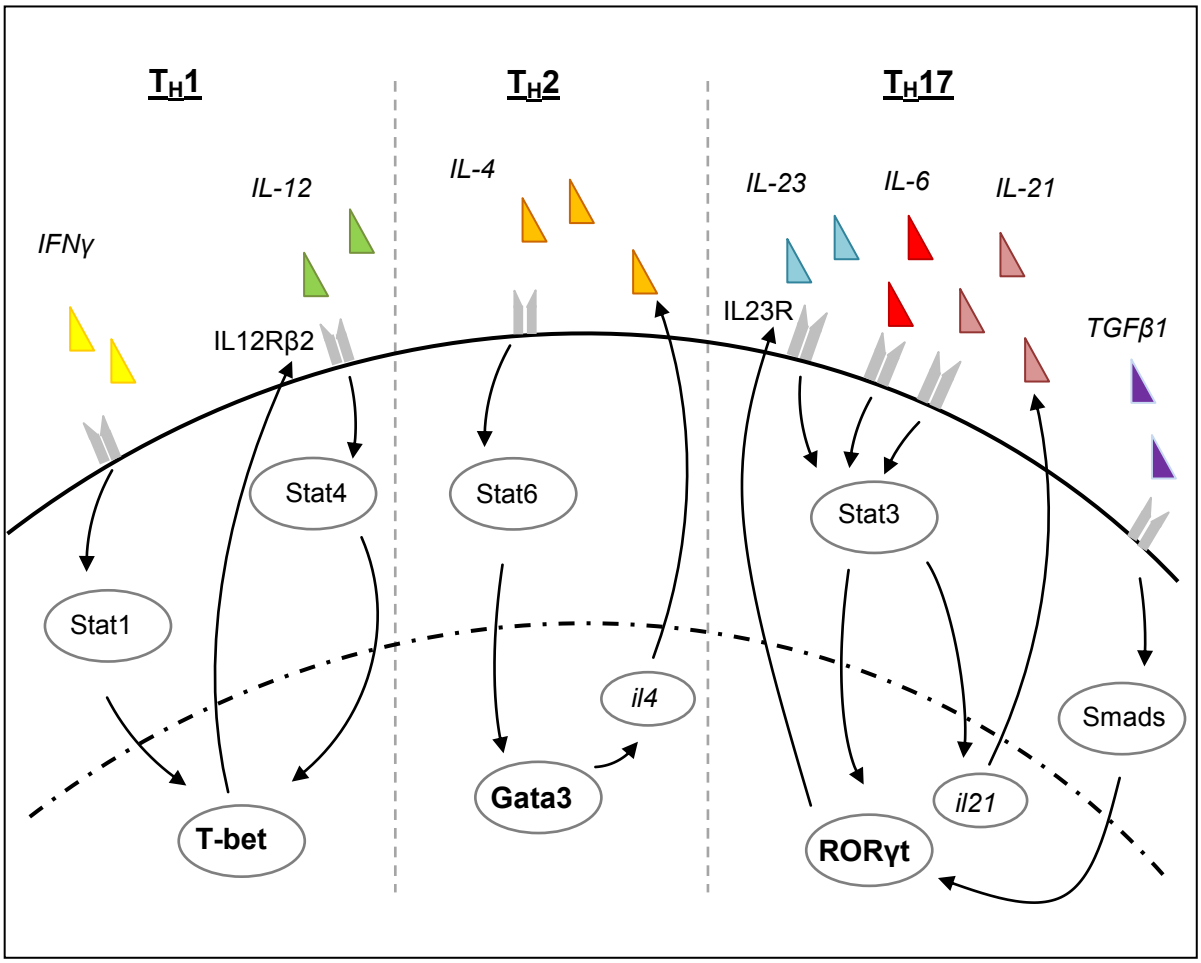


**Figure 2. $T_H$ cell differentiation.** $T_H1$ cells are induced when activated in the presence of IFNγ and IL-12. IFNγ activates Stat1, which induces T-bet. T-bet induces transcription of IL12Rβ2, which allows for Stat4 activation and further induction of T-bet. $T_H2$ cells are induced through IL-4 signaling. Stat6 induces the expression of Gata3 which in turn leads to the transcription of *il4*. IL-6, IL-21 and TGFβ signaling leads to the induction of RORγt. RORγt upregulates IL23R, thus rendering the cell susceptible to IL-23 signaling.

## 3.4 Epigenetic control of $T_H$ differentiation

Several factors contribute to the ability of transcription factors to bind to the DNA, including their concentration, post-translational modifications as well as their subcellular localization, and the state of chromatin and DNA. The latter implies the position and aggregation of nucleosomes, post-translational histone modifications and the methylation status of the DNA. Although the DNA sequence remains unchanged, epigenetic modifications can be heritable, but plastic as the potential for change in response to altered environmental signals is retained.

DNA can be methylated at cytosines in cytosine-phosphate-guanine (CpG) dinucleotides which is the only proven mechanism by which epigenetic information is faithfully propagated from one cell to the next. Methylation of cytosines at gene promoters and at distal regulatory elements can directly inhibit transcription by blocking the binding of transcription factors and regulatory elements and indirectly by generating binding sites for methyl-CpG-binding domain proteins (MBDs). Thereby, recruitment of RNA polymerase II is inhibited.

Each of the core histones contains a 20-35 amino acid long N-terminal tail rich in basic amino acids protruding from the nucleosome. These tails can be post-translationally modified by addition or removal of acetyl, methyl, phosphate, ubiquitin, sumoyl or ADP-ribose groups. Acetylation changes the charge of the histone, thereby reducing the interactions between histones and DNA and enhancing nucleosome mobility. The modifications can also create or remove binding sites for regulatory proteins that enable or restrain transcription. The presence of histones H3 and H4 that are acetylated and H3 lysine 4 (H3K4) modified by one (monomethylated H3K4), two (dimethylated H3K4) or three (trimethylated H3K4) methyl groups are typical characteristics of promoters and enhancers of active or recently transcribed genes. In silent genes these modifications are absent, whereas dimethylated and trimethylated H3K27 and trimethylated H3K9 are present. Interestingly, promoters of genes that are poised to be either activated or silenced either do not have any of these histone modifications or have a bivalent modification pattern – meaning, they have both permissive and repressive histone modifications.

Numerous studies have shown the relevance of epigenetic modifications in $T_H$ cell differentiation and the significance for imprinting cytokine genes for memory expression. When T cell lines were treated with 5-azacytidine, an inhibitor of DNA methylation, this resulted in the production of IL-2 and IFNγ (Ballas, 1984; Young et al., 1994), although the cells were formerly not producing these cytokines. It was also shown that treatment of $CD4^+$ T cells with inhibitors of histone deacetylases (HDACs) augmented the expression of both IFNγ and $T_H2$ type cytokines. Thus, DNA methylation and histone deacetylation dampen the expression $T_H1$ and $T_H2$ cytokines and help to restrict cytokine expression to the appropriate lineage. Information on how regulatory mechanisms and epigenetic processes control $T_H17$ differentiation and memory is so far very limited.

## 3.5 $T_H17$ cells in EAE

Experimental autoimmune encephalomyelitis (EAE) is a $CD4^+$ T cell-mediated demyelinating disease of the central nervous system that is frequently used as a model for the human disease multiple sclerosis (MS; (Sospedra and Martin, 2005)). EAE can be induced in susceptible mice by adoptive transfer of myelin-reactive $CD4^+$ T cells or by immunization with myelin antigens. The course of EAE can be subdivided into an initiation stage involving activation and expansion of myelin-specific T cells in the periphery, which then cross the blood brain barrier (BBB), an effector stage involving re-activation of myelin-specific T cells in the central nervous system, resulting in cytokine-induced chemokine expression in the central nervous system-resident cells and a stage of remission and repair in which the immune response is down-regulated (McFarland and Martin, 2007; Steinman, 2001). Dysregulated $T_H1$ responses have been associated with organ-specific immunity and have been shown to play a critical role in the initiation of inflammatory responses in the central nervous system (Agrawal et al., 2006; Bettelli et al., 2004; Yang et al., 2009). IFNγ expression in the target tissues correlates with clinical signs in EAE. It has been widely accepted that dysregulated IFNγ-producing $T_H1$ cells are pathogenic in EAE, while $T_H2$ cells are thought to be protective (Butti et al., 2008; Kleinschek et al., 2007; Ramirez and Mason, 2000). T-bet- and Stat4-deficient mice are resistant to EAE, and targeting IL-12 with polyclonal antibodies to IL-12 turned out to be an efficient therapy for EAE. However, the $T_H1$ paradigm was put into doubt when it was discovered that mice lacking IL-12p35, IL12Rβ2 or IFNγ were more susceptible to EAE, whereas IL-12p40-deficient mice were resistant to disease (Ferber et al., 1996; Gran et al., 2002; Krakowski and Owens, 1996; Zhang et al., 2003). In 2000 a novel cytokine chain, p19, was discovered (Oppmann et al., 2000). This p19 chain forms heterodimers with the p40 chain of IL-12, together forming a cytokine named IL-23. Thus, all approaches that targeted the p40 chain of IL-12 would affect both IL-12 and IL-23. The discovery of IL-23, and later of $T_H17$ cells, filled an important gap in the understanding of EAE pathogenesis and autoimmunity. By creating IL-23p19 deficient mice and comparing them with IL-12p35 deficient mice, it was demonstrated that IL-23 and not IL-12 was crucial for the induction of EAE (Cua et al., 2003). EAE can be induced in IL-23p19 deficient mice when exogenous IL-23 is delivered into the central nervous system (Cua et al., 2003). Investigating the mechanism underlying the essential role of IL-23 revealed that autoreactive $CD4^+$ T cells producing IL-17 were not induced in IL-23 deficient mice in EAE. $T_H1$ and $T_H17$ cells have been shown in several studies to independently induce EAE with different, albeit overlapping pathological features

(Jager et al., 2009; Kroenke et al., 2008; Lees et al., 2008a; Lees et al., 2008b; Park et al., 2005). $T_H17$ cells are recognized as an important mediator of tissue damage seen in EAE (Axtell et al., 2010; Gyulveszi et al., 2009; Yang et al., 2009). EAE is significantly suppressed in IL-17- and IL-17 receptor-deficient mice and inhibition of IL-17 attenuates inflammation, indicating that IL-17-mediated signaling plays an important role in the effector stage of EAE (Fitzgerald et al., 2007; Gonzalez-Garcia et al., 2009; Komiyama et al., 2006). Recently, the chemokine receptor CCR6, which is highly expressed on $T_H17$ cells, has been implicated in development of EAE (Reboldi et al., 2009; Yamazaki et al., 2008). $CCR6^{-/-}$ mice were resistant to EAE, which was associated with a reduced ability of $T_H1$ and $T_H17$ cells to infiltrate the central nervous system despite normal polarization of both subsets in draining lymph nodes. Inflammation was triggered by a CCR6-dependent infiltration of $T_H17$ cells, followed by a second wave of both $T_H1$ and $T_H17$ cells in a CCR6-independent manner (Reboldi et al., 2009). These data indicate that $CCR6^+$ $T_H17$ cells are crucial in the early phase of disease.

### *3.5.1 Function of IL-17 in EAE*

Although the precise mechanism by which IL-17 participates in EAE is unknown, many functions of how IL-17 induces inflammation have been deciphered. A major function of IL-17 involves coordination of local tissue inflammation through up-regulation of proinflammatory and neutrophil-mobilizing cytokines and chemokines such as IL-6, IL-1, TNFα, G-CSF, CXCL1, CCL2, CXCL2, CCL7, CCL20 and matrix metalloproteases (MMPs), which allow activated T cells to cross the extracellular matrix (Agarwal et al., 2008; Awane et al., 1999; Huang et al., 2007; Jovanovic et al., 1998). IL-17 signals through a heterodimeric receptor complex consisting of IL-17RA and IL-17RC, which are transmembrane proteins expressed on a variety of cells such as astrocytes and microglia (Inoue et al., 2006; Kolls and Linden, 2004; Trajkovic et al., 2001). Act1 has been described as a key component in the signaling of IL-17 (Qian et al., 2007). In a recent study, by using cell-type specific deletion of Act1, it was shown that Act1 deficiency in the central nervous system-resident cells originated from neuroectodermal cells (neurons, oligodendrocytes and astrocytes) delayed the onset and reduced the severity of EAE (Kang et al., 2010).

## 3.6 Project objectives and experimental design

The regulation of IL-17 memory in the newly discovered $T_H17$ cells has so far not been sufficiently investigated. Knowledge about the plasticity of cytokine memory is crucial for the development of new therapies against diseases involving repeatedly activated

$CD4^+$ T cells. These diseases include many autoimmune and allergic inflammatory disorders and are associated with the presence of different subsets of $T_H$ cells that could have a significant influence on these disorders. If $T_H$ cells responses are plastic, it might be possible to reprogram these cells therapeutically. For example in MS it could be of advantage to shift the balance from destructive $T_H17$ and/or $T_H1$ cells towards more benign $T_H2$ cells.

The aim of this work was to 1) analyze the susceptibility of $T_H1$ and $T_H2$ cells to $T_H17$ inducing signals, 2) analyze the susceptibility of $T_H17$ cells to $T_H1$ and $T_H2$ inducing signals and to 3) analyze $T_H17$ cells, which had been generated *in vivo*. In order to investigate the stability of IL-17 expression in $T_H17$ cells, an IL-17 secretion assay was developed in cooperation with Miltenyi Biotec. With this assay it was possible to analyze the plasticity of IL-17 producing cells without the interference of potentially uncommitted T cells from *in vitro* cultures. Also, *in vivo* generated IL-17 producing cells could be isolated and studied in *in vitro* cultures under various conditions. The main focus was directed at the relationship between $T_H17$ and $T_H1$ cells, since $CD4^+$ T cells producing both IFNγ and IL-17 have been observed *in vivo* in both mice and humans.

# 4 Material and methods

## 4.1 Cell Biology

### *4.1.1 Mice and cells*

BALB/c and OVA-TCR transgenic DO11.10 mice, IFNγR-deficient and C57Bl/6 mice were ordered from “Charles River Laboratories” or bred under specific pathogen free (SPF) conditions at the Bundesinstitut für Risikobewertung in Marienfelde in Berlin. T-Bet-deficient mice were a kind gift from J. Penninger (Vienna, Austria). Unless stated otherwise, 6-12 week old mice were used.

### *4.1.2 Cell culture media and buffers*

Murine lymphocytes were cultured in RPMI supplemented with 10% fetal calf serum (Sigma Chemicals, St. Louis, MO), 100 U/ml penicillin, 0,1 mg/ml streptomycin, 0,3 mg/ml glutamine (Invitrogen) and 10 µM β-mercaptoethanol at 37°C in 5% $CO_2$. Unless indicated differently, lymphocytes were in phosphate-buffered saline (PBS) supplemented with 0,5% (w/v) bovine serum albumin (PBS/BSA) during cell-sorting and -isolation.

### *4.1.3 Preparation of a single-cell suspension*

All mice were sacrificed by cervical dislocation. Spleen, peripheral and mesenteric lymph nodes were isolated and pressed through a cell strainer with a pore size of 70 µm (BD Biosciences) and transferred into PBS/BSA.

### *4.1.4 Magnetic cells sorting (MACS)*

Specific cell subsets were isolated by using MACS (high gradient magnetic cell sorting) technology (Miltenyi Biotech, Bergisch-Gladbach, Germany). Cells were specifically labeled with monoclonal antibodies conjugated to superparamagnetic particles and loaded onto a MACS column. The MACS column is surrounded by a magnetic field generated by a permanent magnet. Cells labeled with MACS MicroBeads are retained in the MACS Column, whereas unlabeled cells pass through the column and are depleted. When the MACS column is removed from the magnetic field, the labeled cells can be eluted.

### *4.1.5 Flow cytometric analysis and fluorescent activated cell sorting (FACS)*

Flow cytometry is used to analyze cells, by suspending them in a stream of fluid and passing them by an electronic detection apparatus. A laser light is directed onto the hydrodynamically-focused stream of fluid, where the cells pass by one-by-one. Two detectors detect the light which is scattered by the cell. Light scattered at a slight angle (3-10°) is called forward scatter (FCS) and at a 90° angle, side scatter (SSC). The forward scatter correlates with the size of the cell, whereas the side scatter depends on the granularity of the cell. With these two parameters it is possible to differentiate between for example lymphocytes and granulocytes due to their cell size. Here, a FACSCalibur (BD Biosciences, Heidelberg, Germany) was used, which has four additional detectors (FL1, 530 nm; FL2, 585 nm; FL3, 650 nm; FL4, 670 nm. These detectors are able to measure the light emitted from various fluorescent dyes excited by an argon-laser (488 nm) and a diode-laser (635 nm). A FACSCanto II was also used, which has three lasers (405 nm, 488 nm, 633 nm), and allows the use of six different dyes at the same time.

In order to characterize different lymphocyte subsets, antibodies coupled to fluorescent dyes were used, such as fluorescein isothiocyanate (FITC), AlexaFluor 488 and 405, phycoerythrin (PE), Cy5, Allo-Phyco-Cyanin (APC) and phycoerythrin-Cy5 (PE-Cy5). The data was analyzed using Cellquest software or FlowJo (BD Biosciences).

Different cell populations can also be sorted based on their fluorescent characteristics by fluorescent activated cell sorting. After passing the cells through the laser and acquisition of the fluorescent characteristics, the liquid stream of cells is disrupted into droplets containing only one cell. The cells are given an electrical charge based on their individual fluorescent characteristics and are then deflected into different tubes. In order to sort different cells subsets a FACSAria and a FACSDiva (BD Biosciences) was employed.

### *4.1.6 Antibodies used for flow cytometric analysis and cell culture*

Anti-IL-4 (11B11), anti-IL-12 (C17.18), anti-IFNγ (AN17.18.24) antibodies purified from hybridoma supernatants at the Deutsches Rheuma-Forschungszentrum (DRFZ Berlin, Germany) were used at 20 µg/ml final concentration. FITC-conjugated anti-CD4 (GK1.5, Miltenyi Biotec), Alexa Flour 405-conjugated anti-CD4 (GK1.5, purified and coupled at the DRFZ), PE-conjugated anti-CD62L antibody (clone MEL14, purified and coupled at the DRFZ) and PE-conjugated anti-IL12Rb2 antibody (Clone 305719, R&D Systems) were used for surface staining. FITC-conjugated anti-IL-17 (eBio17B7; eBioscience) Alexa Flour 647-conjugated anti-T-bet (eBio4B10, eBioscience), PE-

conjugated anti-RORγt (AFKJS-9, eBioscience) and PE-Cy7 conjugated anti-IFNγ (BD Biosciences) were used for intracellular stainings. For staining of pStat4 and pStat1, we used an Alexa Fluor 647-conjugated anti-pStat4 (pY693) and anti-pStat1 (pY701) antibody, respectively (both from BD Biosciences).

### *4.1.7 Isolation of CD4$^+$ and CD4$^+$CD62L$^+$ $T_H$ lymphocytes*

Murine CD4$^+$ $T_H$ lymphocytes were isolated by positive selection using anti-CD4 microbeads (Miltenyi Biotec). The single-cell suspension was adjusted to $10^7$ cells/90 µl in PBS/BSA and 10µl/$10^7$ cells of the anti-CD4 microbeads were added and incubated for 20 minutes at 4°C. The cells were washed in PBS/BSA to remove unbound microbeads and loaded onto a LS column (Miltenyi Biotec) which had previously been equilibrated with PBS/BSA. The LS column was situated in a magnetic field (MidiMACS, Miltenyi Biotec) where the labeled cells retain, whereas the unlabeled cells are caught in the flowthrough. CD4$^+$ cells are eluted with PBS/BSA from the column by removing it from the magnetic field. Purity of the subsets was controlled using a FACSCalibur after staining the cells with anti-CD4 FITC (Miltenyi Biotec) for 10 minutes. When the purity was below 97%, the cells were loaded onto an additional LS column.

Naïve $T_H$ lymphocytes were sorted according to their expression of CD4 and CD62L. From spleen and lymph nodes a single-cell suspension was prepared. The cells were stained with anti-CD4 FITC (Miltenyi Biotec) by incubating the cells for 10 minutes at 4-8°C. In order to remove unbound antibodies, the cells were washed with PBS/BSA. The cell-pellet was resuspended in PBS/BSA and incubated with anti-FITC multisort beads (Miltenyi Biotec) for 20 minutes at 4-8°C. Unbound multisort beads were removed by washing the cells with PBS/BSA. Labeled and unlabeled cells were isolated using an LS column. To the purified CD4+ cells 20µl/1ml multisort release reagent (Miltenyi Biotec) was added and incubated at 4-8°C for 30 minutes. The protease contained in this reagent enables the cleavage of the multisort beads from the cells. The cells were loaded onto a LS column. In this process the beads remain in the magnetic field inside to column, whereas the cells are in the flowthrough. The CD4$^+$ cells were adjusted to $10^7$ cells/100 µl with PBS/BSA. Anti-CD62L microbeads (Miltenyi Biotec) was added in the ratio of 1:25 and incubated for 20 minutes at 4-8°C. Unbound microbeads were removed by washing the cells with PBS/BSA. The cells were separated on a LS column and purity was controlled by surface staining with a PE-conjugated anti-CD62L antibody.

### *4.1.8 Cell culture*

$CD4^+$ or $CD4^+CD62L^+$ cells of at least 97% purity were mixed with antigen-presenting cells (APC) in a ratio of 1:5. Irradiated (30 Gy) $CD4^-$ or freshly prepared splenocytes from BALB/c mice were used as APC. The cultures were set up at $2x10^6$ cells/ml. All cultures were done in complete RPMI at 37°C in 5% $CO_2$. $T_H$ cells from TCR transgenic DO11.10 mice were stimulated with the cognate peptide $OVA_{323-339}$ (R. Volkmer-Engert, Humboldt University of Berlin, Berlin, Germany) at 0,5 µM in the presence of APC, whereas $T_H$ from Balb/c mice were stimulated with 1 µg/ml soluble or 3 µg/ml plate-bound anti-CD3 and 1 µg/ml soluble anti-CD28 (BD Biosciences). For $T_H1$ differentiation, the $T_H$ cells were stimulated in the presence of 5 ng/ml recombinant murine IL-12 (R&D Systems) and 20 µg/ml anti-IL-4 antibody (11B11). For $T_H2$ differentiation, the cells were stimulated in the presence of murine IL-4 (100 ng/ml; culture supernatant of HEK293T cells transfected with an expression plasmid encoding murine IL-4), 20 µg/ml anti-IL-12 antibody (C17.8.6) and 20 µg/ml anti-IFNγ antibody (AN18.17.24). $T_H17$ cells were induced by stimulating the cells in the presence of 1 ng/ml TGFβ1, 20 ng/ml IL-6, 20 ng/ml IL-23 (all from R&D Systems), 20 µg/ml anti-IL-4 antibody and 20 µg/ml anti-IFNγ antibody. Dead cells were removed by density gradient centrifugation (Ficoll-Histopaque). Every 6 days viable $T_H$ cells were harvested and re-stimulated under the original conditions, except that 10 ng/ml murine IL-2 (R&D Systems) was added to the $T_H1$ and $T_H2$ cultures.$T_H$ cells stimulated with anti-CD3 and anti-CD28 were harvested every 5 days.

### *4.1.9 Mitogenic re-stimulation and intracellular cytokine staining*

Murine $T_H$ cells were re-stimulated with 10 ng/ml phorbol 12-myristate 13-acetate (PMA) and 1 µg/ml ionomycin (Sigma chemicals) in complete RPMI medium.

Prior to intracellular staining of cytokines, the cells were stimulated for 1 h with PMA/ionomycin and additional 3 h with 5 µg/ml of brefeldin A blocking the secretion of cytokines. Cells were washed twice in PBS and fixed with 2% formaldehyde in PBS for 15 min. The fixation was stopped by washing with PBS, and the cells were transferred into PBS/BSA. For intracellular staining, the cells were permeabilized with 0,5% (w/v) saponin (Sigma) in PBS/BSA supplemented with staining antibodies. After 15 min on ice the cells were washed once with saponin buffer, transferred into PBS/BSA and analyzed by flow cytometry.

For intracellular T-bet and RORγt staining, the Foxp3 staining buffer set (eBioscience) was used according to the manufacturer's instructions. Intracellular IL-17 and IFNγ in murine cells were stained under the same conditions for co-staining of transcription

factors and cytokines. For pStat4 and pStat1 staining, Phosflow Lyse/Perm buffer and Perm Buffer III (BD Biosciences) were used according to the manufacturer's instruction. The cells were stimulated with IL-12 (10 ng/ml) for 30 minutes for pStat4 and with IFNγ (10 ng/ml) for 15 minutes for pStat1 staining. IL12Rβ2 was stained according to the manufactor`s instructions.

#### *4.1.10 Isolation of viable cytokine-secreting $T_H$ cells*

Cells were cultured under IL-17 inducing conditions for 6 days, or $CD4^+CD62L^{lo}$ cells were isolated from spleen of 6-months old ex breeder DO11.10 and Balb/c mice. Cells were harvested and re-stimulated with 10 ng/ml PMA and 1 µg/ml ionomycin for 1.5 hours. The cells were washed twice in ice-cold PBS with 0.5% w/v BSA (PBS/BSA). Cells were labeled for 5 min at 4°C with an IL-17-specific high-affinity capture matrix, i.e., bi-specific Ab-Ab conjugates of an anti-CD45 antibody with an anti-IL-17 antibody (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Cell samples were taken for low control (kept on ice) and high control (incubated with recombinant IL-17 (0,5 µg/ml); Peprotech, Hamburg, Germany), washed after 10 minutes and kept on ice. The rest of the cells were transferred into 37°C warm RPMI medium at a low density ($10^5$ cells/ml) and placed at 37°C. Every 5 minutes the cells were mixed gently. After 30 minutes, the cells were transferred into ice-cold PBS/BSA and kept on ice for 10 minutes. The captured IL-17 was detected with an anti-IL-17 biotin conjugated antibody followed by staining with an APC-conjugated anti-biotin antibody (Miltenyi Biotec) (Figure 3). The IL-17 producing cells and the IL-17 non-producing cells were separated by FACSAria™ cell sorter (BD Biosciences). After sorting, the purity of the sort was confirmed with a FACSCalibur (BD Biosciences). Specificity of the IL-17 secretion assay was confirmed by intracellular staining.

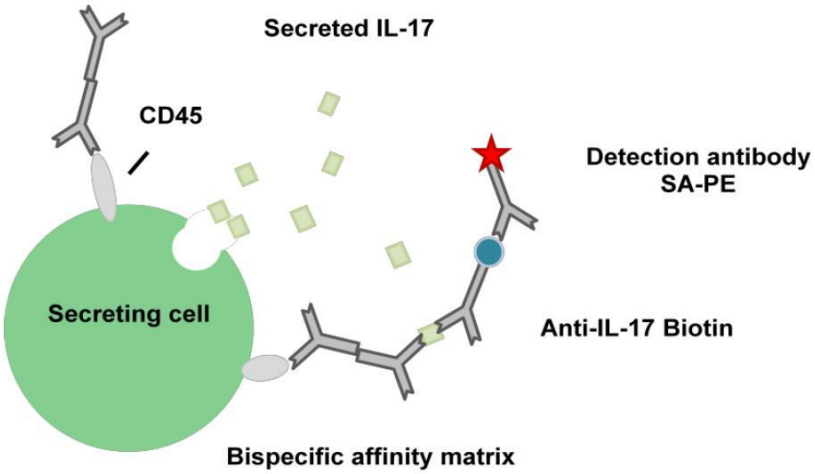


**Figure 3. The principle of the cytokine secretion assay.** A bispecific affinity matrix, the catch reagent, attaches to CD45, which is expressed by all leukocytes. The secreted IL-17 binds to the cytokine catch matrix. Anti-IL-17 Biotin binds to IL-17, and can subsequently be detected by Streptavidin (SA)-PE.

## 4.2 Murine inflammation model

### *4.2.1 Experimental Autoimmune Encephalomyelitis*

Experimental Autoimmune Encephalomyelitis (EAE) is an established murine model of multiple sclerosis (MS) and can be induced in susceptible mouse strains either by immunization with spinal cord homogenate and isolated myelin proteins/peptides (active EAE), or by adoptively transferring lymphocytes pre-activated with the above proteins (passive EAE) into naïve mice. Pre-existing autoreactive T cells are in both protocols activated under pro-inflammatory conditions, they expand and target the central nervous system causing paralysis and ataxia. Here, a protocol for active EAE was applied.

Active EAE was induced in 8- to 12-week-old C57Bl/6 mice by subcutaneous injection of 200 µg $MOG_{35-55}$ peptide (Brustle et al., 2007; Nogai et al., 2005) (mevgwyrspfsrvvhlyrngk; synthesized by Dr. R. Volkmer, Charité, Berlin, Germany) emulsified in complete Freund's adjuvant (containing 1mg/ml *M. tuberculosis* H37RA; Sigma) together with i.v. administration of 200 ng pertussis toxin (Sigma) on days 0 and 2. The additional pro-inflammatory signals through administration of pertussis toxin enhance the inflammatory response; facilitating the invasion of the central nervous system with encephalitogenic MOG-specific $T_H$ cells and with it increases the disease severity. Disease severity was assigned scores daily on a scale of 0–5 as follows: 0, no paralysis; 1, limp tail; 2, limp tail and partial hindleg paralysis; 3, complete hindleg paralysis; 4, tetraparesis; and 5, moribund. Cells were isolated from the spleen of mice at day 7 after immunization. The experiments were performed in collaboration with the group of Thomas Kamradt (Friedrich Schiller University Jena, Germany).

## 4.3 Molecular Biology

### *4.3.1 Real-time PCR*

Real-time PCR was used to quantify the gene expression of selected genes. The cells were lysed, their mRNA was isolated and reverse transcribed into cDNA. In the subsequent PCR with primers specific for the gene of interest (Table 1), the fluorescent compound SYBR green was used to quantify the synthezised DNA. SYBR green intercalates into double stranded DNA. The emitted fluorescence, which is quantified once per amplification cycle, is proportional to the amount of PCR product. The number of cycles that is required until the fluorescence exceeds the threshold (crossing point, cp) is a measure for the starting amount of template DNA.

Total RNA was prepared using NucleoSpin RNA II (Macherey-Nagel) kit. Reverse transcription (TaqMan Reverse Transcription Reagent, Applied Biosystems) was performed in a conventional thermocycler (10 min at 25°C, 40 min at 48°C, and 5 min at 95°C) with 100-500 ng of total RNA and a 1:1 mixture of oligo(dT) and random hexamer primers. Real-time PCR was performed with the LightCycler instrument using the FastStart DNA Master SYBR Green I kit (Roche Diagnostics) and the following cycle conditions: 9 min at 95°C, followed by 45 cycles of 15 sec at 95°C, 15 sec at 60-65°C, 15 sec at 72°C. For the normalization of murine cDNA, the transcripts of the housekeeping genes hypoxanthine guanine phosphoribosyl transferase (HPRT) were quantified. Data were evaluated using Lightcycler software. Relative expression was calculated as follows: $E_t^{\Delta Cp \text{ target gene (reference - sample)}} / E_h^{\Delta Cp \text{ housekeeping gene (reference - sample)}}$. E represents the reaction efficiency. E is calculated after serial dilution of template DNA and plotting the log of amount of template against the cp values as follows: $E = 10^{-1/slope}$.

Primer sequences:

| | |
|---|---|
| HPRT forward | GCTGGTGAAAAGGACCTCT |
| HPRT reverse | CACAGGACTAGAACACCTGC |
| RORγt forward | TGCAAGACTCATCGACAAGG |
| RORγt reverse | AGGGGATTCAACATCAGTGC |
| IL17 forward | TCCAGAAGGCCCTCAGACTA |
| IL17 reverse | AGCATCTTCTCGACCCTGAA |
| IL17F forward | CAAAACCAGGGCATTTCTGT |
| IL17F reverse | ATGGTGCTGTCTTCCTGACC |
| IL22 forward | GTCAACCGCACCTTTATGCT |
| IL22 reverse | CATGTAGGGCTGGAACCTGT |

| | |
|---|---|
| IL21 forward | ATCCTGAACTTCTATCAGCTCCAC |
| IL21 reverse | GCATTTAGCTATGTGCTTCTGTTTC |
| IL21R forward | TGTCAATGTGACGGACCAGT |
| IL21R reverse | CACGTAGTTGGAGGGTTCGT |
| RORα forward | CCCCTACTGTTCCTTCACCA |
| RORα reverse | TGCCACATCACCTCTCT |
| IL23R forward | AACATGACATGCACCTGGAA |
| IL23R reverse | TCCATGCCTAGGGAATTGAC |
| Gata3 forward | CCTACCGGGTTCGGATGTAAGT |
| Gata3 reverse | AGTTCGCGCAGGATGTCC |
| T-bet forward | TCCTGCAGTCTCTCCACAAGT |
| T-bet reverse | CAGCTGAGTGATCTCTGCGT |
| IL12Rβ2 forward | CTGATCCTCCATTACAGAA |
| IL12Rβ2 reverse | CGGAAGTAACGAATTGAGAA |
| IFNγR2 forward | CCGAGTGAAGTACTGGTTTC |
| IFNγR2 reverse | GTGTTTGGAGCACATCATC |

**Table 1.** Real-time PCR Primer sequences for the genes of interest.

### *4.3.2 Microarray analysis*

Oligonucleotide microarrays (GeneChip™, Affymetrix) were used to analyze the gene expression in *in vitro* and *in vivo* generated IL-17$^+$ $T_H$ cells on a genome-wide scale. Those arrays contain spots of short oligonucleotides representative of the coding regions of all genes. The RNA of the cells of interest is isolated, labeled and incubated with the array. Complementary sequences hybridize and the amount of hybridized RNA is later quantified by fluorescence detection after washing and staining.

Total RNA was extracted using NucleoSpin RNA II (Macherey-Nagel). RNA concentration, purity and integrity were assessed with the Agilent 2100 Bioanalyzer and the RNA 6000 Nano LabChip. 300 ng of total RNA was reverse-transcribed, followed by cDNA extraction using a PhaseLock gel (Eppendorf) and precipitation with ethanol and ammonium acetate. Biotinylated cRNA was *in vitro* transcribed using the MEGAscript high yield transcription kit (Ambion) according to the manufacturer's recommendations. Biotinylated cRNA was fragmented, and the hybridization cocktail was prepared according to Affymetrix protocols (15 µg fragmented biotin-labeled cRNA spiked with Eukaryotic Hybridization control). The Murine Genome 430A version 2 GeneChip™ arrays (Affymetrix) was loaded with the hybridization cocktail, hybridized at 45°C for 16 h in a rotisserie motor, washed and stained with streptavidin-phycoerythrin using the Affymetrix GeneChip Fluidics Workstation 400. Arrays were scanned on a Hewlett-Packard Gene Array Scanner (MGU74Av2 arrays) or on an Affymetrix GeneChip Scanner 3000 (MG430Av2 arrays). Data were analyzed using the

Microarray Suite 5.0 software (Affymetrix). Microarrays were globally normalized and scaled to a trimmed mean expression value of 200. Quality checks were performed according to the manufacturer's recommendations. All arrays were compared to each other, and a relational database was generated using Microsoft Access software and including the following parameters: expression heights, call for presence of transcripts, p value for presence or absence of transcripts, $\log_2$ value of fold change and 95% confidence intervals, call for the significance of differentially expression and the p value for that call. For each transcript the significance of differential expression between the groups of arrays was calculated using strict Bonferroni corrected Welch t-tests. Significantly differentially expressed genes were filtered according to the following criteria: mean fold change >= 2 or 1,5; difference of means >= 200; p-value <= 0,05 and excluding immunoglobulin genes. The microarrays were hybridized at DRFZ, and the relational data base for data analysis was created by Joachim Gruen (DRFZ, Berlin, Germany).

### *4.3.3 Bisulfite-based cytosine methylation analysis*

In order to analyze how genes are silenced in the investigated $T_H$ cell subsets, DNA methylation in the IL-17 and RORγt promoter was determined. Only regions conserved between mouse and human as determined by mVista and containing high numbers of CpGs were considered.

DNA of the samples to be analyzed was isolated using a DNeasy Blood and Tissue Kit (Qiagen) and the bisulfite conversion was carried out using EpiTect bisulfite kit (Qiagen). During the bisulfite conversion, sodium bisulfite converts unmethylated cytosine residues into uracil but does not affect 5-methylcytosine. Thus, bisulfite treatment introduces specific changes in the DNA sequence that depends on the methylation status of individual cytosine residues, which can be analyzed by sequencing of the DNA fragment. PCR primers specific for bisulfite modified DNA were designed and the sequence to be analyzed was amplified by nested PCR (Table 2). Nested PCR is a modification intended to reduce the contamination in products due to the amplification of unexpected primer binding sites. Nested polymerase chain reaction involves two sets of primers, used in two successive runs of polymerase chain reaction, the second set amplifying a secondary target within the first run product.

Primer sequences:

| **1st set of Primers:** | |
|---|---|
| IL17 5kb up forward | TATTTTTTTTTTTAAAATGTAGTT |
| IL17 5kb up reverse | ACCTCATAAAAACTAAAACTACTT |
| IL-17 promoter forward | AAGTATTTTTGTTTATCTTTTAA |
| IL-17 promoter reverse | AATACACTTATACCTCATATAAAA |
| RORγt 10kb up foreward | TTGTAATTTTAATGTTTTTATT |
| RORγt 10kb up reverse | TCCCAAAAAAACTATAATATC |
| RORγt 1kb up foreward | AAGTTATTTTATATTTTTATATTTTT |
| RORγt 1kb up reverse | AATATTAAATACCTCAATTCAACA |
| RORγt promoter foreward | GATTAGTAGTTTTGTTTTTAAAG |
| RORγt promoter reverse | CACAAATAACCAAATAACAAC |
| **2nd set of Primers:** | |
| IL17 5kb up forward | TGTGGTTGTTTAAGTTATGTTA |
| IL17 5kb up reverse | CTAAATAAATTCCTCACTAATC |
| IL-17 promoter forward | GAAGTTATGTTTTTTTGTATAGTG |
| IL-17 promoter reverse | AAAATAATACTCCTTTCTCTCTT |
| RORγt 10kb up foreward | GTTGTAATTTTAATGTTTTTATTTT |
| RORγt 10kb up reverse | CAATTAACAACAAAAAAAATCC |
| RORγt 1kb up foreward | TAGTTTTTTTGGGGTTAAGA |
| RORγt 1kb up reverse | TCCTCTACCCAAAATTTAAT |
| RORγt promoter foreward | ATAGAGGGTATTTGTTTGATG |
| RORγt promoter reverse | CATTAAATATAATAACAAACACC |

**Table 2.** Nested PCR primers.

The PCR products were purified from an agarose gel by using NucleoSpin extract II (Macherey-Nagel) and cloned into a pCR®2.1 vector by means of a TA cloning Kit (Invitrogen). The construct was then transformed into competent E.coli cells and the mixture was spread on LB agar plates containing 100 µg/ml ampicillin and X-Gal. The plates were incubated over night at 37°C. Next day 24 white colonies were picked and sequenced (GATC Biotec). The sequences were analyzed with BiQ Analyzer (Bock et al., 2005), a software tool for DNA methylation analysis. Sequences with a conversion rate below 90% or with a high error rate, were excluded from the analysis.

Potential transcription factor binding sites in the here analyzed regions were determined using MatInspector (Genomatix software) (Quandt et al., 1995).

# 5 Results

## 5.1 $T_H1$ and $T_H2$ cells cannot be converted into $T_H17$ cells

To what extend $T_H1$ and $T_H2$ cells could be converted into a $T_H17$ phenotype, identifiable through up-regulation of IL-17 and RORγt, was examined in an *in vitro* culture system. Naïve $CD4^+CD62L^+$ T cells from TCR transgenic DO11.10 mice were activated with their cognate antigen ($OVA_{323-339}$) and antigen-presenting cells (APC), differentiated into $T_H1$ cells with IL-12 and anti-IL-4 antibody, or into $T_H2$ cells with IL-4, anti-IL-12 and anti-IFNγ antibody. The APCs were γ-irradiated, still enabling them to initially present the antigen while precluding their survival. After 6 days the $T_H1$ and $T_H2$ cells were re-stimulated with antigen, this time however under $T_H17$ inducing conditions, i.e. in the presence of TGFβ, IL-6, IL-23, anti-IL-4 and anti-IFNγ antibody. 6 days later, the cells were re-stimulated with PMA/ionomycin, fixed after 4 hours, permeabilized and stained intracellularly for cytokine expression. Induction of IL-17 by TGFβ and IL-6 was not effective in either $T_H1$ (1.6% $IL\text{-}17^+$ cells) or $T_H2$ (4.7%) cells (Figure 4A and 4B). As has been shown in several publications, IFNγ inhibits $T_H17$ differentiation (Harrington et al., 2005). To exclude inhibition of $T_H17$ differentiation by IFNγ in established $T_H1$ cells, we also analyzed cells deficient for the IFNγ receptor (IFNγR-/-). Once the cells had been polarized into $T_H1$ cells, IL-17 expression was not effectively induced (4%) (Figure 5) as compared to naïve cells (25%) (Figure 4C). As shown by real-time PCR, T-bet was up-regulated even under $T_H17$-inducing conditions in $T_H1$ cells (Figure 4D). The transcription factors RORα and RORγt were up-regulated 2-fold and 6-fold, respectively, in $T_H1$ cells under $T_H17$-inducing conditions. In $T_H2$ cells Gata3 was down-regulated 2-3-fold when the cells had been re-stimulated under $T_H17$ inducing conditions and RORγt expression was up-regulated 5-fold. Expression of IL23R and RORα remained unchanged. From these results we concluded that *in vitro* generated $T_H1$ and $T_H2$ cells could not be converted into a $T_H17$ phenotype.

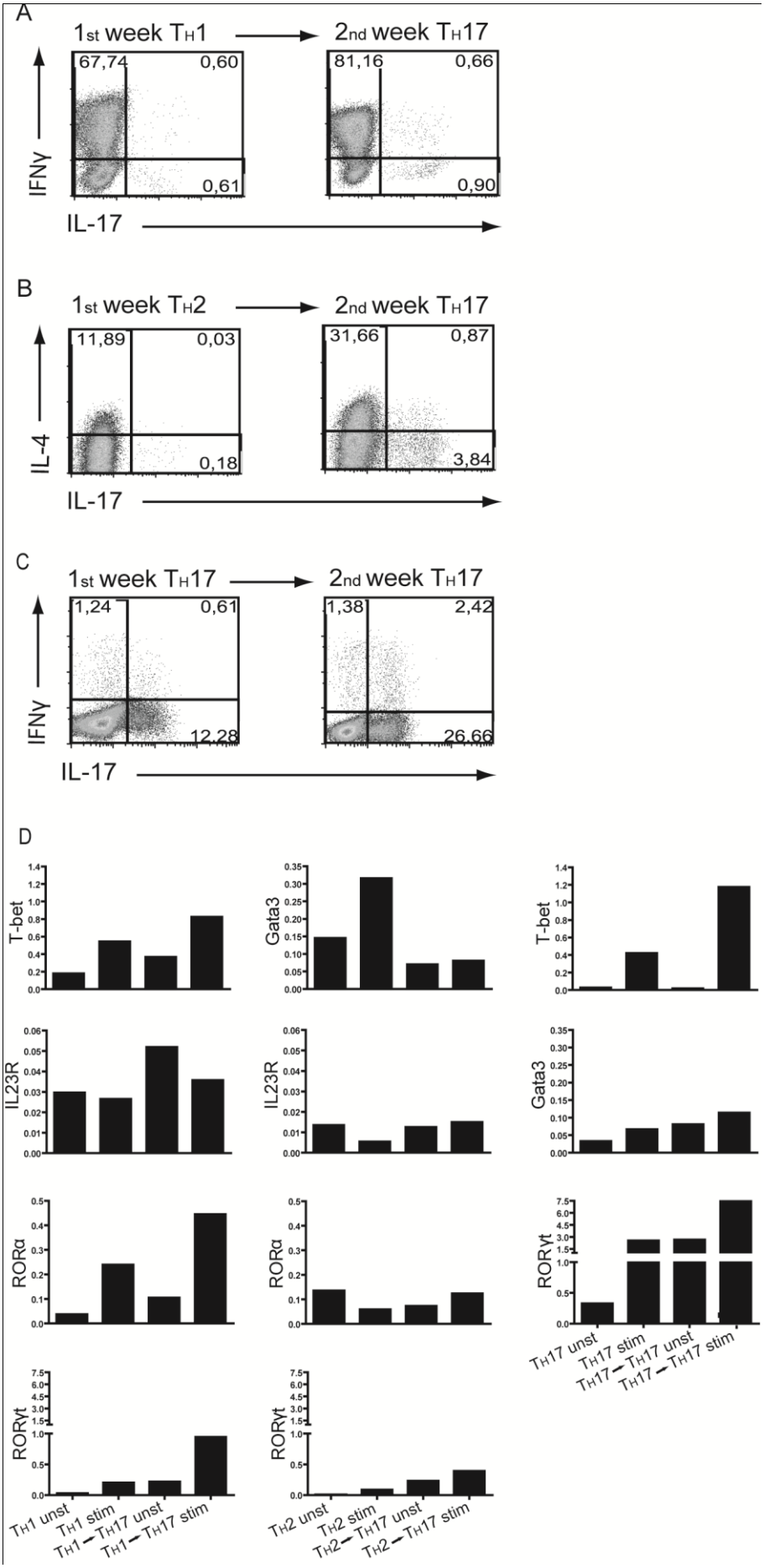
A
1st week TH1
2nd week TH17
67,74
0,60
0,61
81,16
0,66
0,90
IFNγ
IL-17
B
1st week TH2
2nd week TH17
11,89
0,03
0,18
31,66
0,87
3,84
IL-4
IL-17
C
1st week TH17
2nd week TH17
1,24
0,61
12,28
1,38
2,42
26,66
IFNγ
IL-17
D
T-bet
Gata3
T-bet
IL23R
IL23R
Gata3
RORα
RORα
RORγt
RORγt
RORγt
TH1 unst
TH1 stim
TH1→TH17 unst
TH1→TH17 stim
TH2 unst
TH2 stim
TH2→TH17 unst
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**Figure 4. $T_H1$ and $T_H2$ cells are refractory to $T_H17$ polarization.** Naïve $CD4^+CD62L^+$ cells from DO11.10 mice were stimulated with irradiated APC and $OVA_{323-339}$ and **A)** differentiated in the presence of IL-12 and anti-IL-4 antibody to $T_H1$, **B)** in the presence of IL-4, anti-IL-12 and anti-IFNγ antibody to $T_H2$ or **C)** in the presence of TGFβ, IL-6, IL-23, anti-IL-4 and anti-IFNγ antibody to $T_H17$ cells for 6 days. The cells were cultured for another 6 days in the presence of IL-17-inducing conditions (TGF-β, IL-6, IL-23, anti-IL-4 and anti-IFNγ antibody). IFNγ, IL-4 and IL-17 expression was analyzed after 5 hours of re-stimulation with PMA/Ionomycin by intracellular cytokine staining. Data are representative of 4 experiments. **D)** RNA was extracted from unstimulated (unst) cells and cells stimulated (stim) for 3 hours and quantitative real-time PCR was performed. Data are representative of 2 experiments.

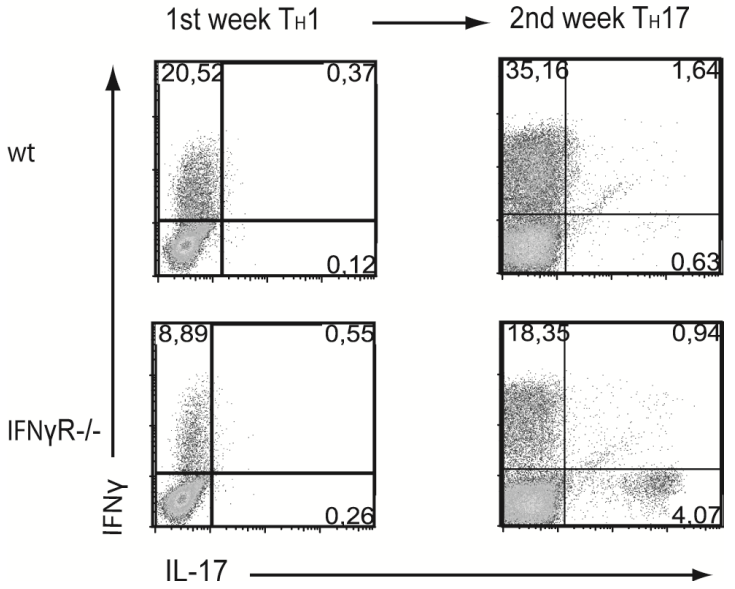


**Figure 5. *In vitro* generated IFNγR-/- $T_H1$ cells cannot be converted into a $T_H17$ phenotype.** $CD4^+CD62L^+$ cells from wt and IFNγ receptor deficient mice were stimulated with anti-CD3 and anti-CD28 antibody and cultured under $T_H1$-inducing conditions (IL-12, anti-IL-4 antibody) for 6 days and then re-stimulated with PMA/Ionomycin for cytokine expression analysis. The $T_H1$ cells were cultured for another 6 days in the presence of IL-17-inducing conditions (TGFβ, IL-6, IL-23, anti-IL-4 and anti-IFNγ antibody). The cells were re-stimulated with PMA/ionomycin, fixed and stained intracellularly for IFNγ and IL-17.

### *5.1.1 IFNγ producing $T_H1$ cells generated in vivo are refractory to $T_H17$ inducing signals*

Next we wanted to analyze if *in vivo* generated $T_H1$ cells behave similarly under $T_H17$ inducing conditions as *in vitro* generated $T_H1$ cells. IFNγ-producing $CD4^+$ T cells were isolated from ex breeder Balb/c mice to a purity of >99% (Figure 6). The cells were stimulated with anti-CD3, anti-CD28 antibody and APC under $T_H17$ inducing conditions (TGFβ, IL-6, IL-23, anti-IL-4, anti-IFNγ and anti-IL-12 antibody) and $T_H1$ conditions (IL-12, anti-IL-4 antibody). After 5 days in culture the cells were re-stimulated with

PMA/Ionomycin, fixed and stained for IL-17, IFNγ, T-bet and RORγt. Under $T_H17$ conditions IL-17 expression was not induced in *in vivo* generated $T_H1$ cells. IFNγ was similarly expressed under both conditions and RORγt was up-regulated under none of the tested conditions. T-bet was slightly down-regulated under $T_H17$ conditions. This is not unexpected as it has been shown that T-bet is suppressed by TGFβ-signalling (Gorham et al., 1998; Lin et al., 2005).

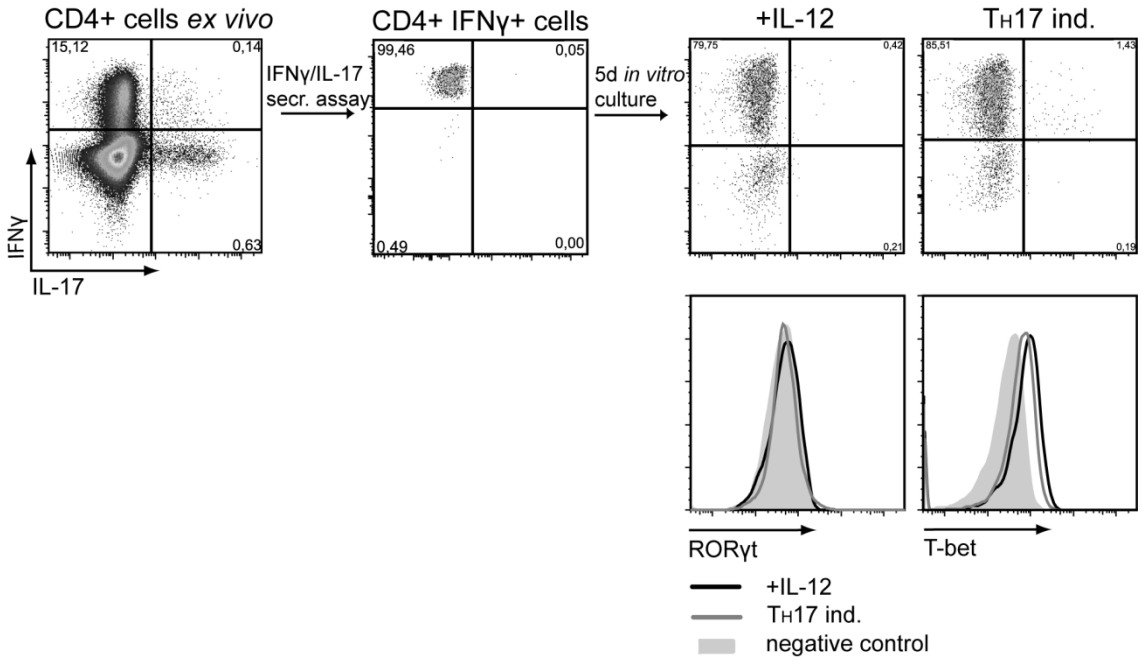


**Figure 6. *Ex vivo* isolated murine IFNγ-producing T cells are refractory to $T_H17$ reprogramming.** IFNγ-producing T cells were isolated *ex vivo* from spleen and lymph nodes from ex breeder Balb/c mice using IFNγ and IL-17 secretion assays and cultured for 5 days under $T_H1$ (+IL-12) or $T_H17$-inducing (+TGFβ + IL-6 + IL-23) conditions. IL-17, IFNγ, RORγt and T-bet expression was assessed by intracellular staining. Data are representative of three independent experiments.

## 5.2 Development of a murine IL-17 cytokine secretion assay

To analyze the stability of IL-17 expression on the single-cell level, we developed a cytokine secretion assay for murine IL-17 in cooperation with Miltenyi Biotech GmbH.

Naïve $CD4^+CD62L^+$ T cells were isolated from spleen and lymph nodes of DO11.10 mice and stimulated with antigen and APC in the presence of TGFβ, IL-6, IL-23, anti-IL-4 and anti-IFNγ antibody to induce $T_H17$ differentiation. Upon PMA/Ionomycin re-stimulation the maximal frequency of IL-17-expressing $T_H$ cells is reached already after 2 hours (Figure 7).

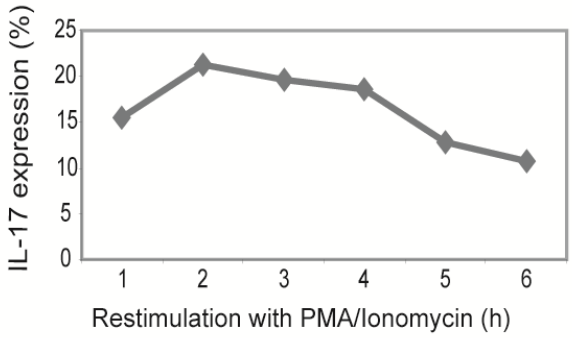


**Figure 7. Kinetic of IL-17 expression in $T_H17$ cells after re-stimulation.** $CD4^+CD62L^{high}$ cells were cultured under IL-17-inducing conditions for 6 days and then re-stimulated with PMA/ionomycin. The cells were fixed and stained intracellularly for IL-17 at different time-points as indicated.

In order to isolate IL-17-producing T cells, $T_H17$ cells were re-stimulated for 2 hours to induce cytokine expression, labeled with the capture matrix and allowed to secrete IL-17 for 30 minutes. The secreted IL-17 bound to the capture matrix was then detected using a fluorochrome-conjugated antibody. The cells were analyzed by flow cytometry or sorted by FACS (Figure 8A). Cells, which had been placed on ice in order to block secretion, were used as a low control (Figure 8B). The capacity of the capture matrix was determined by adding recombinant IL-17 to the cells (Figure 8B). To control for false-positive cells due to cross-feeding of IL-17 from secreting cells to the capture matrix of non-secreting cells, cells were fixed after staining with the detection antibody and stained for IL-17 in the presence or absence of the membrane-permeabilizing agent saponin. All of the cells secreting IL-17 were also positive for IL-17 intracellularly, whereas IL-17 non-secreting cells were negative for IL-17 intracellularly (Figure 8C). Therefore, the IL-17 secretion assay could be used for specific labeling of viable $T_H17$ cells and subsequent sorting of these cells.

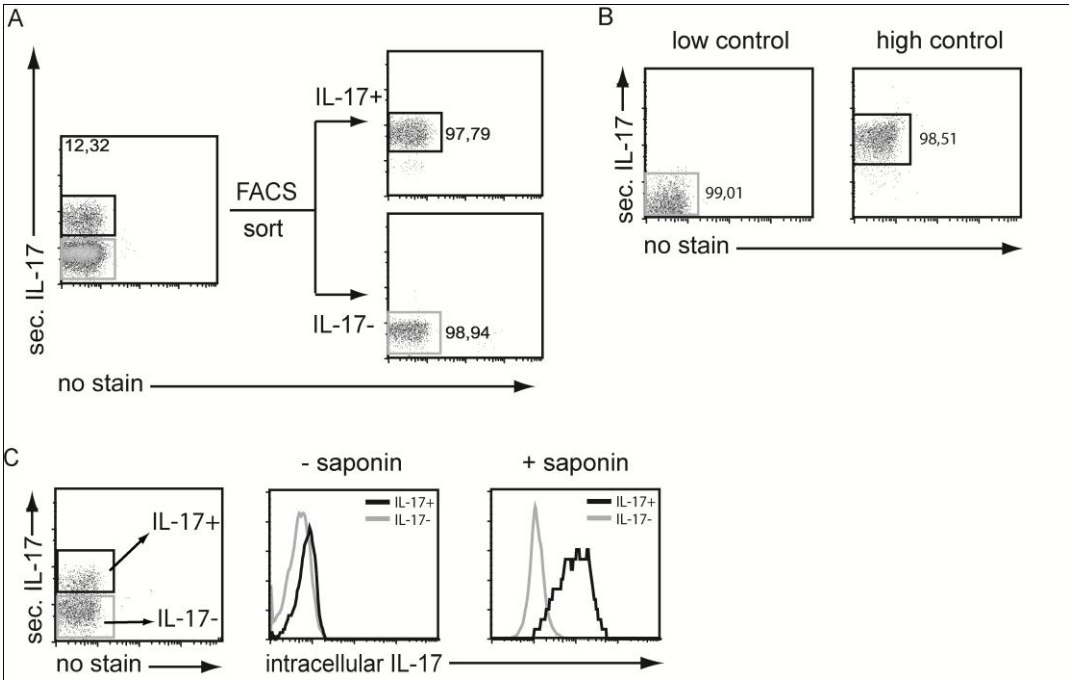


**Figure 8. Isolation of viable IL-17-producing and non-producing cells with the IL-17 secretion assay. A)** Naïve $CD4^+CD62L^+$ cells were differentiated under IL-17-inducing conditions for 6 days and an IL-17 secretion assay was performed. IL-17-secreting and IL-17-non-secreting cells were separated by FACS sorting. **B)** As controls, after labeling with IL-17 capture matrix, cells were either put on ice immediately to prevent secretion or incubated with recombinant murine IL-17 at room temperature for 15 min. IL-17 captured on the cell surface was then detected by an anti-IL-17 biotin conjugated antibody followed by staining with an APC-conjugated anti-biotin antibody. **C)** The staining of secreted IL-17 correlates with the staining of intracellular IL-17. After the IL-17 secretion assay, the cells were fixed in 2% formaldehyde and stained for intracellular IL-17 with a PE-conjugated antibody. To verify that staining of intracellular IL-17 selectively identified intracellular IL-17 and not IL-17 bound to the cell surface, the staining of intracellular IL-17 was performed either in the absence or presence of saponin.

## 5.3 Stability of IL-17 expression in *in vitro* generated $T_H17$ cells

Naïve $CD4^+CD62L^+$ $T_H$ cells were isolated from spleen and lymph nodes of DO11.10 mice and cultured under $T_H17$-inducing conditions. After 6 days in culture, these cells were separated into IL-17-producing and non-producing cells, with a purity >97% (Figure 9A). The sorted $IL\text{-}17^+$ and $IL\text{-}17^-$ cells were re-stimulated with the cognate antigen, cultured under various conditions, and analyzed for IL-17 re-expression. Cell numbers were comparable and viability was above 90% during the culture period. We did not observe selective outgrowth of contaminating cells in any of the tested culture conditions (Figure 10). When cultured in the absence of exogenous cytokines and blocking antibodies, only 13% of the $IL\text{-}17^+$ $T_H$ cells re-expressed IL-17, whereas 49% now expressed IFNγ (Figure 9A). In the presence of blocking antibodies to IL-4 and IFNγ more than 60% of the $IL\text{-}17^+$ cells re-expressed IL-17, irrespective of whether IL-

23 was blocked by adding anti-IL12p40. Addition of TGFβ and IL-6 did not have any effect on the re-expression of IL-17. When IL-23 was added without anti-IL-4 and anti-IFNγ antibody, 35% of the IL-17$^+$ cells re-expressed IL-17, 16% of them re-expressed IFNγ. IL-17 non-expressing cells expressed IFNγ (35%) but no IL-17 (<1%) if no cytokines or antibodies were added. In the presence of IL-23 2% of these cells expressed IL-17. Blocking of IFNγ and IL-4 resulted in the expression of IL-17 in 9% of the IL-17$^-$ cells in the absence of IL-23, 12% in the presence of IL-23 and 18% in the presence of TGFβ, IL-6 and IL-23 (Figure 9A). When comparing mRNA of IL-17$^+$ and IL-17$^-$ cells directly after sorting RORγt and RORα were similarly expressed. mRNA expression of these transcription factors were down-regulated when the cells were cultured without the addition of exogenous antibodies or cytokines. Regulation of RORγt expression correlated with the expression of IL-17, being highly expressed under conditions when IL-17 was expressed (anti-IL-4 and anti-IFNγ antibody with anti-IL12p40 antibody, IL-23 or TGFβ/IL-6). RORα was generally down-regulated upon re-culture, except for a 4-fold up-regulation in the presence of TGFβ/IL-6 compared to cells cultured just in the presence of anti-IL-4, anti-IFNγ antibody and IL-23. T-bet expression in IL-17$^+$ cells was 3-fold enhanced in the presence of endogenous IFNγ and particularly higher in IL-17$^-$ than in IL-17$^+$ cells. IL-17F, IL-22, IL23R and IL-21 were selectively expressed by IL-17$^+$-sorted cells and their expression was down-regulated in the absence of added cytokines or antibodies. IL-23 receptor expression was maintained in the presence of IL-23, but down-regulated in the presence of TGFβ and IL-6. IL-17F expression was only maintained in the presence of TGFβ, IL-6 and IL-23. IL-22 and IL-21 expression was down-regulated under all conditions analyzed (Figure 9B).

The $T_H17$ phenotype of *in vitro* generated $T_H17$ cells was only maintained in the presence of the instructive signals. Under neutral conditions (stim) most of the cells switched to a $T_H1$ phenotype by up-regulating IFNγ (>40%) and T-bet expression.

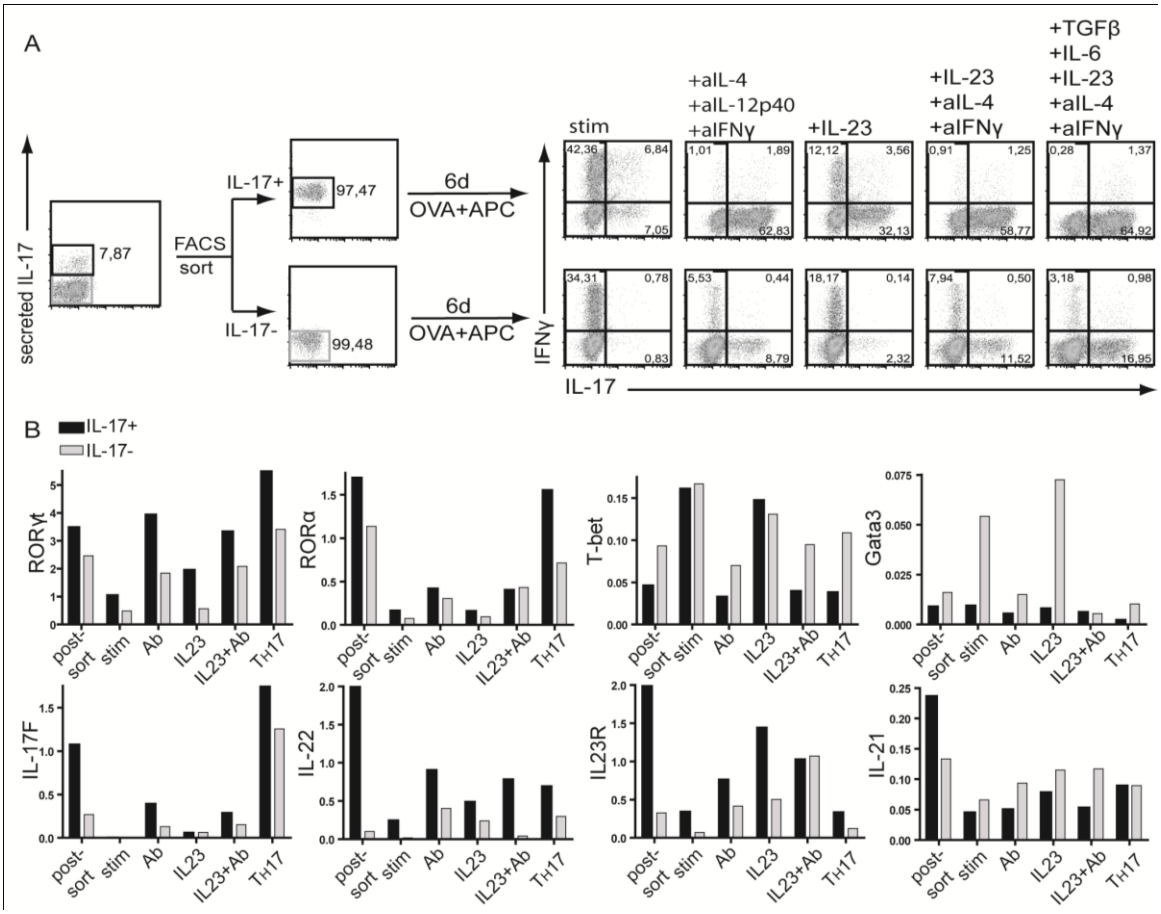


**Figure 9**. ***in vitro*** **generated IL-17 producing $T_H$ cells fail to re-express IL-17.** Naïve $CD4^+CD62L^+$ cells from DO11.10 mice were differentiated under IL-17 inducing conditions for 6 days. **A)** IL-17 producing and non-producing cells were separated and re-cultured for another 6 days in the presence of $OVA_{323-339}$ and irradiated APC only (stim), under neutral conditions (anti-IL-4, anti-IL-12, anti-IFNγ antibody) (Ab) or under different $T_H17$ favoring conditions (IL-23, IL-23 with anti-IL-4 and anti-IFNγ antibody (IL-23+Ab) or TGFβ, IL-6, IL-23, anti-IL-4 and anti-IFNγ antibody ($T_H17$)). Cytokine expression was analyzed by intracellular staining after PMA/ionomycin for 5 h. Data are representative of 3 experiments. **B)** mRNA was extracted from $IL\text{-}17^+$ and $IL\text{-}17^-$ cells directly after the secretion assay and six days later from cells re-stimulated for 2 h. This mRNA was reversely transcribed and quantified by quantitative real-time PCR. Data are representative of 2 experiments.

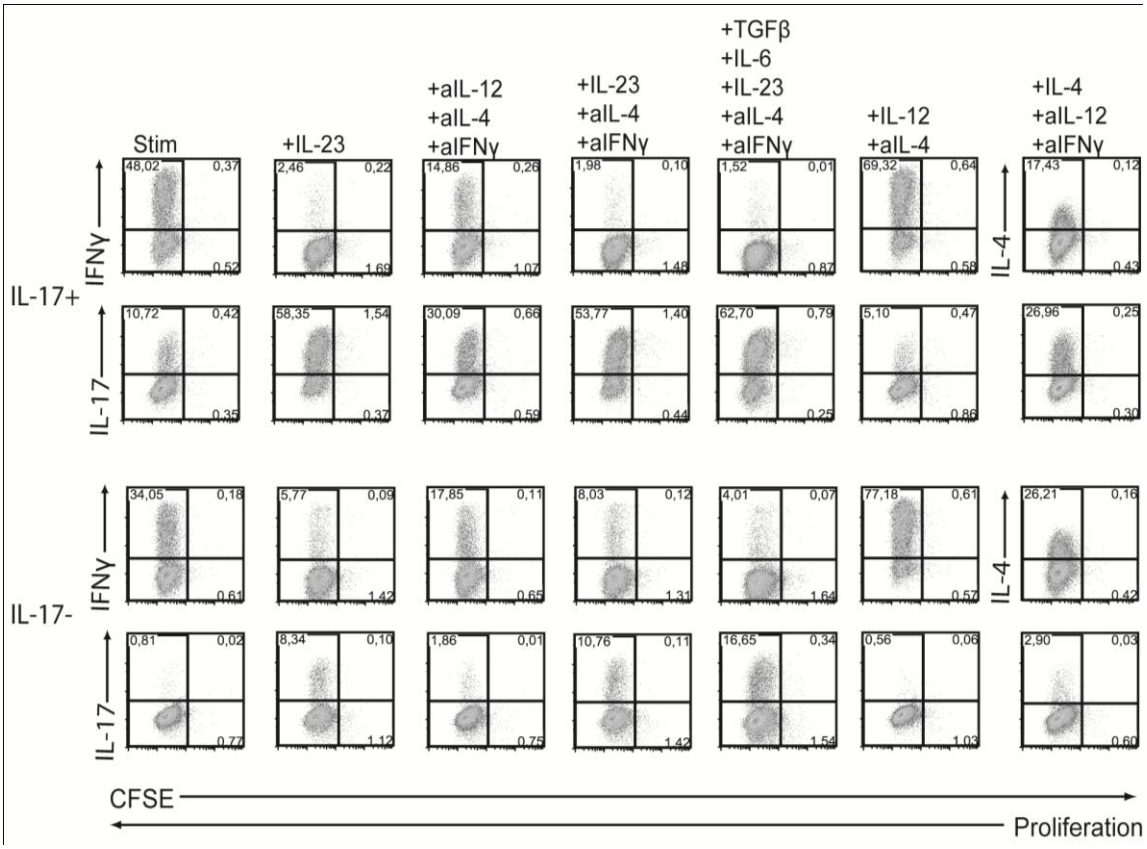


**Figure 10. *In vitro* generated IL-17 producing and non-producing cells have the same proliferative capacity.** $CD4^+CD62L^+$ cells were cultured under $T_H17$-inducing conditions for 6 days, re-stimulated with PMA/Ionomycin and subjected to IL-17 secretion assay. The IL-17 producing and non-producing cells were labeled with CFSE and cultured for another 6 days without addition of antibodies or cytokines (stim), under neutral conditions (anti-IL-4, anti-IL-12, anti-IFNγ antibody), under different $T_H17$ favoring conditions (IL-23, IL-23 with anti-IL-4 and anti-IFNγ antibody or TGFβ, IL-6, IL-23, anti-IL-4 and anti-IFNγ antibody) and $T_H1$- (IL-12 and anti-IL-4 antibody) or $T_H2$-inducing (IL-4, anti-IFNγ and anti-IL-12 antibody) conditions. The cells were re-stimulated with PMA/ionomycin for 5 h for cytokine analysis.

## 5.4 *In vitro* generated $T_H17$ cells can cross-differentiate into $T_H1$ and $T_H2$ cells

Next we wanted to analyze if IL-17-producing $T_H17$ cells could be converted into a $T_H1$ and $T_H2$ phenotype by IL-12 and IL-4 signaling, respectively. Naïve $CD4^+CD62L^+$ $T_H$ cells from DO11.10 mice were stimulated with OVA and APC in the presence of TGFβ, IL-6, IL-23, anti-IL-4 and anti-IFNγ antibody. After 6 days in culture IL-17-producing and non-producing cells were isolated and cultured for additional 6 days under $T_H1$ inducing (IL-12 and anti-IL-4 antibody) or $T_H2$ inducing (IL-4, anti-IFNγ and anti-IL-12 antibody) conditions. $IL\text{-}17^+$ cells re-expressed IL-17 with a frequency of 8% under $T_H1$ conditions

and 26% under $T_H2$ conditions (Figure 11A). IL-17$^+$ and IL-17$^-$ cells started to produce IFNγ (>70% of CD4$^+$ cells) or IL-4 (19% in IL-17$^+$ and 28% in IL-17$^-$ cultures), respectively. RORγt, RORα, IL-17F, IL-22, IL23R and IL-21 expression was down-regulated if cells were cultured under $T_H1$ or $T_H2$ polarizing conditions. Under $T_H1$ conditions the cells up-regulated expression of T-bet 3-8 fold. Under $T_H2$ conditions Gata3 expression was up-regulated 16-20 fold (Figure 11C). Since it has been shown for $T_H1$ and $T_H2$ cells that reversibility of these subsets is lost after long-term stimulation, we tested $T_H17$ cells, which had been cultured under $T_H17$ polarizing conditions for 3 weeks. Every 6 days resting viable $T_H17$ cells were harvested and re-stimulated under the original conditions. After 3 weeks, IL-17$^+$ cells were isolated and further cultured for 6 days under $T_H1$ or $T_H2$ polarizing conditions. $T_H$ memory for IL-17 re-expression was also not stabilized in 3 week old $T_H17$ cells (Figure 11B). Only 9% re-expressed IL-17 under such conditions. 64% of the IL-17$^+$ or IL-17$^-$ cells expressed IFNγ and 12-13% expressed IL-4 under $T_H1$ or $T_H2$ conditions, respectively.

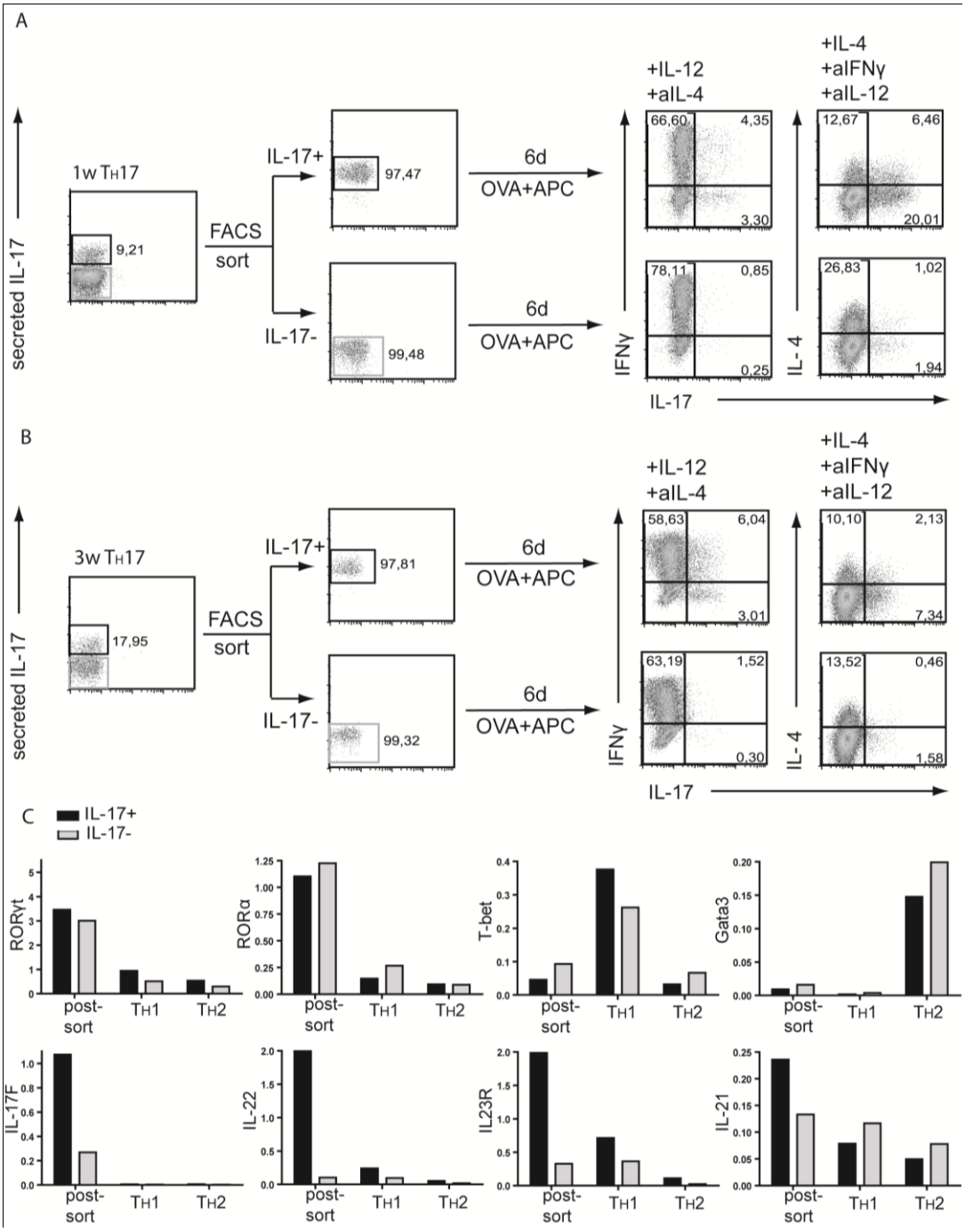


**Figure 11. IL-17-producing $T_H17$ cells can be converted into IL-4-producing $T_H2$ and IFNγ-producing $T_H1$ cells.** Naïve $CD4^+CD62L^+$ cells from DO11.10 mice were differentiated under IL-17-inducing conditions. **A)** After 6 days, the cells were harvested and re-stimulated for IL-17 secretion assay. The IL-$17^+$ and IL-$17^-$ cells were cultured under $T_H1$ (IL-12, anti-IL-4 antibody) and $T_H2$ (IL-4, anti-IL-12, anti-IFNγ antibody) polarizing conditions for 6 days. Cytokine expression was analyzed by intracellular staining after PMA/ionomycin re-stimulation. **B)** Cells were cultured for 18 days under $T_H17$ polarizing conditions (TGFβ, IL-6, IL-23, anti-IL-4, anti-IFNγ antibody). IL-$17^+$ and IL-$17^-$ cells were separated and cultured under $T_H1$ and $T_H2$ polarizing conditions. Data are representative of 3 experiments. **C)** mRNA of 1-week-old IL-$17^+$ and IL-$17^-$ cells directly after isolation and cells cultured under $T_H1$-and $T_H2$-polarizing conditions for 6 days was isolated after 2 h re-stimulation with PMA/ionomycin, reversely transcribed and quantified by quantitative real-time PCR. Data are representative of 2 experiments.

## 5.5 T-bet up-regulation is responsible for the conversion of $T_H17$ into $T_H1$ cells

T-bet is the major transcription factor driving $T_H1$ differentiation. It has been shown that in T-bet KO mice the number of IL-17-producing $CD4^+$ T cells is much higher after immunization compared to wt mice (Park et al., 2005). As $T_H17$ cells readily convert to a $T_H1$ phenotype, we compared T-bet KO to wt $T_H17$ cells to determine the role of T-bet in the conversion of $T_H17$ into $T_H1$ cells. To this end, naïve $CD4^+CD62L^+$ T cells were isolated from T-bet KO and Balb/c wt mice and cultured under $T_H17$-polarizing conditions for 5 days. IL-17-producing cells from these cultures were isolated with the aid of the IL-17 secretion assay and cultured in the presence of IFNγ and IL-12, IFNγ, IL-12 or in the absence of IFNγ and IL-12. Under all conditions, IL-4 was blocked to prevent the induction of a $T_H2$ phenotype.

Under all the tested conditions IL-17 was re-expressed in >40% of the T-bet KO cells, indicating that T-bet is responsible for the conversion of $T_H17$ into $T_H1$ cells. IL-17 expression in wt cells was only stable if all cytokines were blocked (Figure 12).

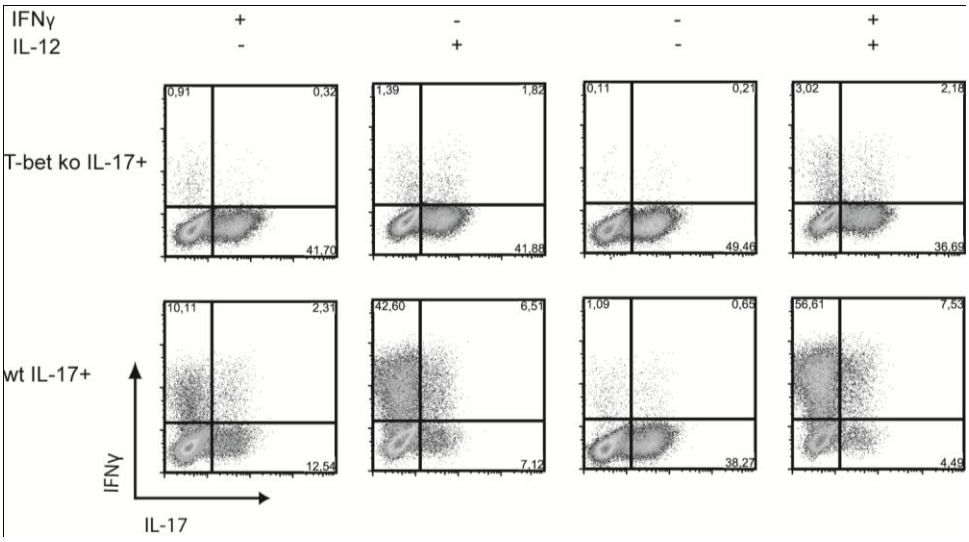


**Figure 12. Instability of IL-17 expression is T-bet-dependent.** Naïve $CD4^+CD62L^+$ cells from T-bet KO mice and Balb/c wt mice were stimulated with anti-CD3, anti-CD28 antibody and APC and cultured under $T_H17$-inducing conditions (TGFβ, IL-6, IL-23, anti-IL-4, anti-IFNγ antibody). IL-17-producing cells were isolated with the aid of the IL-17 secretion assay and cultured under the indicated conditions for 5 days. Data are representative of 2 independent experiments.

## *5.6* $T_H17$ cells generated *in vivo* maintain IL-17-expression *in vitro*

In order to analyze how *in vivo* generated $T_H17$ cells are affected under $T_H1$ and $T_H2$ conditions, we isolated $CD4^+CD62L^{lo}$ splenocytes from 6 month old ex breeder DO11.10 mice and stimulated the cells with PMA/ionomycin and IL-17-expressing cells were isolated with the aid of the IL-17 secretion assay. The cells stimulated with PMA/Ionomycin expressed 32% IFNγ, 0,9% IL-4, and 2,8% IL-17, of which approximately 25% co-expressed IFNγ (Figure 13A). The $IL\text{-}17^+$ and $IL\text{-}17^-$ cells were isolated using an IL-17 secretion assay, stimulated with OVA and APC and cultured under $T_H1$ inducing (IL-12 and anti-IL-4 antibody), $T_H2$ inducing (IL-4, anti-IFNγ and anti-IL-12 antibody), neutral (in the absence of exogenous cytokines and blocking antibodies) or in the presence of IL-23 for 6 days (Figure 13B). In the absence of antibodies or cytokines 72% of the $IL\text{-}17^+$ cells re-expressed IL-17. When the cells were cultured in the presence of IL-23, 83% re-expressed IL-17. Interestingly, *ex vivo* isolated $IL\text{-}17^+$ $T_H$ cells were refractory to $T_H1$- and $T_H2$-polarizing signals. Under $T_H1$-polarizing conditions the frequency of IFNγ expressing cells was 14%. When the $IL\text{-}17^+$ cells were cultured under $T_H2$-polarizing conditions, 4% of IL-4 expressing cells were observed. 75% and 68% of the $IL\text{-}17^+$ cells re-expressed IL-17 under $T_H1$ and $T_H2$ conditions, respectively. Expression of RORγt and RORα was down-regulated 5-fold under $T_H2$-inducing conditions. Gata3 expression was not induced in $IL\text{-}17^+$ cells under any condition. Expression of T-bet was up-regulated 2-fold under $T_H1$-inducing conditions in the $IL\text{-}17^+$ cells. In $IL\text{-}17^-$ cells RORγt and RORα were not up-regulated. T-bet was induced under neutral and $T_H1$ conditions, whereas Gata3 was induced under $T_H2$ polarizing conditions. IL-23 receptor, IL-22 and IL-17F were highly expressed in $IL\text{-}17^+$ cells and their expression was down-regulated upon *in vitro* culture. IL-21 expression was up-regulated at least 2-fold upon *in vitro* culture, except if cultured under $T_H2$-inducing conditions, which led to a 2-fold reduction in IL-21 expression (Figure 13C).

The expression of IL-17 in *in vivo* generated $T_H17$ cells was stably re-expressed under all tested conditions. Some of the $T_H17$ associated genes, such as IL-17F, IL-22 and IL23R, are similarly down-regulated as was observed in *in vitro* generated $T_H17$ cells.

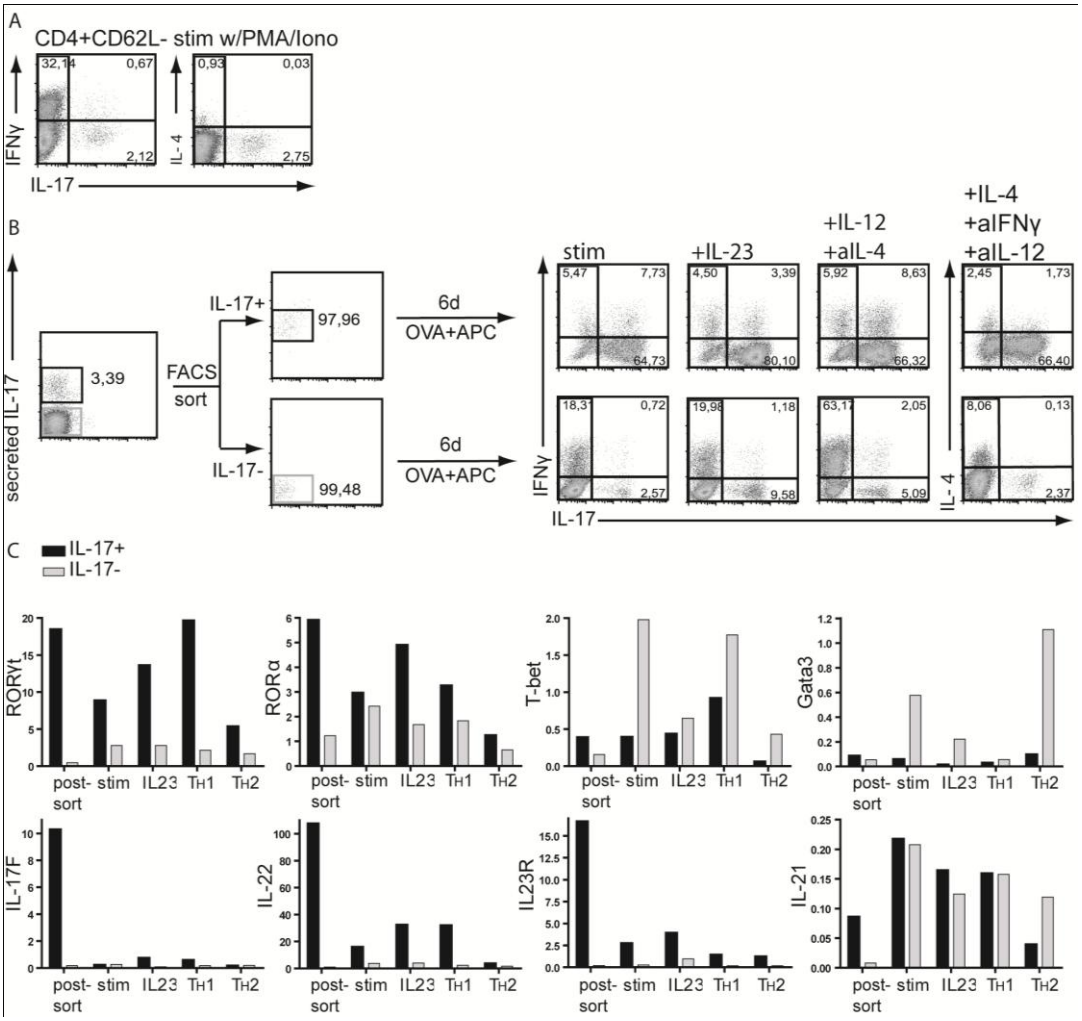


**Figure 13. *In vivo* generated $T_H17$ cells have a stable phenotype for IL-17 re-expression.** $CD4^+CD62L^{low}$ cells from 6 months old DO11.10 mice were isolated and either re-stimulated for **A)** direct analysis of cytokine expression or **B)** IL-17 secretion assay. The IL-$17^+$ and IL-$17^-$ cells were cultured without (stim) or with IL-23 and under $T_H1$- or $T_H2$-polarizing conditions for 6 days. The cytokine expression was analyzed by intracellular cytokine staining after re-stimulation with PMA/Ionomycin for 5 hours. Data are representative of 3 experiments. **C)** mRNA of *ex vivo* isolated IL-$17^+$ and IL-$17^-$ cells (directly after FACS) and cells cultured for 6 days under the indicated conditions was isolated after 2 h re-stimulation with PMA/ionomycin, reversely transcribed and quantified by quantitative real-time PCR. Data are representative of 2 experiments.

## 5.7 Gene expression analysis of *in vivo* and *in vitro* generated IL-17-producing $CD4^+$ T cells

In order to identify differentially expressed genes responsible for the stable phenotype of *in vivo* generated versus *in vitro* generated $T_H17$ cells, we performed global gene expression analysis to compare these two subsets.

Naïve $CD4^+CD62L^+$ T cells from Balb/c mice were activated *in vitro* with splenic APC, anti-CD3 and anti-CD28 antibody under conditions that induce functional differentiation into $T_H17$ cells, i.e. addition of TGFβ, IL-6, IL-23 and blocking antibodies specific for IL-4 and IFNγ. IL-17 producing cells were isolated from $T_H17$ cultures (IL-17+ in vitro) and *ex vivo* from ex breeder Balb/c mice (IL-17+ ex vivo) using an IL-17 cytokine secretion assay.

The transcriptional profiles of *ex vivo* isolated $IL\text{-}17^+$ cells and *in vitro* generated $IL\text{-}17^+$ cells were compared using high-density oligonucleotide microarrays. No genes characteristic for other cell types such as residual APCs or $CD8^+$ T lymphocytes were detected. A total number of 769 genes were differentially expressed by a factor of ten or more in *in vivo* generated versus *in vitro* generated $T_H17$ cells (Figure 14).

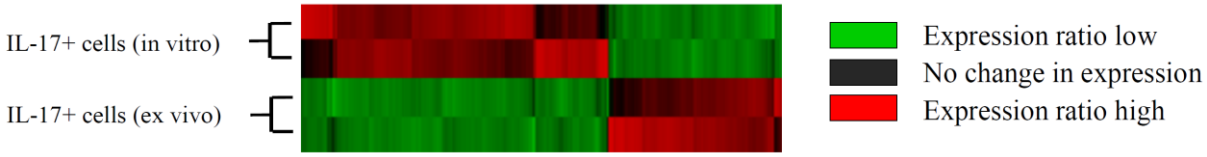


**Figure 14. Genes differentially expressed in *in vitro* generated $IL\text{-}17^+$ versus *ex vivo* isolated $IL\text{-}17^+$ cells.** Naïve $T_H$ cells from Balb/c mice were activated *in vitro* with splenic APC and $OVA_{327\text{-}339}$ under $T_H17$-polarizing conditions. IL-17-producing cells from *in vitro* cultures and *ex vivo* from ex breeder Balb/c mice were isolated using an IL-17 secretion assay. The transcriptional profiles of *in vitro* and *in vivo* generated IL-17-producing cells were compared using Murine Genome 430A version 2 GeneChip™ arrays (Affymetrix). Each group included triplicates of independent cultures/experiments. Genes were filtered according to the following criteria: fold change >= 10; difference of mean signal intensities >= 200; p-value <= 0,05 The microarrays were hybridized in the group of Dr. Thomas Häupl (Charité University Medicine, Department of Rheumatology, Berlin, Germany) and the relational data base for data analysis was created by Joachim Grün, (DRFZ, Berlin, Germany).

To identify the molecular mechanism of how *in vivo* generated $T_H17$ cells are refractory to conversion by IL-12, we here compared expression of genes relevant for IL-12 and IFNγ-signaling by $T_H17$ cells generated *in vitro*, and $CD4^+$ T cells isolated directly *ex vivo* according to secretion of IL-17. Expression of the gene encoding the IL12Rβ2 was up-regulated 2.5-fold in *in vitro* generated $IL\text{-}17^+$ cells compared to *ex vivo* isolated $IL\text{-}17^+$ cells. Although the fold change was relatively low, analysis of IL12Rβ2 is of interest as transcripts of IL12Rβ2 in cells generated *in vivo* were at the detection limit. Since IL12Rβ2 plays a major role in $T_H1$ differentiation and stabilization of the $T_H1$ phenotype, this gene was chosen for further analysis.

## 5.8 Expression of IL12Rβ2 and IFNγR2 in *in vitro* and *in vivo* generated $T_H17$ cells

The relative expression of the lineage-determining transcription factors for $T_H1$ and $T_H17$ cells, T-bet and RORγt, respectively, the IFNγ receptor 2 (IFNγR2) and the inducible IL12Rβ2 chain were analyzed by real-time PCR (Figure 15A). Expression of RORγt in *ex vivo* isolated IL-$17^+$ T cells was 3-fold higher as compared to *in vitro* generated IL-$17^+$ $T_H$ cells. IL12Rβ2 mRNA expression was close to the detection limit in *ex vivo* isolated $T_H17$ cells, confirming the results from the microarray. Increased T-bet mRNA levels (3-fold) were detected in the *ex vivo* isolated IL-$17^+$ $T_H$ cells compared to *in vitro* generated IL-$17^+$ $T_H$ cells (Figure 15A). Directly *ex vivo* isolated IL-$17^+$ $T_H$ cells expressed 5-fold less IL12Rβ2 transcripts than *in vitro* generated $T_H17$ cells (Figure 15A). Allowing the cells to rest did not significantly increase IL12Rβ2 mRNA expression of *ex vivo* isolated $T_H17$ cells (data not shown). This suggests that the expression of the *Il12rβ2* chain in $T_H17$ cells is down-regulated constitutively *in vivo*, and not transiently, as a consequence of TCR activation. *Ex vivo* isolated IL-$17^+$ $T_H$ cells stimulated with IL-12 did not respond by Stat4 phosphorylation, demonstrating the absence of a functional IL-12 receptor on these cells (Figure 15B). *Ex vivo* isolated IL-$17^+$ $T_H$ cells also do not respond to IL-12 by induction of IFNγ expression above the frequencies of pre-existing double-producing cells (Figure13A). *In vitro* generated $T_H17$ cells, on the other hand, responded to IL-12 by phosphorylation of Stat4 in all cells (Figure 15B).

The *Ifnγr2* gene was 3-4-fold higher expressed by $T_H17$ cells, both *in vivo* and *in vitro* generated, than in $T_H1$ cells (data not shown). This is in accordance with previous reports demonstrating up-regulation of *Ifnγr* expression by IL-6 (Sanceau et al., 1992) and down-regulation of *Ifnγr* expression in $T_H1$ cells *in vitro* (Pernis et al., 1995). In *in vitro* generated and directly *ex vivo* isolated $T_H17$ cells the IFNγ receptor is functional since Stat1 phosphorylation was induced upon stimulation with IFNγ (Figure 15B), while $T_H1$ cells did not respond to IFNγ (data not shown).

These data show that, while *in vitro* generated $T_H17$ cells have a functional IFNγ- and IL-12 receptor, *in vivo* generated $T_H17$ cells only have a functional receptor for IFNγ and not IL-12, making them unreactive to IL-12 signaling.

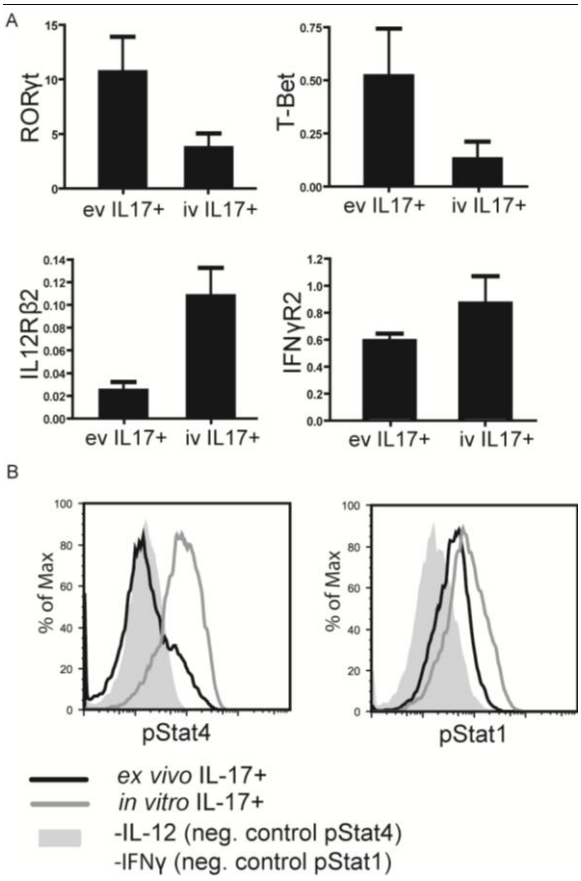


**Figure 15. *In vivo* generated $T_H17$ cells express a functional IFNγR but not IL12Rβ2**. **A)** mRNA expression of RORγt, IL12Rβ2, IFNγR2 and T-bet was determined in IL-17$^+$ cells isolated directly *ex vivo* (ev) and from *in vitro* (iv) induced $T_H17$ cells and normalized to the housekeeping gene HPRT. Data are mean ± SD of 3 independent experiments. **B)** IL-17$^+$ cells isolated *ex vivo* and generated *in vitro* were rested for 2 days in the absence of IL-4, IFNγ and IL-12. The cells were then stimulated for 30 minutes with IL-12 prior to intracellular staining of phosphorylated Stat4 (pStat4) or 15 minutes with IFNγ for staining of phosphorylated Stat1 (pStat1). Cells incubated with culture medium alone served as negative control. Data are representative of three independent experiments.

### *5.8.1 Most $T_H17$ cells do not express IL12Rβ2 in vivo*

It has been shown by Schulz et al. that IL12Rβ2 is down-regulated upon TCR stimulation. So in order to correlate IL12Rβ2 expression with IL-17 expression, we directly isolated splenic CD4$^+$ T cells based on surface expression of IL12Rβ2 (IL12Rβ2$^{high/low}$) (Figure 16A) and measured the expression of IL-17 intracellularly upon reactivation. Within the IL12Rβ2$^-$ CD4$^+$ T cells we could detect 0.66% IL-17 expressing cells, while 0.18% of the IL12Rβ2$^+$ $T_H$ cells expressed IL-17 (Figure 16B). These data show that most $T_H17$ cells do not express the IL12Rβ2 chain, which correlates with the lack of Stat4 phosphorylation in $T_H17$ cells as seen in Figure 15C.

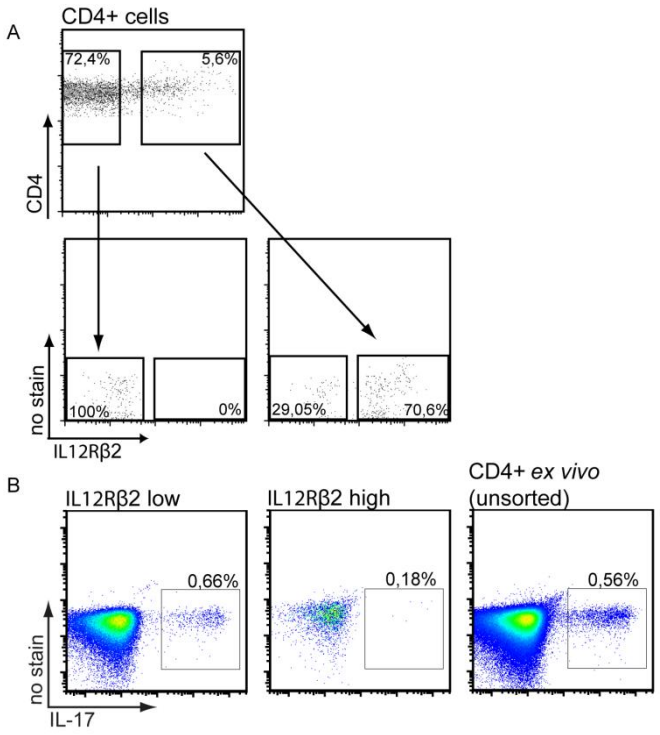


**Figure 16. $T_H17$ cells do not express the IL12Rβ2 chain.** $CD4^+$ T cells from spleen and lymph nodes from ex breeder Balb/c mice were isolated by magnetic cell sorting. **A)** The cells were stained for IL12Rβ2 and, subsequently, $IL12R\beta2^{high}$ and $IL12R\beta2^{low}$ $CD4^+$ $T_H$ cells were isolated by FACS sorting. Data are representative of three independent experiments. **B)** The sorted $IL12R\beta2^{high}$ and $IL12R\beta2^{low}$ $CD4^+$ $T_H$ cells were stimulated with PMA/ionomycin for 4 hours for recall expression of cytokines. The percentage of IL-17-producing cells per spleen was determined by intracellular cytokine staining.

## 5.9 IL12Rβ2 in *in vivo* generated $T_H17$ cells can be induced by IFNγ signalling

IFNγ-induced activation of Stat1 induces T-bet and production of IFNγ in naïve T cells (Afkarian et al., 2002; Schulz et al., 2009). However, culturing directly *ex vivo* isolated $T_H17$ cells in the presence of IFNγ was not sufficient to induce significant IFNγ production despite induction of T-bet (Figure 17A and 17C). IL-12, as shown in Figure 13, did not lead to significant induction of IFNγ expression (Figure 17A), nor responsiveness to IL-12 (Figure 17B) or up-regulation of T-bet expression (Figure 17C). This further confirms our data (Figure 15), as we could not detect any expression of a functional IL-12 receptor in *in vivo* generated $T_H17$ cells by culturing the cells in the presence of IL-12.

However, in *ex vivo* isolated $T_H17$ cells, IFNγ functionally restored responsiveness to IL-12 (Figure 17B) as has been shown for $T_H1$ and $T_H2$ cells (Szabo et al., 1997). When pre-stimulating the cells with IFNγ, IL-12 induced phosphorylation of Stat4 in more than 50% of the cells (Figure 17B). All of the cells had uniformly up-regulated T-bet expression, as determined by intracellular immunofluorescence staining (Figure 17C). Also under all tested conditions, RORγt remained highly up-regulated. Therefore, due to the elevated expression of a functional IFNγ receptor, IFNγ signaling can induce

both expression of T-bet and IL12Rβ2. After the induction of IL12Rβ2, $T_H17$ cells become susceptible to IL-12 signaling, as detected by phosphorylation of Stat4.

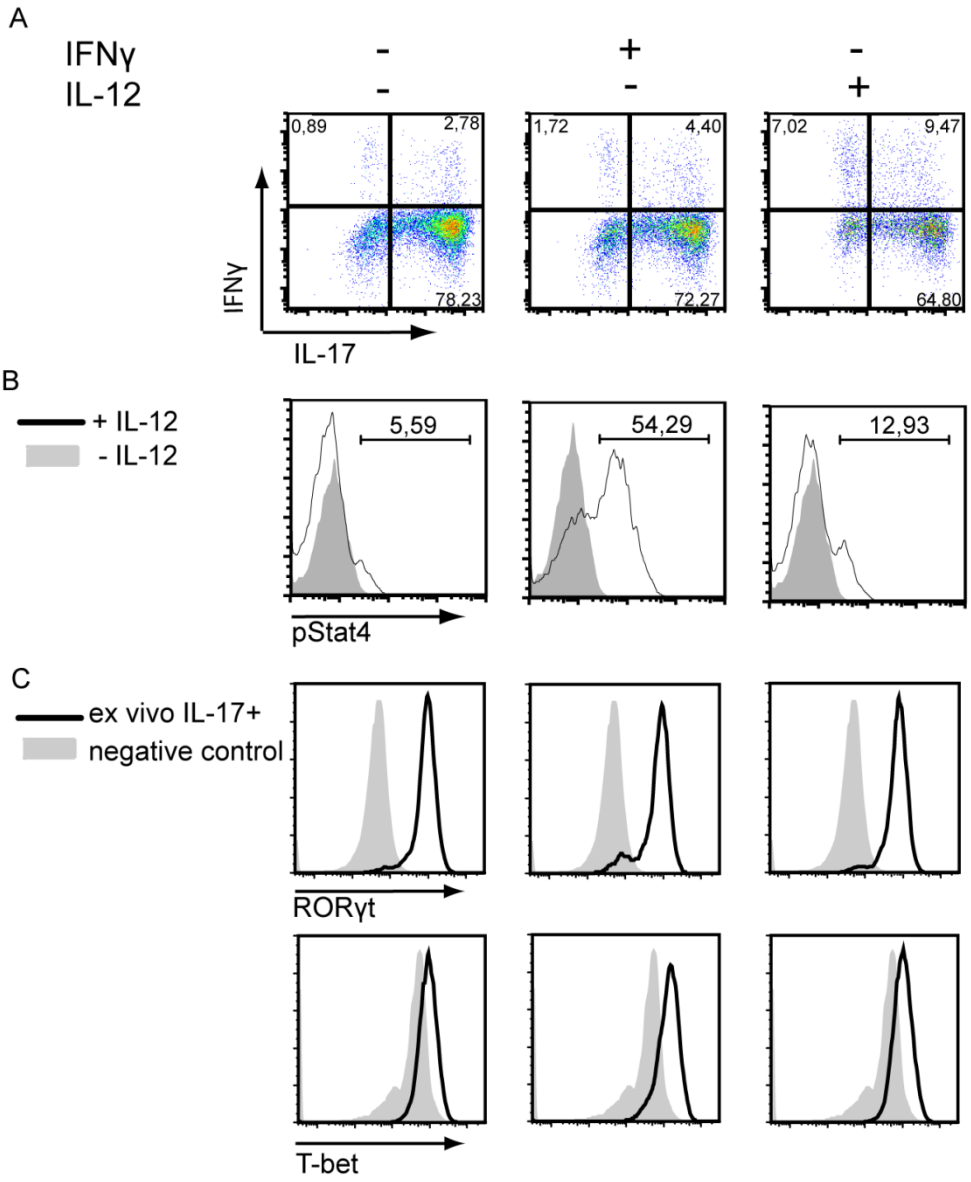


**Figure 17. IL12Rβ2 expression is induced in *ex vivo* isolated $T_H17$ cells by IFNγ. A)** *Ex vivo* isolated IL-17$^+$ cells were cultured in the absence of IFNγ and IL-12 or in the presence of IFNγ or IL-12 only for 5 days and stained intracellularly for IFNγ and IL-17 expression. IL-4 was blocked under all conditions. Data are representative of five independent experiments. **B)** Stat4 phosphorylation (pStat4) in response to IL-12 was measured by intracellular staining in IL-17$^+$ $T_H$ cells cultured under the indicated conditions. Data are representative of three independent experiments. **C)** Intracellular RORγt and T-bet expression was measured after 5 days in cell culture under the indicated conditions. Data are representative of three independent experiments.

## 5.10 *In vivo* generated $T_H17$ become susceptible to IL-12 signaling through IFNγ and adopt a $T_H1/17$ phenotype

To determine the impact of IL-12 signaling on *in vivo* generated $T_H17$ cells, in which the β2 chain of the IL-12 receptor had been up-regulated, we stimulated the IL-17-producing T cells in the presence of IFNγ for 5 days and subsequently stimulated them in the presence of IL-12 for another 5 days. While inducing expression of T-bet and IFNγ in *ex vivo* isolated $T_H17$ cells, combined IFNγ and IL-12 signaling did not suppress expression of RORγt. All cells uniformly continued to express RORγt (Figure 18C). Upon re-stimulation they also re-expressed IL-17. The frequencies of IL-17 producing cells dropped from 75% (±10%) to 38% (±6%) as a result of the IL-12 treatment. In total, 20% of the cells expressed only IL-17, 20% IL-17 and IFNγ and 30% only IFNγ (Figure 18A). This corresponds to unbiased and random co-expression of both cytokines, which is neither linked nor exclusive (Lohning et al., 2002).

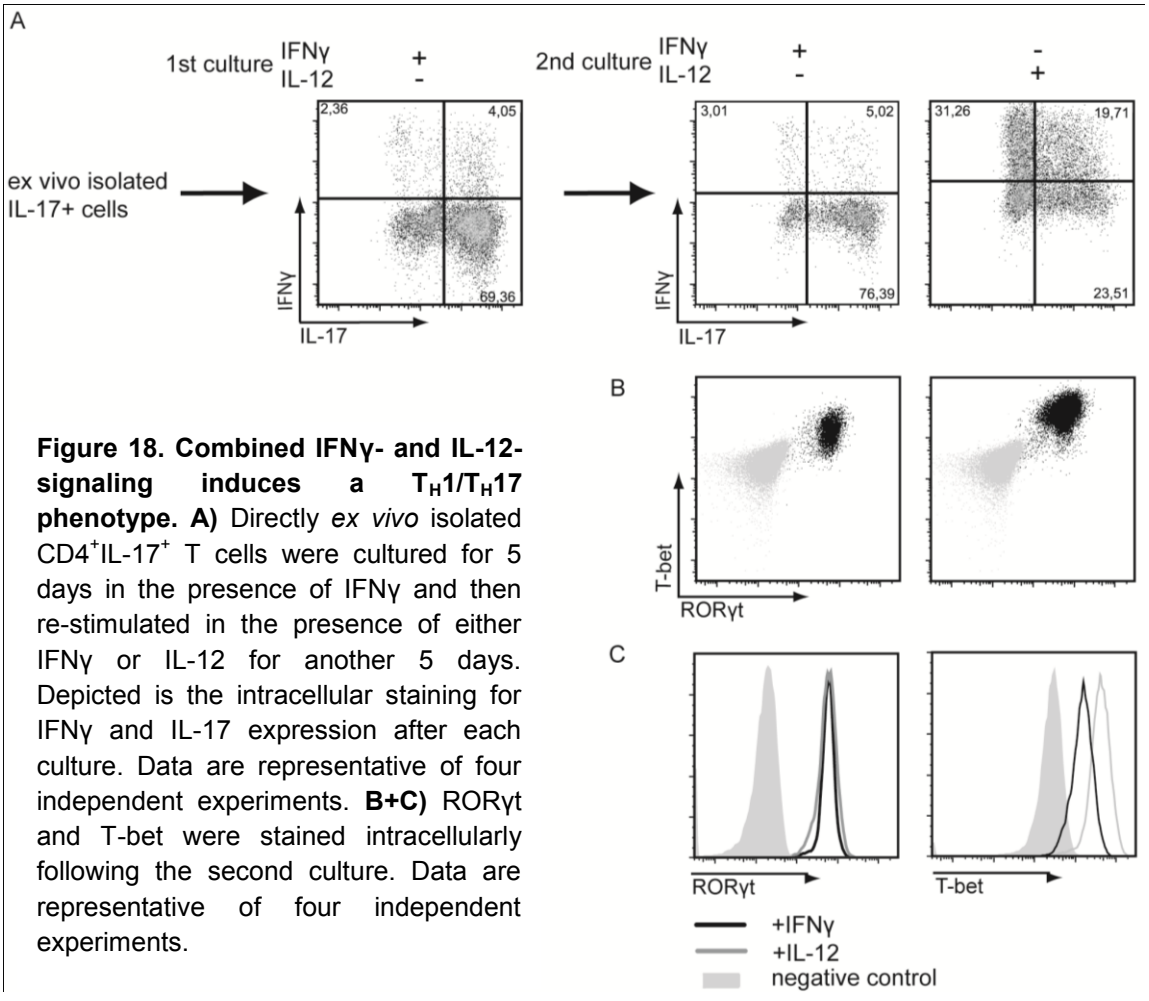


**Figure 18. Combined IFNγ- and IL-12-signaling induces a $T_H1/T_H17$ phenotype. A)** Directly *ex vivo* isolated $CD4^+IL\text{-}17^+$ T cells were cultured for 5 days in the presence of IFNγ and then re-stimulated in the presence of either IFNγ or IL-12 for another 5 days. Depicted is the intracellular staining for IFNγ and IL-17 expression after each culture. Data are representative of four independent experiments. **B+C)** RORγt and T-bet were stained intracellularly following the second culture. Data are representative of four independent experiments.

## 5.11 IL-17/IFNγ-producing $T_H$ cells co-express RORγt and T-bet

To analyze $T_H$1/17 cells generated *in vivo*, we immunized C57Bl/6 wt mice with peptide derived from myelin oligodendrocyte glycoprotein ($MOG_{35-55}$) in complete Freund's adjuvant together with i.v. administration of pertussis toxin on days 0 and 2. On day 7 post-immunization, before onset of clinical symptoms of EAE (Figure 19A), we assessed the expression of the cytokines IFNγ and IL-17 in splenic $CD4^+$ $T_H$ cells. Controls were unimmunized wildtype mice. Following re-stimulation with PMA and ionomycin, 3.29% of the splenic $CD4^+$ $T_H$ cells expressed IFNγ only, while 1.54% expressed IL-17 only. 0.38% of all splenic $CD4^+$ $T_H$ cells co-expressed IL-17 and IFNγ (Figure 19A). Among the PMA/Ionomycin stimulated $CD4^+$ T cells of unimmunized mice on the other hand, 2.25% expressed IFNγ, 0.09% IL-17 and 0.03% expressed both IFNγ and IL-17 (Figure 19A). Using an IFNγ/IL-17 double secretion assay, we isolated splenic $T_H$ cells expressing either IFNγ only, IL-17 only, or co-expressing IL-17 and IFNγ (Figure 19B). $T_H$ cells expressing only IFNγ uniformly expressed elevated T-bet levels (Δ geo mean= "geometric mean of fluorescence intensity" of 1152 compared to negative control) while RORγt expression was almost undetectable. IL-17 only expressing $T_H$ cells expressed high RORγt levels (Δ geo mean of 3387) and detectable levels of T-bet (Δ geo mean of 518). $T_H$ cells expressing both IL-17 and IFNγ uniformly expressed elevated levels of RORγt and T-bet (Δ geo mean of 2368 and 1138, respectively) (Figure 19C). mRNA of $IL\text{-}17^+$, $IFN\gamma^+$ and $IL17^+IFN\gamma^+$ $T_H$ cells was isolated, reversely transcribed and quantified by real time PCR. The expression of the analyzed genes was normalized to the housekeeping gene HPRT. RORγt was elevated in IL-17 single-positive and IL-17-IFNγ double-positive $T_H$ cells, whereas it was hardly detectable in IFNγ single-positive $T_H$ cells. However, RORγt was slightly reduced in the double-producing $T_H$ cells. RORα and IL23R were similarly expressed in the three $T_H$ cell subsets, i.e. high expression in $IL17^+$ cells, reduced expression in double producing cells and low amount in $IFN\gamma^+$ cells. Equally, as had been observed on protein level, T-bet was highly expressed in $IFN\gamma^+$ and $IL\text{-}17^+IFN\gamma^+$ $T_H$ cells, whereas a low expression was detectable in $IL\text{-}17^+$ cells. Relative mRNA expression of IL12Rβ2 was undetectable in $IL\text{-}17^+$ $T_H$ cells, up-regulated in $IFN\gamma^+$ cells and interestingly, highly expressed in $IL\text{-}17^+IFN\gamma^+$ cells. In $T_H$ cells expressing IFNγ, expression of IFNγR2 was low, but detectable, while being elevated in IL-17 single-positive $T_H$ cells.

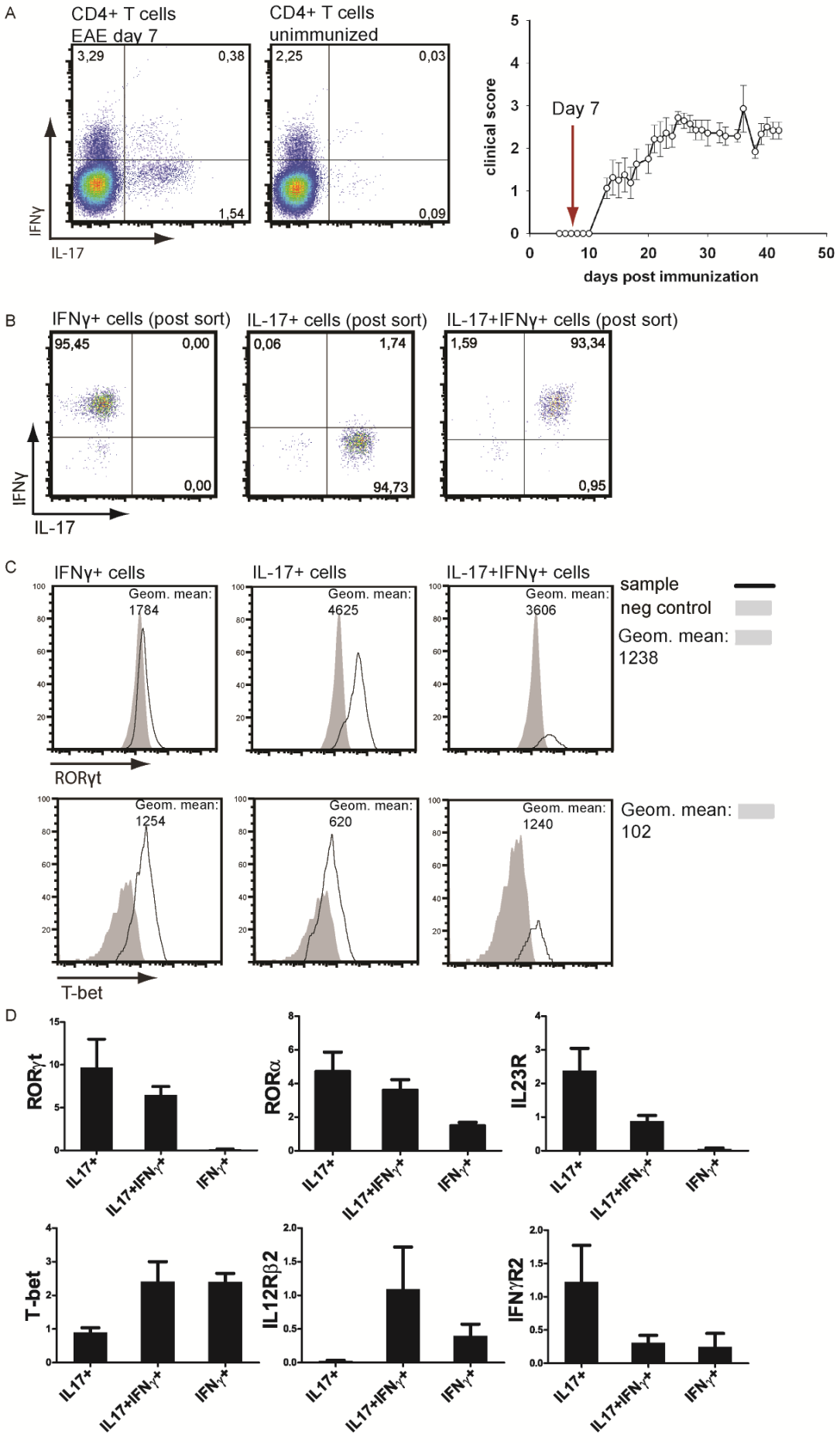
A
CD4+ T cells
EAE day 7
3,29
0,38
1,54
CD4+ T cells
unimmunized
2,25
0,03
0,09
IFNγ
IL-17
clinical score
Day 7
days post immunization
B
IFNγ+ cells (post sort)
IL-17+ cells (post sort)
IL-17+IFNγ+ cells (post sort)
95,45
0,00
0,00
0,06
1,74
94,73
1,59
93,34
0,95
C
IFNγ+ cells
IL-17+ cells
IL-17+IFNγ+ cells
Geom. mean: 1784
Geom. mean: 4625
Geom. mean: 3606
sample
neg control
Geom. mean: 1238
RORγt
Geom. mean: 1254
Geom. mean: 620
Geom. mean: 1240
Geom. mean: 102
T-bet
D
RORγt
RORα
IL23R
T-bet
IL12Rβ2
IFNγR2
IL17+
IL17+IFNγ+
IFNγ+

**Figure 19. *Ex vivo* isolated murine $T_H$1/17 cells co-express RORγt and T-bet A)** EAE was induced in C57Bl/6 mice and cytokine expression on day 7 after immunization was measured in splenic $CD4^+$ T cells after stimulating the cells with PMA/Ionomycin for 4 hours. The cells were analyzed before any clinical score was measureable. Cytokine expression in $CD4^+$ T cells from unimmunized mice were also analyzed by FACS. **B)** $IFN\gamma^+IL\text{-}17^-$, $IL\text{-}17^+IFN\gamma^-$ and $IL\text{-}17^+IFN\gamma^+$ $CD4^+$ T cells were isolated combining the IL-17 and IFNγ secretion assay. **C)** RORγt and T-bet were stained intracellularly in the different subsets. **D)** mRNA was isolated from $IFN\gamma^+IL\text{-}17^-$, $IL\text{-}17^+IFN\gamma^-$ and $IL\text{-}17^+IFN\gamma^+$ $CD4^+$ T cells, reversely transcribed and gene expression was analyzed by real-time PCR. Data are representative of three independent experiments.

Next, we analyzed the stability of $T_H$1/17 cells. Therefore, $T_H$1/17 cells were isolated directly *ex vivo* by using an IL-17 and IFNγ secretion assay and then cultured without the addition of any blocking antibodies and exogenous cytokines (neutral) for 5 or 10 days. Upon re-stimulation with PMA/ionomycin, 23±1% of the cells co-expressed IL-17 and IFNγ, 55±4% only IFNγ and 12±3% only IL-17 after 5 days (Figure 20A). The relative distribution of cytokine producers was maintained at similar levels after day 10 (12±6% co-expressing IL-17 and IFNγ, 59±14% only IFNγ and 14±6% only IL-17) (Figure 20B). T-bet expression was maintained uniformly during the 10 days of culture. RORγt expression was also maintained, although to a lesser degree in some of the cells, most of which corresponded to the IFNγ only producing cells (data not shown). Therefore, $T_H$1/17 cells generated *in vivo* maintain their phenotype, although to a lesser degree compared to $IL\text{-}17^+$ and $IFN\gamma^+$ cells under the same conditions.

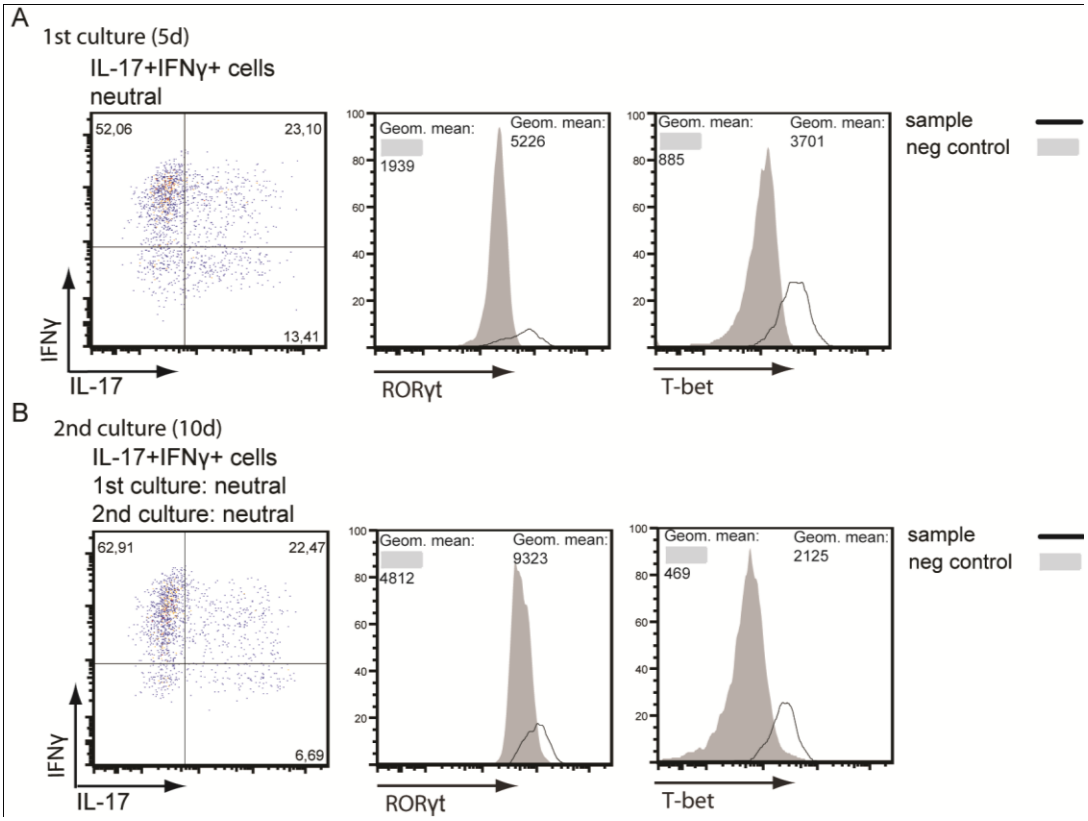


**Figure 20. *In vivo* generated $T_H1/17$ cells maintain their phenotype under neutral conditions. A)** IL-$17^+$IFNγ$^+$ $CD4^+$ $T_H$ cells were stimulated with anti-CD3/anti-CD28 antibody/APC and cultured under neutral conditions (anti-IL-4, anti-IFNγ and anti-IL-12 antibody) for 5 days. The cells were re-stimulated with PMA/Ionomycin for 4 hours and cytokine, T-bet and RORγt expression was measured by FACS. **B)** The cells from **A)** were re-stimulated with anti-CD3/anti-CD28 antibody/APC and cultured under neutral conditions (anti-IL-4, anti-IFNγ and anti-IL-12 antibody) for another 5 days. T-bet, RORγt and cytokine expression was measured after re-stimulation with PMA/Ionomycin for 4 hours. Data are representative of five independent experiments.

## 5.12 DNA methylation pattern of *in vivo* generated $T_H1$, $T_H17$ and $T_H1/17$ cells

To further analyze the plasticity of $T_H1$, $T_H17$ and $T_H1/17$ cells, DNA methylation in the promoter regions of *il17* and *rorγt* was examined in the three T cell subsets. DNA methylation is normally associated with gene silencing. A chemical reaction of sodium bisulfite with DNA converts unmethylated cytosines of CpG dinucleotides to uracil or UpG. However, methylated cytosines will not be converted in this process and primers for PCR are designed to overlap the CpG site of interest which allows one to determine methylation status as methylated or unmethylated.

C57Bl/6 mice were immunized with $MOG_{35-55}$ peptide in complete Freund's adjuvant together with i.v. administration of pertussis toxin on days 0 and 2. 7 days after immunization $IFN\gamma^+$, $IL\text{-}17^+$ and $IL17^+IFN\gamma^+$ T cells were isolated and genomic DNA was purified. Unmethylated cytosines were converted into uracils using a bisulfite kit. Regions of interest were determined based on a high degree of conservation between mouse and human and a high density of CpGs (Figure 21 and 22). A CpG island is a region with at least 200 bp and with a GC percentage that is greater than 50% and with an observed/expected CpG ratio that is greater than 60%. No CpG islands are located within the il17 locus as predicted by a CpG program (http://cpgislands.usc.edu/). A region 5kb upstream of *il17* was selected for further analysis as this has been shown to be a binding site for RORγt and important for high transcription of *il17* (IL-17-1). Furthermore, the promoter region of *il17* was analyzed (IL-17-2) (Figure 21).

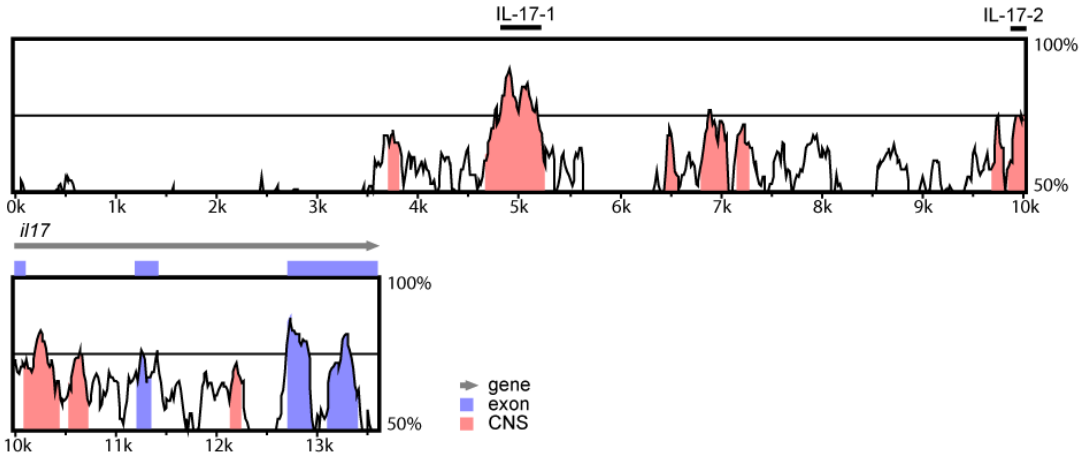


**Figure 21. Genomic organization of the *il17* gene.** Genomic organization of *il17* and the region 10 kb upstream. The regions of interest are depicted above the alignment. The exons are in blue and the conserved non-coding sequences (CNS) in pink.

Both RORγ and RORγt are encoded by *rorc*. RORγt shares a identical nucleotide sequence with RORγ from exon 3 through the last exon. Both alternate RNA splicing (He et al., 1998; Ortiz et al., 1995) and an alternative promoter (Winoto and Littman, 2002) have been suggested to be responsible for the generation of RORγt. Therefore, we analyzed both sequences upstream of *rorc* as well as directly upstream of the first RORγt specific exon. Three regions, 9 kb (RORγt-1), 1 kb (RORγt-2) upstream of *rorc* as well as the potential promoter region of *roryt* (RORγt-3) were analyzed as these regions display a high density of CpGs (Figure 22). Due to the low frequencies of $IL\text{-}17^+IFN\gamma^+$ $T_H$ cells and in order to increase uniformity of all measurements, the same amount of DNA isolated from each $T_H$ cell subset from three experiments were pooled for the analysis.

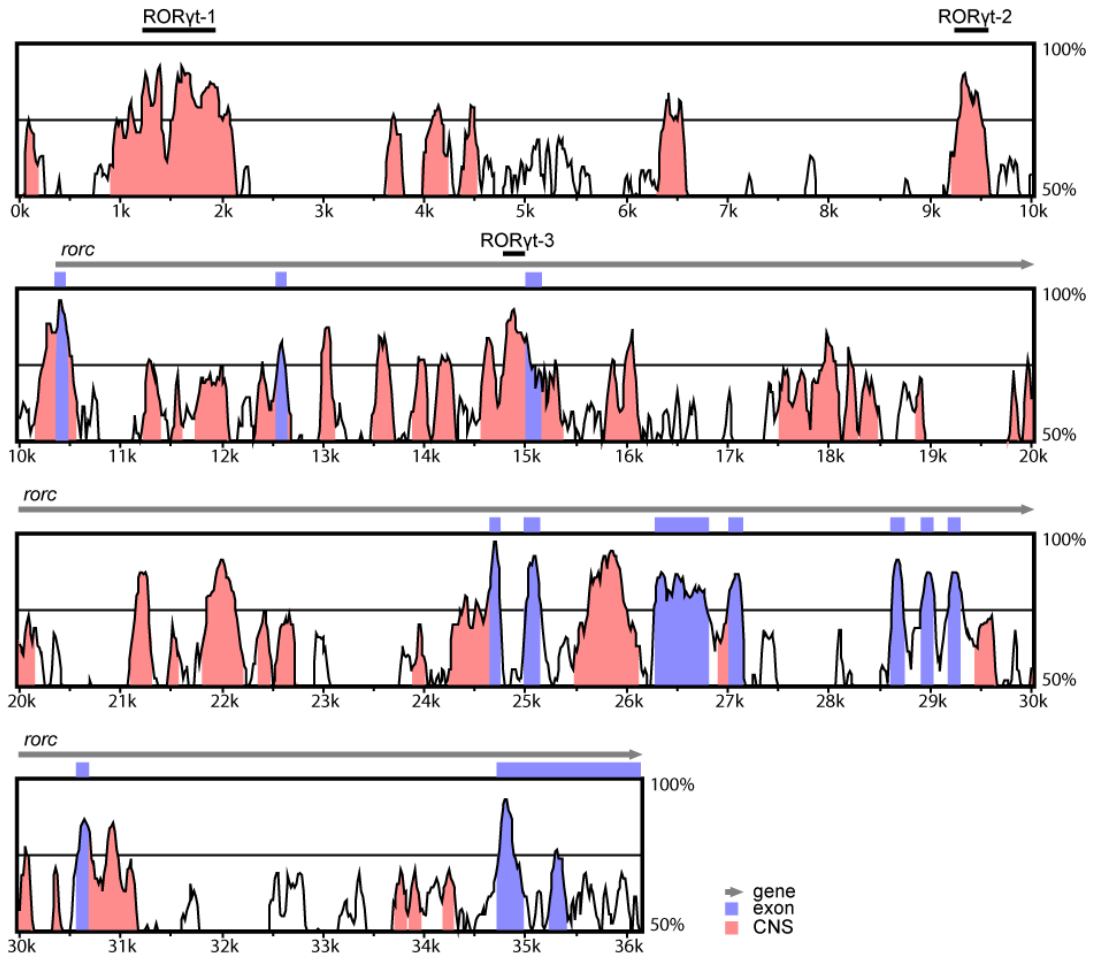


**FIgure 22. Genomic organization of the *rorc* gene.** Genomic organization of *rorc* as well as the region 10.3 kb upstream. RORγ and RORγt are both encoded within the *rorc* locus. The regions of interest are depicted above the alignment. The exons are in blue and the conserved non-coding sequences (CNS) in pink.

The regions of interest were amplified by nested PCR, inserted into the vector pCR®2.1 by a ligation step and transformed into competent cells. 24 positive clones of every $T_H$ cell subset from each region were picked and sequenced. The sequences where then compared to the original sequence and methylated cytosines could hereby be detected, as these were not converted into uracils during the bisulfite conversion reaction.

The region 5kb upstream of *il17* (IL-17-1) was completely demethylated in IL-17$^+$ $T_H$ cells, whereas more than 50% of the CpGs were methylated in IFNγ$^+$ $T_H$ cells (Figure 23). In IL-17$^+$IFNγ$^+$ $T_H$ cells we detected some methylated CpGs.

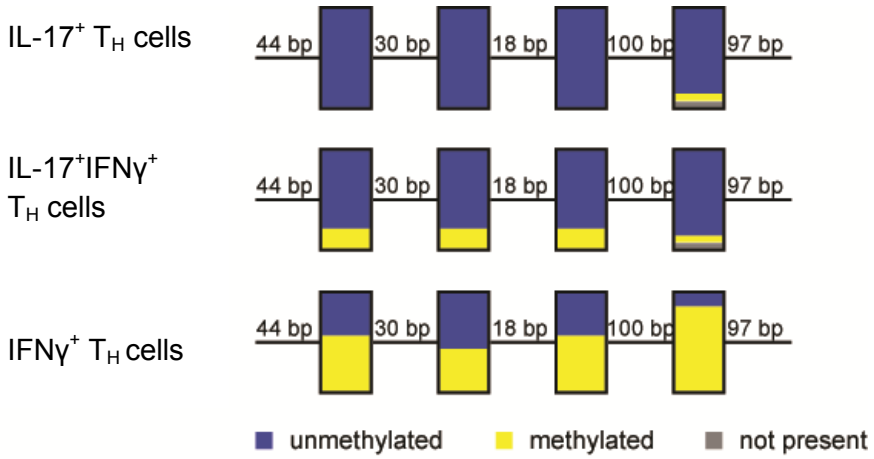


**Figure 23. 5 kb region upstream of *il17* (IL-17-1).** 4 CpGs were analyzed in 18 clones of each subset for cytosine methylation. Each box represents one CpG.

The methylation pattern of the *il17* promoter was similar as observed for the region 5 kb upstream. No cytosine methylation in IL-17$^+$ $T_H$ cells, some in IL-17$^+$IFNγ$^+$ $T_H$ cells and high CpG methylation in IFNγ$^+$ $T_H$ cells (Figure 24). Therefore, methylation of regions upstream of *il17* seems to correlate with the lack of IL-17 expression, as in IFNγ$^+$ $T_H$ cells, whereas unmethylated regions correspond with active expression of IL-17.

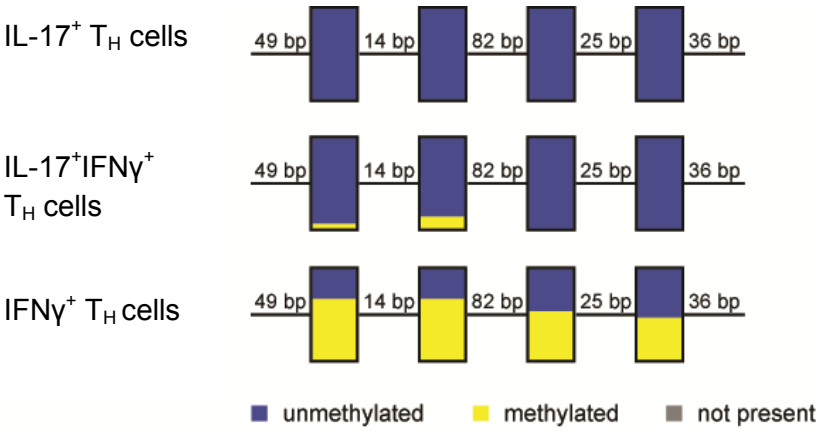


**Figure 24. Promoter region upstream of *il17* (IL-17-2).** 4 CpGs were analyzed in 18 clones of each subset for methylation of cytosines.

The CpG island 9 kb upstream of *rorc* was mostly unmethylated in IFNγ$^+$ and IL-17$^+$IFNγ$^+$ $T_H$ cells, whereas unexpectedly, in IL-17$^+$ $T_H$ cells this region was strongly methylated, even though these cells have a high expression of RORγt (Figure 25).

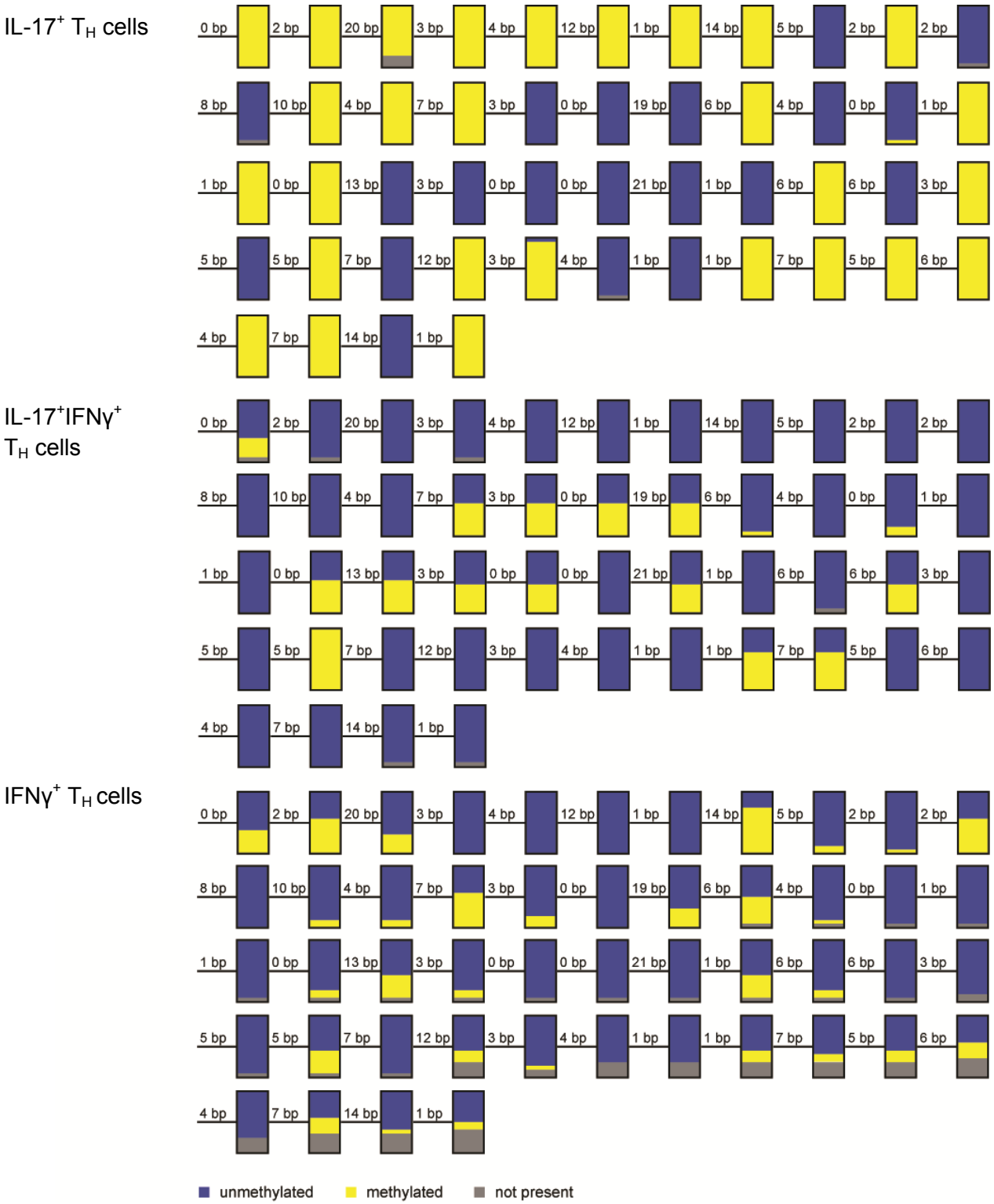


**Figure 25. 9 kb region upstream of *rorc* (RORγt-1.)** 48 CpGs were analyzed in 16 clones of each subset for cytosine methylation.

Potential transcription factor binding sites were analyzed for RORγt-1 by using MatInspector (Genomatix software). 2 putative T-bet binding sites, as well as 7 CTCF binding sites were detected (Figure 26). However, if these transcription factors are able to bind in this region and whether CpG methylation has an impact on the ability to bind, remains to be determined.

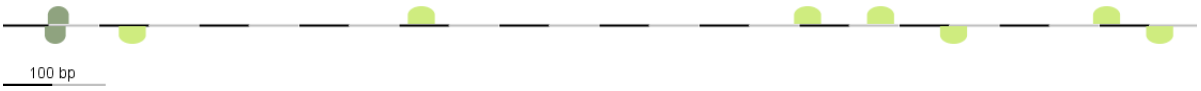


**Figure 26. Putative binding sites in the complete CPG island 9 kb upstream of *rorc*.** 2 potential T-bet binding sites (dark green) and 7 CTCF binding sites (green) were detected using MatInspector.

For the region 1 kb upstream of *rorc* methylated cytosines were highly enriched in all tested $T_H$ cell subsets (Figure 27).

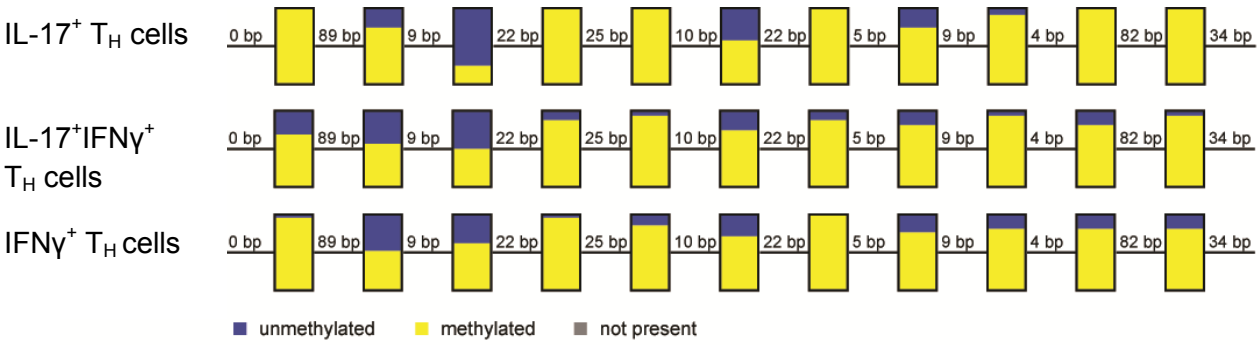


**Figure 27. 1 kb region upstream of *rorc* (RORγt-2).** 11 CPGs were analyzed in 15 clones of each subset for cytosine methylation.

Interestingly, the region directly upstream of the first *rorγt* exon showed nearly no methylation of CpGs in all of the tested $T_H$ cell subsets (Figure 28). As methylation is associated with silencing of genes, this suggests a silenced RORγ on the one hand and an active RORγt on the other.

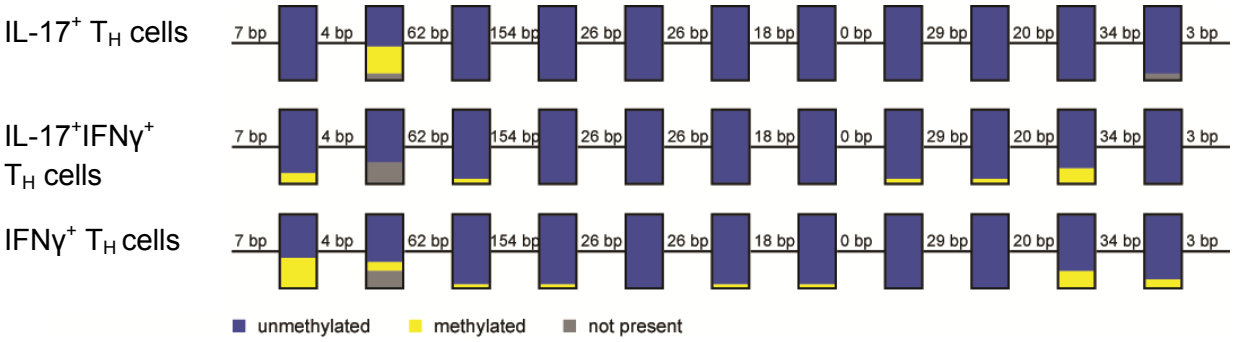


**Figure 28. Potential promoter region upstream of *rorγt* (RORγt-3).** 11 CPGs were analyzed in 19 clones of each subset for cytosine methylation.

Whereas DNA methylation of the regions upstream of *il17* correlated with IL-17 expression, the *rorc* locus seems to be modified in a bivalent manner. A potential regulatory element was identified 9 kb upstream of *rorc*, which has to be analyzed further.

# 6 Discussion

$CD4^+$ T cells are critically involved in autoimmunity, allergy and asthma and the concept of distinct $T_H$ cell lineages has provided a simple model for the conceptualization of $CD4^+$ cell differentiation. However, are the different $T_H$ cell subsets really distinct lineages, and if they are, how stable or plastic are they? Commitment of $T_H$ cells into distinct lineages during differentiation was proposed to involve stable programs of gene expression correlating with epigenetic changes at the loci of cytokine genes (Ansel et al., 2003; Bird et al., 1998; Murphy et al., 1996). Consistently, cytokine genes were shown to be stably expressed even under conditions, which favor differentiation of other effector lineages (Assenmacher et al., 1998; Lohning et al., 2002; Murphy et al., 1996). However, many recent publications have challenged the existing paradigm concerning stable, inconvertible committed $T_H$ cells lineages (Hegazy et al., 2010; Zhou et al., 2008).

IL-17 expressing $T_H$ lymphocytes have been recognized as a separate lineage of $T_H$ cell differentiation, distinct from the $T_H1$ and $T_H2$ lineages (Harrington et al., 2005; Park et al., 2005). Stability and plasticity of the cytokine memory of $T_H17$ memory effector cells has been a matter of debate, especially in light of reports describing $T_H$ cells expressing both IL-17 and IFNγ (Acosta-Rodriguez et al., 2007; Annunziato et al., 2007; Infante-Duarte et al., 2000; Suryani and Sutton, 2007). Therefore, we concentrated on the interconversion between $T_H17$ and $T_H1$ cells. We analyzed the plasticity of $T_H17$ cells by generating an IL-17 secretion assay in cooperation with Miltenyi Biotec GmbH in order to isolate viable $T_H17$ cells, either from *in vitro* cultures or directly *ex vivo*.

Understanding the molecular mechanisms, which stabilize $T_H$ cell subsets and the conditions allowing plasticity of these subsets, is of major importance for the development of new therapies. Transfer of Tregs in some mice with autoimmunity has been shown to ameliorate disease. Therefore, administration of human Tregs in patients has been considered as a treatment for various human autoimmune diseases. In a series of studies investigating the behavior of Tregs in the intestine however, it was shown that Treg cells have the propensity to differentiate into pro-inflammatory $T_H17$ cells (Xu et al., 2007). This could have disastrous consequences, if this was the case during treatment of an autoimmune disease. Thus, gaining more knowledge on the

stability and plasticity of $T_H$ cell subsets might revise our approaches for treating such diseases.

## 6.1 $T_H1$ and $T_H2$ cells cannot cross-differentiate into $T_H17$ cells

Almost a quarter of a century ago, it was recognized that cytokine production by $T_H$ cells was not stochastic, but could rather be divided into two subsets, $T_H1$ and $T_H2$ cells producing IFNγ and IL-4, respectively (Mosmann et al., 1986). Stable cytokine production was only observed after a set number of cell divisions under polarizing conditions, which was interpreted as a requirement for establishing a stable transcriptional program (Bird et al., 1998; Grogan et al., 2001). Also, the requirement for specific master transcription factors, T-bet for $T_H1$ and Gata3 for $T_H2$ cells, further supported the lineage model. Richter et al. showed that the initial S phase of T cell activation is required for the instruction of $T_H$ cells to express IL-4 or IL-10 upon re-stimulation (Richter et al., 1999). This observation points to a decisive role of epigenetic modifications of cytokine genes as a molecular correlate of the memory to express particular cytokines. For the cytokine memory of $T_H1$ and $T_H2$ cells molecular mechanisms have been described which prevent the differentiation of $T_H1$ into $T_H2$ cells and vice versa. The commitment to the $T_H1$ cell lineage is associated with the expression of the β2 chain of the IL-12 receptor complex, thereby conferring responsiveness to IL-12. Induction of IL12Rβ2 is dependent on T-bet induction through IFNγ and Stat1 signaling (Afkarian et al., 2002; Mullen et al., 2001; Schulz et al., 2009). IL-4 has been shown to repress IL-12 signaling through inhibition of IL12Rβ2 expression, thereby antagonizing $T_H1$ differentiation (Szabo et al., 1997). Furthermore, Gata3 has been shown to down-regulate Stat4 expression and thereby stabilizing the $T_H2$ phenotype (Usui et al., 2003). $T_H1$ cytokines on the other hand, repress $T_H2$ differentiation through a feed-forward mechanism. IFNγ induces T-bet, which subsequently induces expression of Runx3. T-bet then cooperates with Runx3 to further promote IFNγ production, while at the same time silencing the *il4* gene in $T_H1$ cells through binding to the *ifnγ* promoter and the *il4* silencer, respectively (Ansel et al., 2004; Djuretic et al., 2007; Naoe et al., 2007). However, cross-regulation during $T_H1$ and $T_H2$ differentiation has been demonstrated in several studies. In a recent study, $Gata3^+T\text{-}bet^+$ and $IL\text{-}4^+IFN\gamma^+$ $T_H$ cells were described *in vivo* (Hegazy et al., 2010). $Gata3^+$ $T_H2$ cells could be re-programmed by type I and type II interferons plus IL-12 *in vitro*. This lineage re-programming was T-bet-dependent and resulted in $T_H$ cells co-expressing Gata3 and T-bet and producing both IFNγ and IL-4. These cells were stably maintained *in vivo* for months, suggesting lineage-like properties.

To what extent $T_H1$ and $T_H2$ cells are refractory to $T_H17$ inducing signals was not clear so far. We show here that *in vitro* generated $T_H1$ and $T_H2$ cells cannot convert into $T_H17$ cells by the bona fide $T_H17$ instructive signals TGFβ, IL-6, and IL-23, simultaneously blocking IL-4 and IFNγ. Interestingly $T_H1$ cells polarized under $T_H17$ conditions up-regulate RORα and RORγt 2- and 6-fold on mRNA level, respectively. However, this up-regulation of $T_H17$ lineage master transcription factors is apparently not sufficient for the induction of IL-17 expression in such $T_H1$ cells. This may be due to even further up-regulation of T-bet in $T_H1$ cells under $T_H17$ polarizing conditions, as T-bet has been described as a negative regulator of $T_H17$ differentiation (Gocke et al., 2007; Mathur et al., 2006). In $T_H2$ cells, Gata3 is down-regulated 2-3-fold and RORγt up-regulated 4-fold upon re-stimulation in a $T_H17$ inducing cytokine milieu. Expression of IL-23 receptor and RORα was not up-regulated. It has been shown that ectopic expression of RORγt in combination with RORα in $T_H1$ and $T_H2$ cells can lead to expression of IL-17 (Martinez et al., 2008). However, in those experiments RORγt and RORα were expressed in $T_H$ cells that may have been not fully committed to the $T_H1$ or $T_H2$ lineage. Another possibility is that the concentration of RORγt and RORα achieved under these conditions is too low compared to cells in which these transcription factors are over-expressed. Accordingly, higher expression of RORγt and RORα might “overrun” the negative effects T-bet has on $T_H17$ differentiation.

As IFNγ has been shown to exhibit negative effects on $T_H17$ differentiation and thus, could inhibit the conversion of $T_H1$ to $T_H17$ cells, we generated $T_H1$ cells *in vitro* from IFNγR deficient mice and cultured them under $T_H17$-inducing conditions (Harrington et al., 2005). As there was only minor expression of IL-17 in $T_H1$ cells from IFNγR deficient mice compared to wt $T_H1$ cells, we concluded that IFNγ signaling is not responsible for the lack of IL-17 induction in $T_H1$ cells under $T_H17$ conditions.

Suryani et al. have suggested that IL-17/IFNγ co-expressing cells are derived from $T_H1$ cells gaining the ability to express IL-17 (Suryani and Sutton, 2007). While we do not exclude this option here, the conversion of $T_H1$ cells into $T_H1/17$ cells must require signals different from the canonical $T_H17$ differentiation signals TGFβ and IL-6. Neither *in vitro* generated $T_H1$ cells, nor *ex vivo* isolated $T_H1$ cells, as was show here, can be induced to express IL-17 by combined action of TGFβ, IL-6 and IL-23. Although T-bet was slightly reduced in *in vivo* generated $T_H1$ cells under $T_H17$ inducing conditions, we could not detect any up-regulation of RORγt and also no induction of IL-17. Down-regulation of T-bet by TGFβ has been described in several publications (Lin et al., 2005; Yang et al., 2008c). For instance, TGFβ can prevent the development of autoimmune disease by restraining the development of autoreactive $T_H1$ cells. TGFβ

was shown to inhibit $T_H1$ development in part by suppressing the expression of T-bet, but exactly how TGFβ suppresses T-bet is incompletely understood. Furthermore, upon adoptive transfer, $T_H1$ cells are not converted into $T_H1/17$ cells *in vivo* (Lim et al., 2008). Interestingly, as was recently published, in the absence of T-bet, IFNγ production and $T_H1$ differentiation are susceptible to inhibition by IL-6 and TGFβ (Yang et al., 2008c). As a result, $T_H17$ development is strongly favored, the threshold for TGFβ requirement is lowered and IL-6 drives $T_H17$ differentiation, illustrating an important role for T-bet in directing T cell differentiation to $T_H1$ vs $T_H17$.

From our experiments and observations from other groups we conclude that $T_H1$ cells are especially averse to $T_H17$ inducing signals and that $T_H$ cells producing both IL-17 and IFNγ probably are not generated from $T_H1$ cells. However, to what extent $T_H1$ cells maintain their phenotype *in vivo* during immune reactions favoring $T_H17$ differentiation has not been sufficiently investigated so far.

## 6.2 *In vivo* generated $T_H17$ cells are refractory to $T_H1$- and $T_H2$-inducing signals

Next, we wanted to analyze the stability and plasticity of the $T_H17$ phenotype. It had already been shown that both IFNγ and IL-4 could inhibit the induction of IL-17 under $T_H17$ inducing conditions (Harrington et al., 2005), but it was still not clear how already established $T_H17$ cells react to $T_H1$ or $T_H2$ inducing conditions. Since $T_H17$ cells induced *in vitro* were heterogeneous with respect to IL-17 expression, it was necessary to develop an IL-17 secretion assay allowing the isolation and analysis of IL-17 expression on the single cell level. This assay was used to analyze the memory of $T_H17$ cells for re-expression of IL-17 in $T_H17$ cells generated *in vitro* as well as *in vivo*. Isolated IL-17-expressing cells generated *in vitro* by activation of naïve $T_H$ cells in the presence of TGFβ, IL-6 and IL-23 with concomitant blockade of IFNγ and IL-4, failed to re-express IL-17 upon later reactivation, if the original inducing signals were lacking, or if IFNγ and IL-4 were not neutralized. Isolated IL-$17^+$ or IL-$17^-$ $T_H$ cells showed the same proliferation and survival during re-culture. We could therefore exclude the possibility that contaminating cells were outgrowing IL-$17^+$ cells and thereby give the false impression that a subpopulation was responsible for the observed loss of IL-$17^+$ cells. As it has been shown that the plasticity of $T_H1$ and $T_H2$ cells is dependent on their differentiation state, we analyzed $T_H17$ cells that had been primed once (6d) or three times (18d) (Murphy et al., 1996). IL-$17^+$ $T_H$ cells, even after three weeks of repeated instruction for IL-17 expression could still be converted into IFNγ-expressing $T_H1$ cells with IL-12 or into IL-4-expressing $T_H2$ cells with IL-4. As $T_H17$ cells are readily

converted into $T_H1$ cells, we analyzed the role of T-bet, the $T_H1$ master transcription factor, by using T-bet KO mice. IL-17$^+$ T-bet$^-$ $T_H$ cells were stable when stimulated with IFNγ only, IL-12 only or both cytokines. This indicates that T-bet is responsible for the conversion of $T_H17$ into $T_H1$ cells. Since both IFNγ and IL-12 signaling had no impact on IL-17 re-expression, we conclude that in the absence of T-bet, Stat4 and Stat1 cannot inhibit IL-17 expression. The molecular mechanism, by which T-bet inhibits IL-17 expression is not clear and has to be analyzed further.

In contrast to *in vitro* generated $T_H17$ cells, IL-17 expressing $T_H$ cells isolated *ex vivo* maintained a memory for IL-17 expression *in vitro*, even in the presence of IL-12 or IL-4. These results indicate that there may be epigenetic differences between *in vitro* and *in vivo* generated $T_H17$ cells.

These results also suggest that the currently available *in vitro* protocols for the induction of IL-17 expression in naïve $T_H$ cells lack signals for the induction of a stable cytokine memory for IL-17 re-expression. Identification of *in vitro* culture conditions that mimic *in vivo* environments would be of great aid to further dissect the signaling pathways and the transcriptional regulatory networks of $T_H$ cell differentiation. Although IL-17 expression was induced efficiently *in vitro* by TGFβ, IL-6, IL-23 and anti-IFNγ and anti-IL-4, incomplete blocking of IFNγ or IL-4 during subsequent re-stimulation and culture led to the loss of IL-17 re-expression in cells which once had expressed IL-17. In consistence with this result, both IFNγ and IL-4 had been described as negative regulators of IL-17 expression (Harrington et al., 2005; Park et al., 2005). Expression of both RORγt and RORα was down-regulated in *in vitro* generated $T_H17$ cells under conditions where the cells did not re-express IL-17 and in particular under $T_H1$- or $T_H2$-polarizing conditions. Unlike the memory for IL-10 expression in $T_H2$ cells, requiring multiple rounds of stimulation by IL-4 for stability (Chang et al., 2007; Lohning et al., 2003), repeated *in vitro* stimulation in the presence of TGFβ, IL-6 and IL-23 did not lead to a stable commitment for IL-17 expression. The cells were still plastic and responded to the presence of IL-12 or IL-4 with differentiation into IFNγ-expressing $T_H1$ or into IL-4-expressing $T_H2$ cells, respectively. Expression of IL-17F in the *in vitro* generated as well as in the *ex vivo* isolated $T_H17$ cells did not correlate with the re-expression of IL-17 after re-culture. IL-17F is highly homologous to IL-17 and the *il17f* gene neighbors the *il17* gene (Starnes et al., 2001), suggesting a coordinated expression. While being highly expressed initially in cells sorted for IL-17 expression, IL-17F expression was only maintained in the presence of TGFβ and IL-6. Interestingly, IL-17F expression was also not stable in *ex vivo* isolated IL-17 expressing cells, either indicating that the memory for IL-17F re-expression is conditional and depends on

different or additional signals than that for IL-17 re-expression, or that maintained RORγt and RORα expression may be required (Martinez et al., 2008), but may not be sufficient for IL-17F expression. This observation was confirmed by a study showing that TGFβ is the critical cytokine for IL-17F expression (Lee et al., 2009). However, whether TGFβ-signaling through Smads is directly or indirectly contributing to the maintenance of IL-17F is not known.

Recent publications have shown that $T_H17$ cells transferred into immunodeficient mice are also plastic and convert to $T_H1$ cells (Lee et al., 2009). The same was observed when BDC2.5 $CD4^+$ T cells were polarized into $T_H17$ cells and subsequently adoptively transferred into NOD-SCID mice (Bending et al., 2009). Here the $T_H17$ cells caused β-cell destruction and diabetes, but were also converted into $T_H1$ cells. However, these *in vivo* studies involved transferring cells into lymphopenic hosts, in which homeostatic proliferation may influence the stability of $T_H$ differentiation. Interestingly, in a recent publication the phenotype of *in vitro* generated $T_H17$ cells adoptively transferred into normal mice was maintained, even in the absence of antigen and inflammation (Nurieva et al., 2009). It remains to be shown which factors contribute *in vivo* to maintain IL-17 expression, as well as identifying how *in vivo* generated $T_H17$ cells are able to resist conversion into $T_H1$ cells.

Here, we have demonstrated that IL-17 expressing cells generated *in vitro* are not functionally imprinted for IL-17 re-expression. Their re-expression of IL-17 depends on the continued presence of the canonical $T_H17$-inducing signals. The conversion of *in vitro* generated IL-17-expressing $T_H17$ cells into IFNγ-expressing $T_H1$ cells is T-bet-dependent. IL-17 expressing $T_H$ cells generated *in vivo* on the other hand, are a stable lineage of effector memory cells, distinct from $T_H1$ and $T_H2$ cells, and functionally imprinted for re-expression of IL-17 upon TCR stimulation, even in the presence of $T_H1$ or $T_H2$ inducing conditions.

## 6.3 $T_H1/17$ cells are induced from $T_H17$ cells by subsequent IFNγ- and IL-12- signaling

To determine which factors contribute to the stability of *in vivo* vs *in vitro* generated $T_H17$ cells, we compared expression of genes relevant for IL-12- and IFNγ-signaling in a global gene expression analysis. We decided to concentrate on genes crucial for $T_H1$ differentiation as $T_H$ cells producing both IL-17 and IFNγ have been described *in vivo* in several autoimmune diseases in both mice and humans (Aarvak et al., 2000; Annunziato et al., 2007; Infante-Duarte et al., 2000). Expression of the gene encoding IL12Rβ2 was 2.5-fold up-regulated in *in vitro* compared to *in vivo* generated IL-$17^+$ $T_H$

cells. This observation was further confirmed by real-time PCR. Expression of T-bet and RORγt were higher in *ex vivo* isolated $T_H17$ cells, whereas IFNγR2 was slightly increased in *in vitro* generated $T_H17$ cells. Both subsets responded to IFNγ signaling by induction of Stat1 phosphorylation, pointing to expression of a functional IFNγ receptor. Although differential expression of IL23R and IL12Rβ2 has been thought to distinguish $T_H17$ and $T_H1$ developmental programs, we found that whereas IL12Rβ2 expression is diminished in $T_H17$ cells, it remains fully functional in *in vitro* generated $T_H17$ cells. Correspondingly, *in vivo* generated $T_H17$ cells were refractory to IL-12 signaling. The lack of IL12Rβ2 on *ex vivo* isolated $T_H17$ cells was further confirmed by surface staining. Therefore, *in vivo* generated $T_H17$ cells are refractory to $T_H1$-inducing signals because these cells cannot respond to IL-12-signaling due to down-regulation of IL12Rβ2. However, they still expressed the IFNγ receptor and responded to IFNγ signaling. IFNγ signaling induced the expression of T-bet and of the IL12Rβ2 chain in naïve $T_H$ cells (Afkarian et al., 2002; Mullen et al., 2001; Schulz et al., 2009). Schulz et al. showed that IFNγ induced initial T-bet expression, whereas IL12Rβ2 was repressed by TCR signaling. After termination of TCR signaling, IL12Rβ2 expression was up-regulated by T-bet and subsequent IL-12 signaling was essential for maintenance of T-bet expression. This late expression of T-bet, together with the up-regulation of the transcription factors Runx3 and Hlx was required to imprint the $T_H$ cell for IFNγ re-expression (Schulz et al., 2009). When *ex vivo* isolated IL-$17^+$ $T_H$ cells were cultured in the presence of IFNγ, a functional IL-12 receptor as well as T-bet was induced. RORγt was neither affected by IFNγ- nor by IL-12-signaling. Sequential activation of *in vivo* generated $T_H17$ cells with IFNγ and IL-12, however, induced further up-regulation and maintenance of T-bet expression. Expression of IFNγ was induced as well, and the *Ifnγ* gene was imprinted for re-expression. No expression of IL12Rβ2 and high expression of IFNγR2 are typical features of naïve $CD4^+$ T cells (Groux et al., 1997; Tau et al., 2000). Thus, *in vivo* generated $T_H17$ cells behave like naïve $T_H$ cells (Schulz et al., 2009), except that they maintain also their enhanced expression of RORγt and expression of IL-17.

Taken together, we here provide a molecular mechanism for the generation of a distinct $T_H$ cell population characterized by the additive phenotypes of $T_H1$ and $T_H17$ cells, the $T_H1/17$ cells. $T_H1/17$ cells are characterized by the co-expression of the cytokines IFNγ and IL-17 and the lineage-defining and -determining transcription factors T-bet and RORγt. $T_H1/17$ cells develop from $T_H17$ cells upon synergistic action of IFNγ, required for the up-regulation of the IL12Rβ2 chain, and IL-12. IL12Rβ2 signaling is crucial for stable imprinting of $T_H1$ cells (Schulz et al., 2009). It remains to

be shown how expression of the IL12Rβ2 chain is down-regulated in $T_H17$ cells *in vivo*. Evidence has been provided that IL-17 itself directly (Toh et al., 2009) or indirectly, by inducing antigen-presenting cells to release as yet unidentified factors (Nakae et al., 2007), down-regulates IL12Rβ2 chain expression in activated $T_H$ cells.

In order to analyze $T_H1/17$ cells generated *in vivo*, we isolated IL-$17^+$IFN$\gamma^+$ $T_H$ cells *ex vivo* from mice immunized with MOG peptide. IL-$17^+$ and IL-$17^+$IFN$\gamma^+$ $T_H$ cells are induced upon immunization in the spleen of these mice and can be readily isolated. As expected, IFN$\gamma^+$ $T_H$ cells expressed high amounts of T-bet and no RORγt, IL-$17^+$ $T_H$ cells expressed high amounts of RORγt and slightly increased levels of T-bet, whereas IL-$17^+$IFN$\gamma^+$ $T_H$ cells expressed high amounts of both T-bet and RORγt. This was confirmed on mRNA level by real-time PCR. RORα was up-regulated in both subsets producing IL-17 and low expression was detectable in IFN$\gamma^+$ $T_H$ cells. Interestingly, the IL-23 receptor was strongly down-regulated in IL-$17^+$IFN$\gamma^+$ compared to IL-$17^+$ $T_H$ cells, whereas it was not detectable in IFN$\gamma^+$ $T_H$ cells. The IL-23 receptor has been shown to be up-regulated by IL-6-signaling and RORγt and down-regulated by T-bet (Gocke et al., 2007; Maggi et al., 2010; Yang et al., 2007). As $T_H1/17$ cells have a high expression of both T-bet and RORγt, it implies that the elevating effect for IL-17 expression by RORγt can overrule the inhibitory effect of T-bet, as these cells are still able to produce IL-17. IL12Rβ2 was undetectable in IL-$17^+$ $T_H$ cells, but highly up-regulated in IL-$17^+$IFN$\gamma^+$ and IFN$\gamma^+$ $T_H$ cells. IFNγR2 was up-regulated in IL-$17^+$ $T_H$ cells, but only low expression was detectable in $T_H$ cells producing IFNγ. Consequently, down-regulation of IL23R with simultaneous up-regulation of IL12Rβ2 in IL-$17^+$IFN$\gamma^+$ $T_H$ cells could lead to “preferential” skewing towards a $T_H1$ phenotype of these cells. Nevertheless, IL-$17^+$IFN$\gamma^+$ $T_H$ cells are characterized by expression of cytokines and transcription factors known to be hallmarks of both $T_H17$ and $T_H1$ cells.

Next we analyzed the stability of IL-$17^+$IFN$\gamma^+$ $T_H$ cells by culturing these cells under neutral conditions. Although IL-17 expression was strongly down- and IFNγ up-regulated within 2 rounds of stimulation, expression of T-bet and RORγt remained stable. RORγt was however, slightly down-regulated. Simultaneous expression of T-bet and RORγt appears to be a stable feature in $T_H1/17$ cells. Re-expression for IL-17 on the other hand is less faithful in these cells. To what extent $T_H1/17$ cells are still able to convert back into $T_H17$ cells or if these cells are preferentially inclined towards a $T_H1$ phenotype, as indicated here, has not been investigated so far.

## 6.4 DNA methylation of $T_H17$, $T_H1/17$ and $T_H1$ cells

In several publications silencing of the *il4* gene in $T_H1$ or the *ifnγ* gene in $T_H2$ cells has been demonstrated (Grogan et al., 2001; Jones and Chen, 2006). The methylation of single CpG sites in cytokine gene promoters has been shown to silence gene expression by preventing binding for TCR responsive transcription factors (Jones and Chen, 2006; Murayama et al., 2006). So far, the regulatory mechanisms and epigenetic processes that control $T_H17$ cell differentiation have only been partially characterized. In the 8 described conserved non-coding sequences as well as in the promoter of *il17*, permissive H3 acetylation is induced in naïve $CD4^+$ T cells that are cultured under $T_H17$ polarizing conditions compared to $T_H1$ or $T_H2$ conditions (Akimzhanov et al., 2007). As we could not find any CpG islands in these conserved non-coding sequences, we analyzed a region which has been reported as a RORγt-dependent enhancer located 5 kb upstream of the *il17* transcriptional start site (IL-17-1) (Zhang et al., 2008) and the proximal promoter of *il17* (IL-17-2). These regions contained several CpGs that were analyzed for methylation. The methylation upstream of *il17* corresponded with expression of IL-17, that is, the regions upstream of *il17* were completely unmethylated in IL-$17^+$, somewhat methylated in IL-$17^+$IFN$\gamma^+$ and strongly methylated in IFN$\gamma^+$ $T_H$ cells. However, if methylation of the 5 kb-upstream region is blocking the binding of RORγt and thereby the production of IL-17, remains to be shown.

RORγ and RORγt are both encoded by *rorc*. RORγt shares an identical nucleotide sequence with RORγ from exon 3 through the last exon. Winoto and Littman et al. suggested an alternative promoter, whereas Ortiz et al. proposed alternate splicing to be responsible for the generation of RORγt (He et al., 1998; Ortiz et al., 1995; Winoto and Littman, 2002). The putative promoter region of the *rorγt* gene was unmethylated in all three subsets, whereas the region 1 kb upstream of *rorc* was characterized by a high quantity of methylated CpGs. This could support the model of an alternative promoter, instead of alternative splicing as was proposed by Ortiz et al. The methylated region 1kb upstream of *rorc* could be the reason why RORγ is silenced in all $T_H$ cell subsets, as RORγ is highly expressed only in thymus, kidney, liver, muscle, brown fat, but not in cells of the immune system (Hirose et al., 1994; Jetten, 2009; Kurebayashi et al., 2000). It would be interesting to test if this region is unmethylated in cells known to express RORγ.

Interestingly, whereas the CpG island 9 kb upstream of *rorc* in IL-$17^+$IFN$\gamma^+$ and IFN$\gamma^+$ $T_H$ cells was only partially unmethylated, this region displayed a distinct pattern of

highly methylated CpGs in IL-17$^+$ $T_H$ cells. This was surprising as IL-17$^+$ $T_H$ cells highly express RORγt. Assuming this region plays a role in the regulation of RORγt, there could be several explanations for why a region upstream of a gene known to be expressed by the same cell is methylated. One possibility is through methylation of this region transcriptional repressors are unable to bind and therefore cannot suppress *rorc*/*rorγt* transcription. Interestingly, in this region, seven potential binding sites for CCCTC-binding factor (CTCF) were found. The 11-zinc finger protein CTCF is an ubiquitously expressed and highly conserved transcriptional regulator implicated in many key processes within the nucleus, including promoter activation and repression (Ohlsson et al., 2001). CTCF has been shown to bind in the vicinity of insulators, elements that affect gene expression by preventing the spread of heterochromatin, and to inhibit inappropriate interactions between regulatory elements on adjacent chromatin domains, thus acting as an enhancer blocker (Bell et al., 1999; Wallace and Felsenfeld, 2007). It has been shown that CTCF preferentially associates with unmethylated CpG dinucleotides (Kanduri et al., 2000; Ling et al., 2006). As the analyzed region is strongly methylated in IL-17$^+$ $T_H$ cells, it would be possible that CTCF is therefore unable to bind and consequently, cannot block potential enhancer elements further upstream. Since this region is mostly unmethylated in IL-17$^+$IFNγ$^+$ and $T_H$ cells, CTCF would possibly be able to bind, thereby inhibiting an enhancer. This could explain the lower expression of RORγt in these cells.

Furthermore, two potential T-bet binding sites were found in this region. High methylation could affect the binding to its motif; thereby T-bet would be unable to perform potential negative effects on *rorc*/*rorγt* transcription. This could also explain the difference of stability observed between IL-17$^+$ and IL-17$^+$IFNγ$^+$ $T_H$ cells as well as the lower expression of RORγt in IL17$^+$IFNγ$^+$ $T_H$ cells.

Whether T-bet or CTCF are principally able to bind in this region would have to be tested in chromatin immunoprecipitation experiments.

Interestingly, it has been shown that whereas histones in the *tbx21* locus are modified in a bivalent manner in $T_H$ cells subsets, *rorc* and *il17* are repressed in $T_H$1 cells (Wei et al., 2009). In contrast to histone modifications providing labile transcriptional repression, DNA methylation is a highly stable silencing mark that is not easily reversed. It was suggested, in consideration of the bivalent modifications in the *tbx21* locus, that it would be possible to convert $T_H$17 cells into a $T_H$1 cells. As both *rorc* and *il17* were shown to have repressive marks in $T_H$1 cells, it was proposed that it would be more difficult to induce a $T_H$17 phenotype in established $T_H$1 cells. For both hypotheses

evidence has been provided here. We could not find any $T_H1$ specific DNA methylation pattern in the *rorc* locus, however. Although DNA methylation and histone modification are carried out by different chemical reactions and require different sets of enzymes, a biological relationship between the two systems has been shown. It has been described that DNA methylation and specific histone modifications might influence each other (Cedar and Bergman, 2009). The relationship has been suggested to work in both directions: histone methylation can help to direct DNA methylation patterns, and DNA methylation might serve as a template for some histone modifications after DNA replication. However, the presence of histone methylation at H3K9 or H3K27 does not always lead to *de novo* methylation and vice versa. Our results are therefore not necessarily in conflict with their data (Wei et al., 2009). Furthermore, whereas we analyzed $T_H$ cell subsets isolated *ex vivo*, they used *in vitro* generated $T_H$ cells for the analysis. This could further explain the discrepancy observed between our experiments.

## 6.5 Conclusion and Perspective

What significance do $T_H1/17$ cells have? The physiological advantage of $T_H1/17$ cells over $T_H1$ and $T_H17$ cells could be their combined effector repertoire on the single cell level, co-expressing IFNγ and IL-17, but also chemokine receptors of both $T_H1$ and $T_H17$ cells (Lim et al., 2008), i.e. CCR2, CCR5 and CXCR3 of $T_H1$ and CCR4 and CCR6 of $T_H17$ cells, allowing them to deliver their cytokines at non-canonical locations. E.g. $T_H1/17$ cells could deliver IL-17 into inflamed tissue, attracted by the CXCR3 ligands CXCL9, 10 or 11 (Janke et al., 2010). In consistence with this assumption it was suggested that $T_H1/17$ cells have an advantage over $T_H17$ and $T_H1$ cells in crossing the blood-brain barrier (BBB) and in accessing the central nervous system (Kebir et al., 2009). Evidence has been provided that IFNγ up-regulates the expression of ICAM-1 on the surface of BBB-endothelial cells, which is an important adhesion molecule that controls $T_H17$ lymphocyte migration across the BBB. As $T_H1/17$ cells express IFNγ and possess a $T_H17$ phenotype, this might positively affect the capacity of these cells to enter the central nervous system.

What implications do plastic/mixed $T_H$ cell subsets have for classification of $T_H$ cells subsets? This work and other reports have shown that differentiated effector $T_H$ cells have are a lot more flexible than previously thought (Hegazy et al., 2010; Lee et al., 2009; Lexberg et al., 2008; Xu et al., 2007). Hegazy et al. for instance described that stably committed $Gata3^+$ $T_H2$ cells could adopt a $Gata3^+$ $T\text{-}bet^+$ and $IL\text{-}4^+IFN\gamma^+$ "$T_H2+1$" phenotype that was maintained *in vivo* for months. The emergence of an increasing

number of new $T_H$ cell subsets as well as $T_H$ cells displaying a mixed phenotype has raised the question to what extent the classification of $T_H$ cell subsets based on the selective expression of cytokines is reasonable. $T_H$ cell lineages express lineage-defining transcription factors, but as has been shown, transcription factor expression can be dynamic and a particular subset, such as $T_H1/17$ cells can express more than one master regulator. In addition, the expression of a master transcription factor can be lost or induced in committed $T_H$ cells. Therefore, it might be more accurate to view cytokine-producing subsets in probabilistic terms. Certain factors increase the probability of stably producing a certain cytokine, such as expression/suppression of receptors, expression of transcription factors, as well as epigenetic modifications. All these factors have to be taken into account when predicting whether a $T_H$ cell will behave as a differentiated cell. Another factor worth considering is the level and ratio of transcription factors in $T_H$ cells. Recently, it has been shown that RORγt is inhibited by Stat4 and T-bet during IL-12-signaling, and that constitutive expression of RORγt has an essential role in maintenance of the *il17* locus (Mukasa et al., 2010). Hence, enforced RORγt expression strongly inhibited IL-12-induced down-regulation of IL-17 through a mechanism which blunted Stat4 activation and induction of T-bet expression. Therefore, rather than viewing transcription factor expression as an “all or none” proposition, it may be more appropriate to consider that a range of $T_H$ cells exist with varying ratios of different transcription factors and hence graded properties.

The plasticity and unstable phenotype of different $T_H$ cell subsets, for instance of $T_H17$ cells, will have important biological implications for designing therapeutic regimens to combat infections and control autoimmunity. Future experiments will have to clarify how pronounced the plasticity of $T_H$ cell subsets is *in vivo* and how relevant this is for the regulation of immune responses.

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# 9 Erklärung

Ich erkläre an Eides Statt, dass die vorliegende Dissertation in allen Teilen von mir selbstständig angefertigt wurde und die benutzten Hilfsmittel vollständig angegeben worden sind.

Teile der Dissertation wurden im European Journal of Immunology in 2008 veröffentlicht (siehe Schriftenverzeichnis).

Weiter erkläre ich, dass ich nicht schon anderweitig einmal die Promotionsabsicht angemeldet oder ein Promotionseröffnungsverfahren beantragt habe.

Die Bestimmungen der Promotionsordnung sind mir bekannt.

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# 10 Curriculum vitae

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**Publications**

Mariani L, Schulz E, **Lexberg MH**, Helmstetter C, Radbruch A, Löhning M, Höfer T. **Short-term Memory in Gene Induction Reveals the Regulatory Principle behind Stochastic IL-4 Expression.** Molecular Systems Biology. 2010 Apr 13;6:359

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**Miscellaneous**

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# 11 Schriftenverzeichnis

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