The role of EBI2 for encephalitogenic T_H17 cells in EAE and in MS

Dissertation zur Erlangung des Grades

Doktor der Naturwissenschaften

am Fachbereich Biologie

der Johannes Gutenberg-Universität Mainz

vorgelegt von

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geb. am 24. Mai 1984 in Kirchheim/Teck, Deutschland

Tag der mündlichen Prüfung: 06.06.2016

Für meine Familie

“Die Grenze des Wahren ist nicht das Falsche, sondern das Sinnlose”

- René Thom -

Acknowledgements

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1. Summary

Epstein-Barr virus-induced gene 2 (EBI2), also termed GPR183, and its ligand 7α,25-dihydroxycholesterol (7α,25-OHC) direct leukocyte migration and localization in secondary lymphoid organs. Using a novel reporter-knockin/knockout (KO) mouse model, we found that IL-23 and IL-1β induced expression of EBI2 in T_H17 cells and that its expression by myelin oligodendrocyte glycoprotein (MOG)-specific T_H17 cells promotes CNS inflammation in a transfer model of experimental autoimmune encephalomyelitis (EAE). In addition, we found that the enzymes CH25H and CYB7B1, synthesizing 7α,25-OHC from cholesterol, dramatically change expression in the spleen and spinal cord in the course of EAE, being reduced in the spleen and elevated in the CNS upon immunization. Our findings indicate that the distribution of 7 α,25-OHC changes from the periphery to CNS during EAE which fosters transmigration of encephalitogenic T_H17 cells into the inflamed CNS via EBI2.

2. Introduction

2.1 Multiple sclerosis (MS)

Multiple sclerosis is the most common inflammatory disease of the central nervous system in Europe. In Germany about 122.000 to 138.000 persons are affected, depending on different statistics (Hein and Hopfenmüller, 2000). The first reports of this disease reach back to the 13th century (Gold et al., 2006) and the first medic to give a detailed description of MS was William MacKenzie in 1840. Although many efforts in research have been done, the exact reasons for the occurrence of this disease remain to be elucidated. However studies suggest that genetic, as well as environmental factors may play a role in the pathogenesis of MS. Additionally viral infections may also increase the risk for MS, as shown for Epstein-Barr Virus (EBV) infections in children (Alotaibi et al., 2004). Initially, symptoms often occur as one isolated syndrome, which is therefore called Clinically isolated Syndrome (CIS). Depending on the localization of the inflammation in the CNS, different outcomes appear, e.g. sight disorders (Apel et al., 2006) or paralysis. The diagnosis of MS is done according to the McDonald criteria (Ghaffar and Feinstein, 2007; McDonald et al., 2001) and relies on imaging techniques as well as laboratory diagnostic. Inflammatory plaques within the brain or the spinal cord are hallmarks of MS and can be visualized by magnetic resonance imaging. Additionally, so called shadow plaques arise representing scarred regions from previous inflammations with a lower degree of myelination. Laboratory diagnosis is done for the blood and the liquor cerebrospinalis in terms of leukocyte numbers, level of c-reactive protein (CRP) (Apel

et al., 2006), as well as antibodies forming oligoclonal bands in sodium dodecyl sulfate polyacrylamide gels. It is important to perform multiple techniques for the diagnosis of MS, as it has to be distinguished from a variety of other diseases showing related symptoms, including Neuroborreliosis or HIV infection.

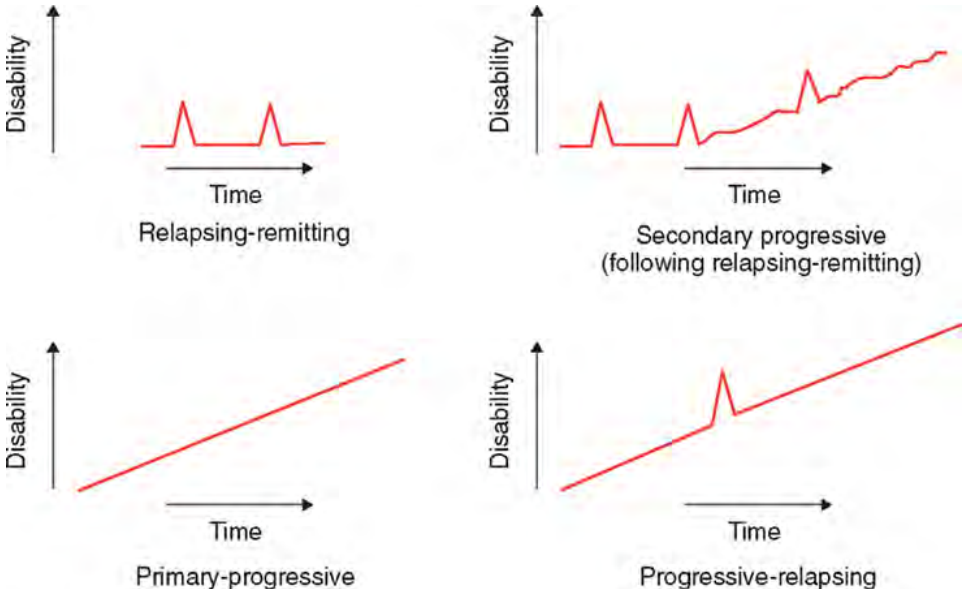

Figure 1: Forms of multiple sclerosis (MS)
The different forms of MS have been classified by the National Multiple Sclerosis Association into four main forms (Lublin FD *et al*.; Neurology; 1996)

The clinical symptoms occur in different forms and were classified by the National Multiple Sclerosis Society into four different types depicted in Figure 1:

1) Relapsing-remitting

2) Secondary progressive

3) Primary-progressive

4) Progressive-relapsing

The relapsing-remitting form shows peaks of the disease, which appear after periods of only weak or even no symptoms. In the secondary progressive form, the disease peaks disappear during further development, leading to a continuous course. The primary-progressive form is characterized by a continuously rising course of the disease from the beginning on, whereas the progressive-relapsing form is additionally accompanied by severe disease peaks. It is still not known how the innate part of the immune system initiates the activation of the adaptive immune system through antigen presenting cells like DCs and contributes to the onset of disease. As a result, the integrity of the blood-brain-barrier becomes affected during the onset of MS and allows lymphocytes and auto-antibodies to pass this hurdle into the CNS. During the pathology of MS, demyelination of the axons occurs along with axonal injury or even loss (Ferguson et al., 1997; Trapp et al., 1998) mostly dominant in the white matter of the brain. The inflammatory process is guided by $CD4^+$ T cells which dominate the lesions, at least in early stages (Flügel et al., 2001). It was shown that myelin-reactive T cells from MS patients are present in an effector or memory phenotype, whereas T cells from healthy donors show a naïve phenotype (Lovett-Racke et al., 1998; Scholz et al., 1998) and differ in cytokine secretion and expression of chemokine receptors (Crawford et al., 2004; Kivisäkk et al., 2004). Myelin-reactive T cells from MS patients show rather a T_H1 like cytokine profile compared to the T_H2-like cytokine profile of T cells from healthy donors (Lock et al., 2002). Additionally, $CD8^+$ T cells are found in high frequency and seem to expand in high rates (Babbe et al., 2000; Booss et al., 1983; Gay et al., 1997; Hayashi et al., 1988) in lesions of MS patients. As MHC class I molecules are highly expressed within the inflamed CNS by

neurons and glia cells (Höftberger et al., 2004). Interestingly granzyme B expressing $CD8^+$ T cells have been found in close proximity of demyelinated axons or oligodendrocytes (Neumann et al., 2002). It was additionally shown that MS patients show lower frequencies of $CD4^+$ $CD25^+$ regulatory T cells (Balashov et al., 1995; Viglietta et al., 2004) and reduced titers of IL-10, which may contribute to the pathology. Recent studies indicate the relevance of B cells and plasma cells in MS pathology. Both are present in the CNS of patients with progressive MS and are organized in structures which resemble B cell follicles containing B cells, plasma cells and DCs. It was shown that cytokines, involved in lymphatic tissue formation like BAFF or CXCL13 are differentially expressed and may contribute to the formation of these structures (Meinl et al., 2006). Characterization of the cerebrospinal fluid of MS patients revealed increased B cell proliferation and mutation rate. These auto-reactive B cells are not present in the periphery and it is suggested that they respond to CNS specific antigens (Owens et al., 2003; Qin et al., 1998). An interesting fact in MS pathology is, that resulting demyelination may be partially reversed by remyelination (Kornek et al., 2000) within the plaques or the whole white matter lesion (Prineas and Connell, 1979; Prineas et al., 1993). However, resulting remyelinated shadow plaques show reduced myelin density. Oligodendrocyte progenitor cells seem to be involved in the process of remyelination and are recruited to the site of inflammation (Lucchinetti et al., 1999; Prineas et al., 1989; Raine et al., 1981). This process of recovery is still not well understood and has to be further investigated. The heterogeneity of MS (Kurschus et al., 2011) and the complex processes involved in the pathology make it difficult to find appropriate

medication for this disease. However therapy with e.g. IFN-ß (Rebif®) shows beneficial results since years and new approaches have been made towards new concepts of medication. This requires extensive research, including the use of animal models for this disease and intense clinical trials.

2.2 Experimental autoimmune encephalomyelitis (EAE)

First investigations towards the pathogenesis of MS in animal models reach back to the first half of the 20th century. Koritschoner and Schweinburg showed in 1925 that injection of human spinal cord and sheep brain homogenates into rabbits may cause limb paralysis. The disease model was initially called acute disseminated encephalomyelitis, which was later changed to the term experimental autoimmune (or allergic) encephalomyelitis (EAE), used today. Immunization methods were improved by addition of Freund's adjuvant (CFS) (Freund and McDermott, 1942) and pertussis toxin (Munoz et al., 1984), enabling the induction of the relapsing-remitting form similar to MS pathology. First experiments were made in guinea pigs (FREUND et al., 1947) and monkeys (Kabat et al., 1947). Until the 1980s rats were used for investigation of MS pathogenisis, with the Lewis rat as most popular model (Croxford et al., 2011; Yang et al., 2008b). This model has the opportunity of not being dependent on pertussis toxin and therebye reduces the variety of compunds used to trigger autoimmunity. However, Lewis rats show no signs of demyelination in EAE and the sites of inflammation are mainly localized in the spinal cord, which is in contrast to human pathology (Croxford et al., 2011). Most laboratories changed from rats to mice (OLITSKY and YAGER, 1949), as they are easier to breed and therefore

much cheaper. The C57BL/6 and SJL/J strains are usually subjected to EAE induction, but also BALB/c mice are susceptible to EAE, when immunized properly (Määttä et al., 1998). The next important step in improving the model, was to reduce the complexity of the material used for immunization, to allow exact reproducibility. Today, peptides from myelin proteins localized at different positions within the myelin sheath are widely used for immunization and include myelin basic protein (MBP) (EINSTEIN et al., 1962), myelin oligodendrocyte glycoprotein (MOG) (Lebar et al., 1986; Wei et al., 2007) and proteolipid protein (PLP) (Tuohy et al., 1988). However, there is evidence for various other autoantigens to be involved in the pathogenesis of MS and EAE. Besides the direct immunization of mice with autoantigens emulsified in CFA, viral models may be used for induction of neuroinflammation. The most commonly used model is induced by infection with Theiler's virus (Theiler, 1937), but even expression of lymphocytic choriomeningitis virus proteins triggers demyelination and inflammation in the CNS of mice (Evans et al., 1996). Both the viral model and the model depending on immunization with auto antigens emulsified in CFA require an effective induction of the host immune system to induce autoimmunity. Adoptive transfer of either total lymph node cells (PATERSON, 1960), antigen specific T cells (Ben-Nun et al., 1981) or IL-23 dependent antigen-specific T_H17 cells (Langrish et al., 2005) offer the possibility to study the pathogenicity of different cell types in healthy recipient mice. The generation of specific knock-out animals or mice overexpressing different proteins, enabled a better understanding of gene functions and their interplay in the pathogenesis of the disease. One of the first knock-out mice to be used for studies in the EAE model, was

deficient in IL-6 and showed complete resistance towards disease induction (Mendel et al., 1998; Okuda et al., 1998; Samoilova et al., 1998). Also mice expressing transgenic T cell receptors (TCRs) specific for antigens relevant in MS pathology were created on different backgrounds, e.g. C57BL/6 or SJL/J background. Strikingly mice with transgenic TCR specific for the MOG_{35-55} peptide (2D2 mice), generated by Bettelli *et. al* (Bettelli et al., 2003), develop spontaneous EAE in low frequency and are often used for adoptive transfer experiments. However, the different animal models do not show all pathological outcomes of MS and the results may not reflect the actual situation in patients. Multiple studies demonstrate compounds with beneficial effects in the mouse model failed to reproduce the same results in clinical trials. Nevertheless, some of these studies led to the development of approved medication and show beneficial effects in MS therapy, like Glatiramer acetate (Copaxone), IFN-ß treatment (Rebif), VLA-4 blocking (Tysabri), anti-CD20 antibodies (Rituximab) or S1PR antagonist (Fingolimod). Compounds with possible implications for MS treatment may be analyzed in the mouse model prior to human application in order to clarify their relevance and reveal possible side effects and are therefore essential for clinical research.

2.3 T cells

T cells are part of the adaptive immune system and arise from hematopoietic stem cells in the bone marrow. Precursor cells migrate into the thymus, where they develop their TCR specificity by rearrangement of the receptor genes. T cells express additionally co-receptors supporting the recognition of antigens presented on MHC

molecules. Depending on recognition of MHC molecules during thymic development, T cells express either of the two co-receptors. Thus they are restricted to recognize antigens presented on MHC I (CD8 co-receptor) or MHC II (CD4 co-receptor) molecules. Precursor cells arriving in the thymus do not express CD4 or CD8 co-receptors. However in later developmental stages T cells co-express both CD4 and CD8 and become single positive for one of the two receptors during further maturation. This process goes along with positive and negative selection by antigen presenting cells (APCs) for functionality and autoreactivity in the thymic stroma (Stutman, 1978). Autoreactive and non-functional cells are eliminated by induction of apoptosis. This maturation from precursor cells to naïve T cells results in two different populations, $CD4^+$ helper T cells and $CD8^+$ T cells. Naïve T cells are released into the periphery and migrate to adjacent lymph nodes in order to meet APCs presenting their cognate antigen. Both T cell subsets are activated by DCs, which are the only APCs shown to stimulate naïve T cells effectively. During this process, T cells are stimulated to proliferate and differentiate into distinct phenotypes depending on the cytokine milieu, the extent of TCR signaling and different expression of stimulatory/inhibitory molecules by DCs (Constant and Bottomly, 1997; Constant et al., 1995; O'Garra, 1998). $CD8^+$ T cells are primed for cytotoxic activity during this process and may eliminate infected or malignant cells by various mechanisms, e.g. the perforin /granzyme pathway. The effector phenotypes of $CD4^+$ T cells may be further divided into different subpopulations according to their function and cytokine expression.

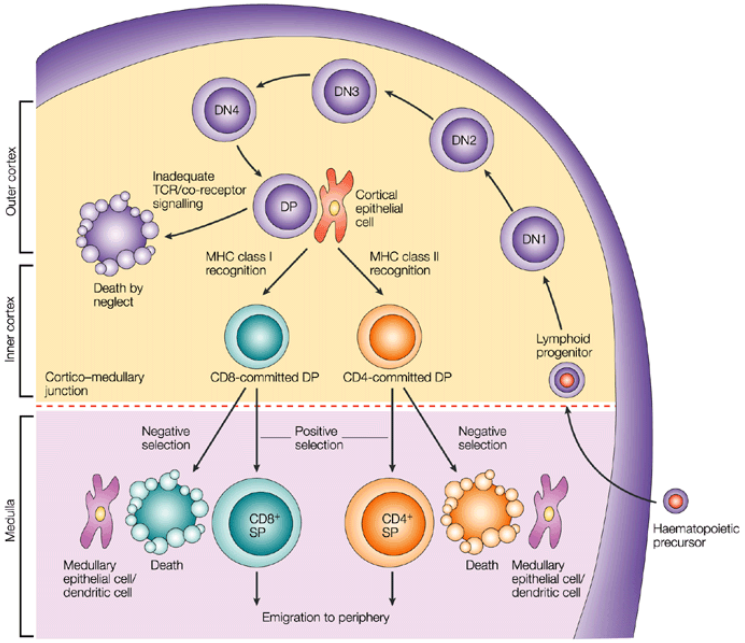

Figure 2: T cell development in the thymus

T cell precursors migrate from the bone marrow into the thymus, were they acquire their antigen specificity and expression of co-receptors. Arriving T cell precursors do not express co-receptors (DN). In later stages they initially express CD4 together with CD8 and are called "double-positive" (DP). During positive and negative selection in the thymic stroma, they stop expression of either CD4 or CD8 and migrate into the periphery. (Germain RN *et al.*; Nature Reviews Immunology; 2002)

2.3.1 T_H1 cells

The hallmark cytokine produced by T_H1 cells is IFNγ, which is essential for immune responses to intracellular pathogens such as viruses or mycobacteria. It is important for the activation of mycobacteria-infected macrophages, contributes to the activation of cytotoxic $CD8^+$ T cells and initiates antibody class-switching to IgG isotypes. Therefore, animals with deletion of the IFNγ receptor suffer from severe mycobacteria infections, as they fail to control the pathogen properly. Additionally, activated T_H1 cells express IL-2, which triggers proliferation in an autocrine manner, thereby amplifying the response.

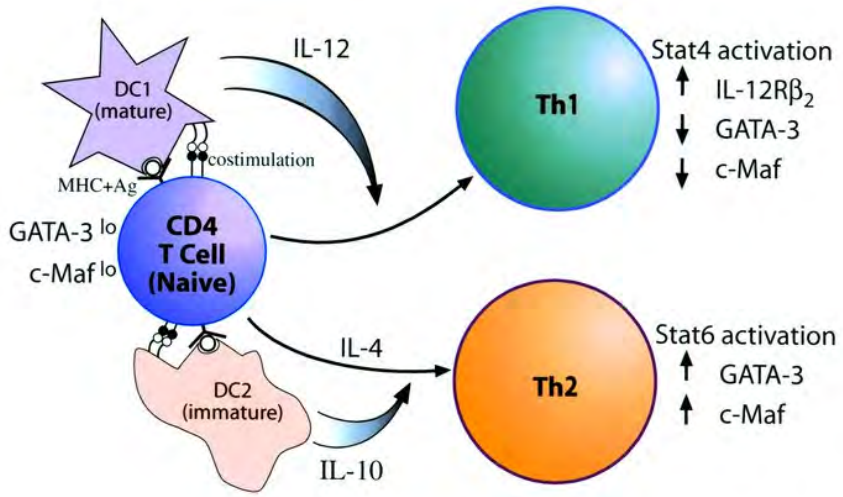

Figure 3: T_H1 and T_H2 differentiation
Naïve $CD4^+$ T cells are stimulated by DCs presenting their cognate antigen on MHC II molecules. Different cytokines influence the commitment to the different T cell subsets by influencing the main transcription factors T-Bet and GATA-3, as well as expression of respective cytokine receptors. (Anuradha Ray *et al.* ; J Clin Invest.; 1999)

The development of this subset requires IL-12 (Hsieh et al., 1993) and IFNγ (Lighvani et al., 2001) besides activation of the TCR. As IL-12 is mainly secreted by activated macrophages the positive feedback-loop of IFNγ triggers differentiation of other T cells towards this phenotype, while inhibiting T_H2 differentiation. On the molecular level the transcription factor T-Bet has been shown to be essential for T_H1 development (Szabo et al., 2000), as it triggers high expression of IFN-γ while inhibiting T_H2 specific genes. Mice lacking T-Bet are unable to clear intracellular infections (Szabo et al., 2000) comparable to IFNγ deficient mice, showing the tight connection of these two genes. The expression of T-Bet leads to commitment of T cells to the T_H1 phenotype, as it inhibits GATA3, which is the key transcription factor for T_H2 differentiation (Hwang et al., 2005). It has been shown that T-Bet is required for proper differentiation even when GATA 3 is blocked, indicating further functions in T_H1 development (Zhang and Boothby, 2006). Besides the blockade of other

differentiation directions, T-Bet directly triggers expression of the IL-12 receptor ß2 chain (IL-12rb2), thereby increasing IL-12 responsiveness (Afkarian et al., 2002). The initial expression of T-Bet is accomplished via IFNγ signaling and activation of the TCR, leading to expression of IL-12rb2 and IFNγ. The resulting feedback-loop further enhances T_H1 differentiation and contributes to clonal expansion by IL-2 secretion (Mullen et al., 2001). Recent studies additionally suggest that IFNγ producing T_H1 cells are an important source of IL-10, thereby executing a regulatory role. It is suggested that IL-10 production is the final step in T_H1 development and occurs after several periods of restimulation and effector activity. This process results in anergized cells, which have stopped expression of effector cytokines, but maintain IL-10 production and thus control themselves.

2.3.2 T_H2 cells

The hallmark cytokine produced by T_H2 cells is IL-4, but also other cytokines, including IL-5 and IL-13 are expressed by this subset. In contrast to T_H1 cells they do not produce IFNγ and lymphotoxin (Zhu et al., 2010). The main function of T_H2 cells is to direct the immune system against extracellular pathogens, e.g. parasites. They coordinate antibody responses, favoring isotype switching to IgE and initiate eosinophil expansion and activation. However, T_H2 cells are additionally associated with allergic disease, by stimulating elevated secretion of IgE autoantibodies, leading to activation of mast cells and eosinophils. It was shown that besides TCR engagement, IL-4 and IL-2 are needed for T_H2 differentiation *in vitro* (Cote-Sierra et al., 2004; Le Gros et al., 1990). Initial IL-4 may be derived by basophilic granulocytes,

mast cells and NKT cells, but may be also produced by naïve T cells prior to first antigen recognition. The main transcription factor for T_H2 differentiation is GATA3 induced by IL-4 mediated STAT6 signaling (Kurata et al., 1999; Zhu et al., 2001). Activation of STAT5 is additionally required to induce effective IL-4 production by up-regulating the expression of IL-4Rα (Liao et al., 2008) and is critical to maintain GATA3 expression in later stages. Expression of GATA3 in naïve $CD4^+$ T cells leads to the commitment to T_H2 differentiation inhibiting the T_H1 direction. GATA3 down-regulates STAT4 expression which is important for IL-12 signaling (Usui et al., 2003). T-Bet expression is further inhibited by a constitutively active form of STAT5 (Zhu et al., 2003) and direct interactions with GATA3. Although IL-4 induces GATA3 *in vitro*, other factors may be involved in the differentiation of T_H2 cells *in vivo*, as IL-4 induced GATA3 is not required under several conditions. This suggests that GATA3 expression may be up-regulated by other factors than IL-4 signaling or STAT5 activation.

2.3.3 T_H17 cells

T_H17 cells have been characterized by their expression of IL-17A, but they also express IL-17F, IL-21 and IL-22. They seem to be required to support T_H1 and T_H2 cells to handle difficult pathogens and are crucial for clearance of Staphylococcus aureus and Candida albicans infections. However it was shown that they are also associated with many experimental and human autoimmune diseases. They were first discovered by the identification of IL-23 which shares the p40 subunit with IL-12 (Becher et al., 2002; Oppmann et al., 2000) but binds to p19 (IL-23) instead of p35

(IL-12) and is crucial for T_H17 cell differentiation. Moreover, myelin specific T_H17 cells generated in the presence of IL-23 may induce EAE when adoptively transferred into naïve wildtype mice (Langrish et al., 2005). However, naïve $CD4^+$ T cells do not express the receptor for IL-23, therefore it seems that it is up-regulated upon activation and during differentiation (Bettelli et al., 2006). T_H17 cells may be effectively differentiated from naïve $CD4^+$ T cells *in vitro* by activation in the presence of TGF-ß and IL-6 (Bettelli et al., 2006; Mangan et al., 2006; Veldhoen et al., 2006), or TGF-ß and IL-21 (Korn et al., 2007; Thornton and Shevach, 1998). Further experiments showed that TGF-ß is dispensable for human and murine T_H17 development and IL-1ß together with IL-6 / IL-23 (Acosta-Rodriguez et al., 2007; Wilson et al., 2007) may compensate the lack of TGF-ß signaling. The exact differentiation process of T_H17 cells remains to be clarified although the steroid receptor-type nuclear receptor RORγt seems to be the major transcription factor for differentiation of T_H17 cells and is essential for IL-17 production (Ivanov et al., 2006). Additionally, RORα is selectively expressed in T_H17 cells and was shown to be able to fulfill similar roles as RORγt (Yang et al., 2008b). Expression of RORγt is STAT3 dependent which is activated by IL-6, IL-21 or IL-23 and is important for IL-17 production in T cells (Mathur et al., 2007; Yang et al., 2007; Zhou et al., 2007) as it binds directly to the *Il17* and *Il21* promoters (Wei et al., 2007). It was shown that RORγt also cooperates with yet unidentified transcription factors. The interferon regulatory factor 4 (IRF4) plays an important role in T_H1 and T_h2 differentiation (Lohoff et al., 2002; Rengarajan et al., 2002), but seems also to be required for T_H17 differentiation as IRF4 KO mice fail to raise a T_H17 response(Brüstle et al., 2007).

Differentiated T_H17 cells have additionally been shown to be transient in nature, as they shift towards an IL-17 and IFNγ producing phenotype when transferred into naïve WT mice (Kurschus et al., 2010).

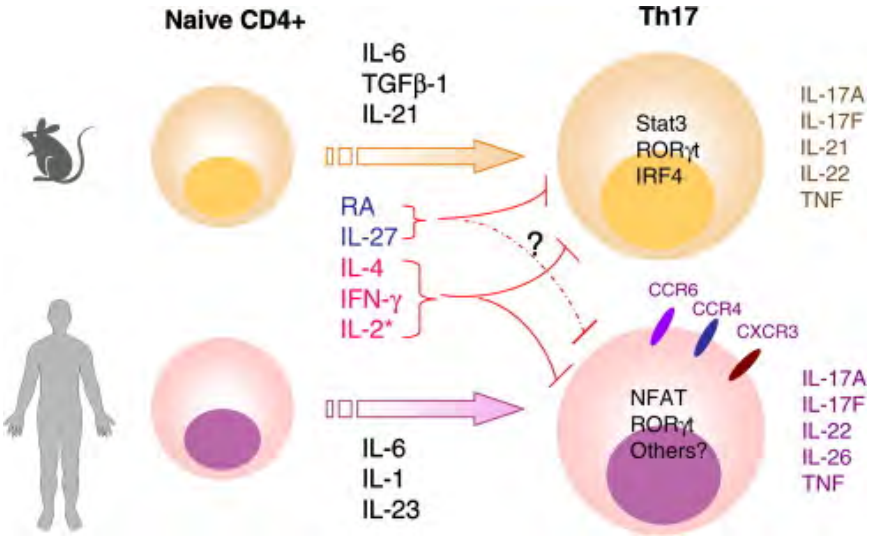

Figure 4: T_H17 differentiation in mice and humans
In mice TGF-ß, IL-6 and IL-21 are involved in T_H17 development, whereas in humans IL-1, IL-6 and IL-23 are needed for effective differentiation. (Zhi Chen *et al.*, National Institutes of Health, Bethesda, MD, USA; 2007)

Recent studies also raise the possibility that T_H17 cells may trans-differentiate into T_R1 like cells, being anti-inflammatory and to less extend acquire a T_{reg} phenotype (Gagliani et al., 2015). These findings suggest a mechanism of T_H17 cells to balance and resolve immune reactions which might be similar to plasticity and fate of T_H1 cells expressing IL-10 at later stages. Further experiments need to clarify the exact differentiation process, elucidate the difference between TGF-ß and IL-1ß differentiated $T_{H1}7$ cells and to define the functions and molecular mechanism of T_H17 plasticity.

2.3.4 Regulatory T cells (T_{reg})

Regulatory T cells protect the body from overwhelming immune responses. They occur as natural T_{regs} (nT_{reg}) which develop in the thymus and are specific to self-antigen, or inducible Tregs (iT_{regs}) generated in the periphery being specific to environmental antigens. They can be further classified into naïve, effector or memory phenotypes which show different properties (Huehn et al., 2004). It was shown that effector- and memory-T_{regs} may express effector cytokines like IL-17 of IFNγ under different conditions (Feng et al., 2011; Koenen et al., 2008). This suggests a heterogeneous population of nT_{regs} showing either a committed phenotype or plasticity. Regulatory T cells specific for tissue self-antigen reside in draining lymph nodes (Samy et al., 2005) to become activated while other T_{regs} may migrate to inflammatory sites or tumors (Belkaid et al., 2002). In mice both subsets show similar functions *in vivo* and *in vitro* (DiPaolo et al., 2007), whereas human iT_{regs} fail to demonstrate activity in functional *in vitro* assays (Tran et al., 2007). This might be due to the fact that expression of the main transcription factor forkhead box P3 (FoxP3) can be induced in human $CD4^+$ T cells upon activation without differentiation to a suppressive phenotype and suggests different functions of FoxP3 expression in humans and mice (Miyara et al., 2009; Tran et al., 2007). The importance of regulatory T cells in controlling immune responses has been shown as depletion of T_{regs} leads to severe autoimmune disease, as well as immune reactions to the bacteria flora in the intestine resulting in inflammatory bowel disease (IBD) (Singh et al., 2001). Furthermore T_{regs} have moved into focus of transplantation and cancer research concerning their suppressive role (Wood and Sakaguchi, 2003; Yamaguchi

and Sakaguchi, 2006). About 5-10 % of $CD4^+$ T cells are nT_{regs}, characterized by the expression of CD25 and FoxP3, but also express a variety of other stimulatory or inhibitory surface molecules, including CD28 and cytotoxic T lymphocyte antigen 4 (CTLA-4). Immune suppressive effects are mediated by secretion of different anti-inflammatory cytokines, e.g. IL-10 or TGF-ß. It was shown that the expression of FoxP3 is crucial for the development of nT_{regs} and retroviral transduction of the FoxP3 gene into $CD4^+$ $CD25^-$ T cells leads to conversion into $CD4^+$ $CD25^+$ T_{regs} showing suppressive activity *in vitro* and *in vivo*. They are characterized by expression of other markers, e.g. CTLA-4 or neuropilin-1. The expression of initial FoxP3 is induced during the maturation in the thymus in the late double-positive stage and requires TCR engagement but also co-stimulatory signals as well as cytokine-signaling (Kim et al., 2009; Samon et al., 2008). It was show that IL-2 is a major cytokine required for nT_{reg} development (Fontenot et al., 2005), but also TGF-ß seems to play a role in nT_{reg} homeostasis and suppressive function due to induction of FoxP3 expression (Chen et al., 2003; Liu et al., 2008). In contrast to nT_{regs} other suppressive regulatory T cell populations have been described including IL-10 secreting T_R1 cells and TGF-ß induced T_H3 cells (Mills and McGuirk, 2004). However T_H3 cells seem to be $FoxP3^+$, whereas T_R1 cells do not express FoxP3 but secrete IL-10. In addition T_R1 cell show the closest relation to nT_{regs}, demonstrated by their lower proliferation rate and IL-2 secretion, as well as cell contact-dependent suppression (Vieira et al., 2004).

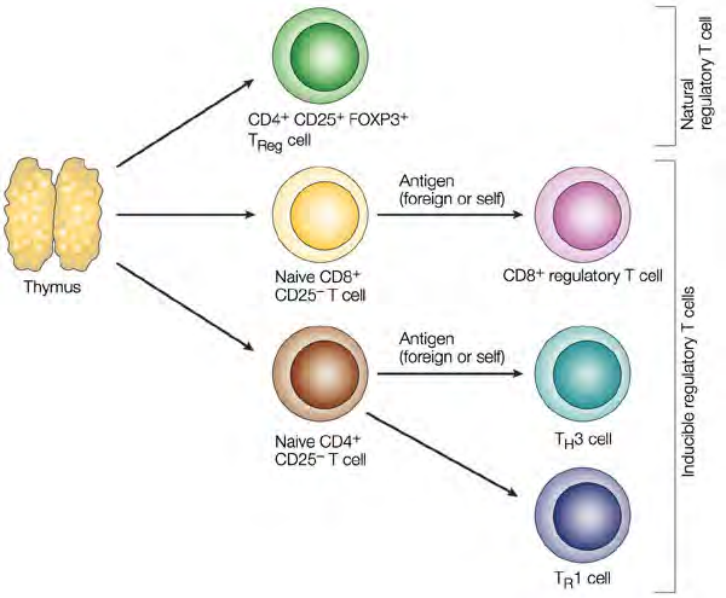

Figure 5: Regulatory T cell development

Regulatory T cells are divided into thymus derived natural occurring Tregs and subsets which are primed in the periphery and are therefore called inducible regulatory T cells. This group includes $CD8^+$ T_{regs}, T_H3 and T_R1 cells. (Mills *et al*.; Nature Reviews Immunology; 2004)

Regulatory T cells are able to suppress the proliferation of antigen stimulated naïve T cells *in vitro* (Thornton and Shevach, 1998), or inhibit autoimmune disease like IBD when transferred into T_{reg} depleted mice (Sakaguchi et al., 1995; Singh et al., 2001). Additionally, regulatory T cells suppress cytokine production (e.g. IL-2) by antigen specific $CD4^+$ and $CD8^+$ T cells as well as $CD8^+$ cytotoxicity. It is suggested that cell contact is essential for suppression of responder T cells, as culture of both populations separated by a membrane does not inhibit the proliferation of responder cells (Takahashi et al., 1998; Thornton and Shevach, 1998). Furthermore culture supernatant of regulatory T cells failed to inhibit proliferation of responder cells. T_{regs} may induce apoptosis via the perforin/granzyme B pathway (Cao et al., 2007; Gondek et al., 2005), or interact with B7 expressed by the responder T cells (Paust et al., 2004). Moreover, interaction with APCs lead to down-regulation of CD80 and CD86 expression and lower co-stimulatory capacity of the APCs.

Additionally, they may trigger up-regulation of indoleamine-2,3-dioxygenase (IDO) expression in APCs. IDO catalyzes the conversion of tryptophan to kynurenine, which is toxic to T cells and limits their proliferation (Fallarino et al., 2002; Grohmann et al., 2002). Beside these cell contact dependent mechanisms of suppression, other humoral pathways may be involved and include IL-10 or TGF-ß secretion by regulatory T cells. However, neutralization of IL-10 or TGF-ß does not alter *in vitro* suppression of T_{regs} (Takahashi et al., 1998; Thornton and Shevach, 1998) but show importance *in vivo*, as IL-10 deficient mice are unable to suppress IBD. Furthermore, combined blockade of IL-10R with TGF-ß neutralization abrogates T_{reg} mediated suppression of the disease. TGF-ß may act as a membrane bound form and contribute to maintenance of FoxP3 expression and suppressive function. Yet another novel cytokine, IL-35 may also contribute to immune suppression, as IL-35 deficient T_{regs} are less suppressive *in vivo* as well as *in vitro* (Collison et al., 2007).

2.4 G protein coupled receptors (GPCRs)

G protein coupled receptors represent the largest superfamily of cell surface receptors with around 1000 members and exist in most eukaryotes (Vassilatis et al., 2003). The different ligands range from small molecules to large proteins. Interestingly, agonistic / antagonistic compounds for GPCRs constitute about one third of currently available pharmaceutical drugs (Wise et al., 2002). In general, these receptors consist of a highly conserved seven-transmembrane domain and are coupled to G proteins. Initial studies on rhodopsin (Downer and Cone, 1985; Liebman and Entine, 1974), muscarinic (Dadi and Morris, 1984) and ß-adrenergic

(Lefkowitz et al., 1972) GPCRs suggested monomeric forms of the receptors, however later experiments indicate that a fraction may also be present in lipid raft regions (Barnett-Norris et al., 2005; Insel et al., 2005a; 2005b) of the plasma membrane as dimers (Angers et al., 2002; Bulenger et al., 2005; George et al., 2002; Lee et al., 2003) or oligomers (Dadi and Morris, 1984; Lefkowitz et al., 1972). Signaling via these receptors is initiated by conformational change of the heterotrimeric G protein complex consisting of G_{α} and $G_{\beta\gamma}$ subunits. Upon ligand binding, GDP is exchanged by GTP through catalytic activity of the G_{α} subunit acting as guanine exchange factor (GEF) and results in its dissociation from the $G_{\beta\gamma}$ dimer (Wall et al., 1998). Further signal transduction depends on interaction with downstream proteins. G protein dependent signaling is divided into sub-classes depending on the G_{α} subunit being involved. Different sub-classes ($G_{\alpha s}$, $G_{\alpha i/o}$, $G_{\alpha q/11}$ and $G_{\alpha 12/13}$) were distinguished by sequence homology and include multiple proteins (Wall et al., 1998). GPCRs show preferential signaling via distinct G_{α} subunits although they may activate other subtypes as well. However each sub-class activates pathways being dependent on different effector proteins. In addition to catalytic activity of the G_{α} subunit as GEF, it also harbors intrinsic guanine triphosphatase (GTPase) activity. The resulting hydrolysis of GTP to GDP renders the G_{α} subunit into its inactive state, thereby terminating signaling interactions (Ford et al., 1998; Li et al., 1998).

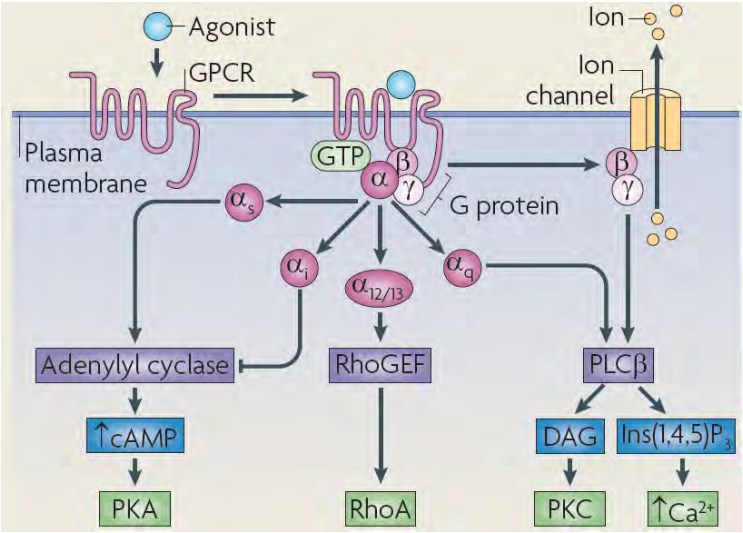

Figure 6: G protein mediated signaling

Signaling via GPCRs is triggered via GTP mediated dissociation of the G_{α} subunit from the trimeric complex. Shown are different pathways which are involved in the cascade and are activated by the respective sub-classes of the G_{α} subunit. (Hall *et al.*; Nautre Reviews; 2009)

2.4.1 $G_{\alpha s}$ / $G_{\alpha i/o}$ signaling pathway

This pathway leads to activation ($G_{\alpha s}$) (BERTHET et al., 1957; Ross and Gilman, 1977; SUTHERLAND and RALL, 1958) or inhibition ($G_{\alpha i/o}$) (Hildebrandt and Birnbaumer, 1983; Hildebrandt et al., 1983; Hsia et al., 1984; Smith and Limbird, 1982) of adenylate cyclase which catalyzes the conversion of ATP to cAMP. Signaling via different GPCRs coupled to these subunits may therefore counteract each other. Intracellular levels of cAMP stimulate ion channels, as well as protein kinase A (PKA) acting as second messenger and secondary effector respectively.

2.4.2 $G_{\alpha q/11/14/16}$ signaling pathway

Signaling by $G_{\alpha q/11}$ coupled GPCRs involves activation of phospholipasce Cβ (PLCβ) and triggers cleavage of phosphatidylinositol-4,5-biphosphate (PIP2) into inositol (1,4,5) trisphosphate (IP3) and diacetylglycerol (DAG) (Rhee, 2001). The second

messenger IP3 binds to receptors at the endoplasmatic reticulum and stimulates release of Ca^{2+} from the ER into the cytosol. Additionally, DAG activates protein kinase C (PKC) localized at the plasma membrane. Further signaling is transduced by Ca^{2+} binding proteins called calmodulins which bind and activate $Ca2^{+}$/calmodulin-dependent kinases (CAMKs).

2.4.3 $G_{\alpha 12/13}$ signaling pathway

GPCR signaling this pathway involves Guanin exchanging factor (GEFs) Rho A which acts as small cytosolic GTPase when bound to $G_{\alpha 12/13}$ subunits (Worthylake et al., 2000). The resulting exchange of GDP by GTP activates Rho A and regulates other proteins e.g. Rho-kinase (Fukuhara et al., 2001; Martin et al., 2001; Whitehead et al., 2001; Zohn et al., 2000).

2.4.4 $G_{\beta\gamma}$ signaling pathways

Initially, it was suggested that $G_{\beta\gamma}$ subunits inhibit G_{α} mediated signaling as they display guanine nucleotide dissociation inhibitor (GDI) activity. Interestingly, it is now clear that $G_{\beta\gamma}$ dimers activate individual effectors after dissociation of the G_{α} subunit. The first identified interaction partner was G protein-regulated inward-rectifier K^{+} channels (GIRK) where the G_{α} subunit binds directly to the N- and C-termini (Doupnik et al., 1996; Huang et al., 1995; Inanobe et al., 1995; Lei et al., 2000). Furthermore, activation of several kinases, e.g. ERK1/2, JNK, PI3K and p38 mitogen activated protein kinases (MAPKs) by $G_{\beta\gamma}$ dimers has been demonstrated (Coso et al., 1996; Crespo et al., 1994; Faure et al., 1994; Yamauchi et al., 1997; Yi et al., 2012).

2.4.5 G protein independent signaling pathways

Additionally, G protein independent signaling pathways have been shown. The C-terminus of most GPCRs is rich in serine and threonine residues, which show high affinity for ß-arrestins when phosphorylated. Recruitment of ß-arrestins to the phosphorylated C-terminus prevent coupling to G proteins and lead to assembly of other signaling complexes which trigger activation of the extracellular-signal regulated kinase (ERK) pathway or results in internalization of the receptor. Furthermore, several GPCRs have been shown to interact with proteins of the janus kinase (JAK) family of tyrosine kinases upon agonist binding, transmitting signals via signal transducers and activators of transcription (STAT) family members (Godeny et al., 2007; Liang et al., 1999; Yi et al., 2012).

2.4.6 Termination of GPCR signaling

Termination of GPCR mediated signaling may be triggered by phosphorylation of the receptor via serine / threonine specific GPCR kinases (GRKs: GRK1-7) (Ferguson, 2001; Hausdorff et al., 1991; Penela et al., 2006; Suan et al., 2015) and through association with arrestins (Chalmin et al., 2015; Hanyaloglu and Zastrow, 2008; Moore et al., 2007), leading to internalization. As an example, agonistic stimulation of β_2-adrenergic receptors was found to decrease their surface distribution by internalization into the cytosol (Chuang and Costa, 1979; Reboldi et al., 2014). GPCR internalization is mediated via clathrin-coated or un-coated vesicles, called caveolae. After GRK induced phosphorylation of GPCRs, β–arrestin is recruited and stimulates the machinery providing clathrin-coated vesicles (Goodman et al., 1996; Laporte et

al., 1999; Rutkowska et al., 2015). In this process GPCRs are ubiquitinated by three enzymes (E1-E3) (Hershko and Ciechanover, 1998; Rutkowska et al., 2015), which leads to degradation in lysosomes after internalization (Hanyaloglu and Zastrow, 2008).

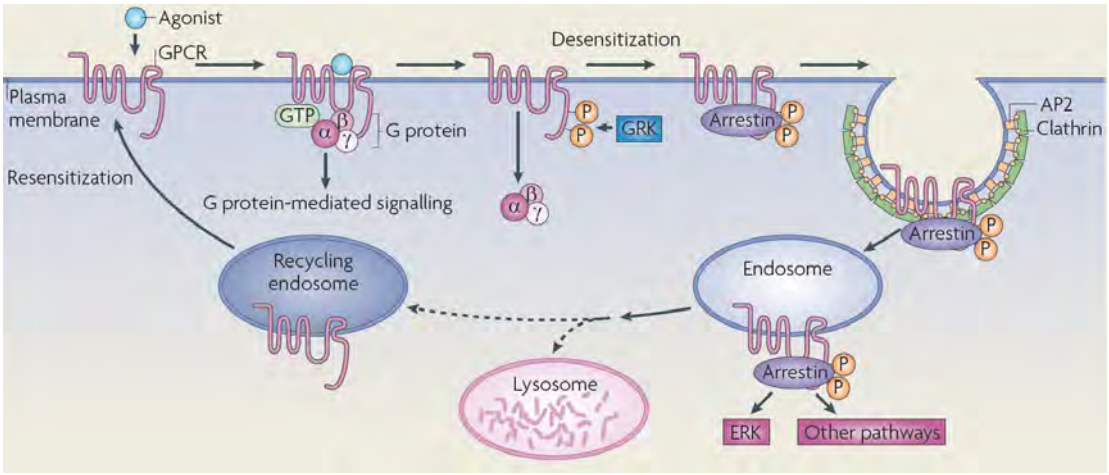

Figure 7 Arrestin mediated GPCR signaling and degradation
Agonistic stimulation of GPCRs is regulated and shut down by arrestin mediated engulfment of the receptor complex. This desensitization process is induced by recruitment of arrestins to phosphorylated sites in the N-terminus of the protein. GPCRs enclosure into endosomes either leads to degradation of the protein in lysosomes or to receptor recycling. (Hall *et al*.; Nature Reviews; 2009)

However ubiquitination of β-arrestin has been shown to determine the stability of the binding to the respective GPCRs and defines them as class A or class B GPCRs. Class A GPCRs display fast separation of β-arrestin due to rapid deubiquitination, whereas Class B GPCRs show stable coupling as a result of enduring ubiquitination (Shenoy and Lefkowitz, 2003). Upon internalization, receptors are sorted for multiple pathways leading to recycling or degradation and my trigger G protein independent signaling pathways. These processes thereby control desensitization and resensitization of GPCR mediated signaling.

2.5 Epstein Barr Virus induced gene 2 (EBI2)

Epstein Barr virus induced gene 2 (EBI2), also termed GPR183 was identified in 1993 among other genes to be induced in a Burkitt's lymphoma cell line after infection with EBV (Birkenbach et al., 1993). Signaling via EBI2 is mediated by pertussis toxin sensitive G proteins of the $G_{\alpha i/o}$ sub-class. Ligand binding therefore results in calcium mobilization, cAMP reduction and ERK activation (Gatto et al., 2011). For a long time EBI2 remained orphan and even ligand independent activation processes were speculated. However, in 2011 two groups independently de-orphanized EBI2 with 7α,25-dihydroxycholesterol (7α,25-OHC) as most potent ligand (Hannedouche et al., 2011; Liu et al., 2011). It is generated from cholesterol as an intermediate of the alternate pathway of hepatic bile acid synthesis and was shown to be regulated by differential expression of three enzymes. Cholesterol hydoxylase (CH25H) mediates hydroxylation of cholesterol to 25-hydroxycholesterol (25-HC) (Russell, 2003). Surprisingly, CH25H is hardly present in the liver in contrast to other enzymes involved in bile acid synthesis. However CH25H is abundant in many other tissues, suggesting roles outside the liver (Russell, 2003; 1998) and in different processes than cholesterol metabolism. In a second step CYP7B1 converts 25-HC to 7α,25-dihydroxycholesterol (7α,25-OHC) (Russell, 2003). This enzyme belongs to the cytochrome p450 family of proteins and is highly expressed in the liver and in other tissues. Furthermore, the biologically active ligand may be metabolized by 3β-hydroxy-Δ^5-C_{27} steroid oxidoreductase (HSD3B7) into its 3-oxo derivative, resulting in loss of EBI2-specific ligand activity.

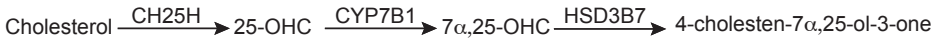

Figure 8: EBI2 ligand synthesis pathway
Cholesterol 25-Hydroxylase (CH25H) mediates hydroxylation of cholesterol, in a second step CYP7B1 driven hydroxylation of 25-hydroxycholesterol generates the active EBI2 ligand 7α,25-dihydroxycholesterol (7α,25-OHC). The active ligand may be degraded by HSD3B7 into its 3-oxo derivate.

These enzymes play an important role in the regulation of bile acids, as deficiency for HSD3B7 leads to vitamin deficiency and cholesterol malabsorption. The different intermediates exhibit lipophilic properties and may easily traverse the cell membrane. Therefore the different enzymes involved in generation and inactivation of the active EBI2 ligand may be expressed by different cells (Yi et al., 2012).

2.5.1 Role of EBI2 in B cells

To mount an appropriate antibody response with long term memory, B cells need to undergo several maturation steps taking place in different compartments of lymphoid follicles. Homing to the follicles and movements within this structure are guided by sequential expression of chemokine receptors on B cells and varying distribution of the respective ligands. It is known, that expression of CXCR5 is high in naïve B cells and crucial for migration to the follicle, subsequently disturbance of the CXCR5-CXCL13 axis results in loss of normal lymphoid structures. After antigen encounter, B cells up-regulate CCR7 expression mediating migration to the T-B boundary by expression of the chemokines CXCL19 and CXCL21. After interaction with T cells, CCR7 expression is down-regulated and B cells migrate to the inner and outer follicle. Recently, Gatto *et al.* found EBI2 expression to be involved in B cell positioning by regulation of EBI2 expression levels. They could show, that naïve B

cells express EBI2 although it is not involved in homing to the follicles like expression of CXCR5 (Gatto et al., 2011; 2009; 2013).

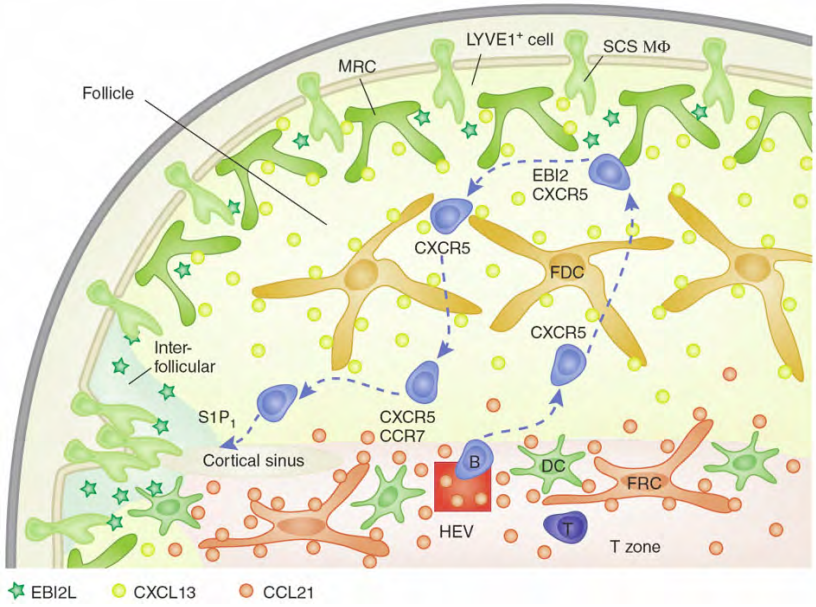

Figure 9: B cell movements in lymphoid follicles

Naïve B cells enter lymphoid follicles by CXCR5:CXCL13 mediated chemotaxis. Upon antigen stimulation migration to the outer follicle is guided via EBI2 and CXCR5. Egress from lymphoid follicles via the cortical sinus is dependent on $S1P_1$ signaling. (Cyster J.; Nature Immunology; 2010)

However upon antigenic stimulation, EBI2 expression is transiently up-regulated in an NFκB dependent manner. Deficiency of EBI2 leads to accumulation of B cells in the follicle center, suggesting an important role of EBI2 in early activation of B cells. EBI2 mediated positioning to the outer follicle is followed by CCR7 directed movement to the T-B boundary. After interaction with T cells and CD40 engagement, EBI2 expression is again elevated accompanied by down-regulation of CCR7 expression, leading to migration of B cells to the outer follicle. Subsequently, deficiency for EBI2 or enzymes involved in ligand synthesis, results in accumulation of B cells in the follicle center along with delayed antibody responses and impaired plasma cell development after immunization (Hannedouche et al., 2011; Pereira et al., 2009). Barroso *et al.* further reported that EBI2 may form heterodimers with

CXCR5 resulting in decreased binding affinity for CXCL13, thereby regulating B cell migration independent of 7α,25-OHC (Yi et al., 2012).

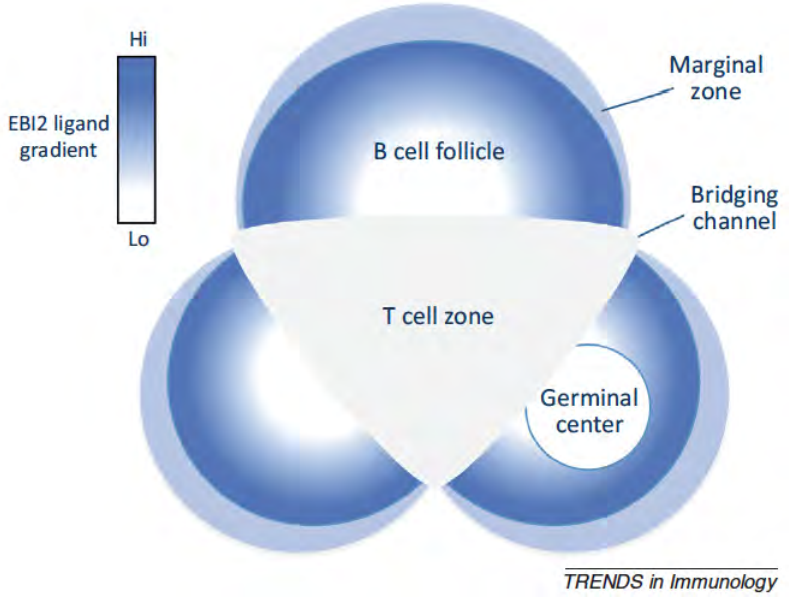

Figure 10: Distribution of EBI2 ligand in lymphoid follicles
Enzymes involved in generation of the EBI2 ligand 7α,25-OHC are differentially expressed in lymphoid follicles in the spleen. This leads to high distribution of the ligand in the outer follicle areas and low concentration in the germinal centers and the T cell zone. (Gatto et *al.*; *Nature*; 2013)

Furthermore, it was shown that 7α,25-OHC is distributed in a gradient-like fashion with high concentrations in the outer follicle and low concentrations in the germinal centers (Yi et al., 2012) Concentrations of 7α,25-OHC correlate with EBI2 expression of B cells within these compartments. Different cells participate in the generation of this gradient. It was shown, that lymphoid stromal cells express CH25H and CYP7B1 to generate 7α,25-OHC, whereas stromal cells expressing HSD3B7 regulate its distribution by inactivating the ligand. Furthermore, follicular dendritic cells (FDCs) seem to be involved in this network as well, although their exact contribution needs to be elucidated (Yi et al., 2012).

2.5.2 Role of EBI2 in dendritic cells (DCs)

Two groups showed that splenic $CD4^+$ DCs express high levels of EBI2 in contrast to migratory $CD8^+$ DCs. Interestingly they are strongly diminished in EBI2 deficient mice (Gatto et al., 2013; Yi and Cyster, 2013). Furthermore, they suggest that 7α,25-OHC concentration is high at marginal zone (MZ) bridging channels within the lymphoid follicles due to high expression of CH25H and CYP7B1. Hence $CD4^+$ DCs position at these sites which is abrogated when EBI2 or 7α,25-OHC generating enzymes are absent. It is suggested, that EBI2 deficiency does not influence differentiation from pre-DCs, as the phenotype could not be reversed by application of Flt3 ligand or GM-CSF. However remaining $CD4^+$ DCs in $EBI2^{-/-}$ mice showed elevated levels of LTβR and treatment with agonistic antibodies partially restored the numbers of this subset. Due to their localization close to the red pulp of the spleen, MZ bridging channels are an important site to capture blood borne antigen. Upon antigen contact residing $CD4^+$ DCs migrate to the T cell zone in a CCR7 dependent manner. In the absence of appropriate, 7α,25-OHC directed positioning of $CD4^+$ DCs, immunization with T cell dependent antigens leads to reduced proliferation and activation of responding T cells and B cells. These results demonstrate that expression of EBI2 as well as the generation of 7α,25-OHC play an important role in dendritic cell (DCs) homeostasis, positioning and function (Gatto et al., 2013; Yi and Cyster, 2013).

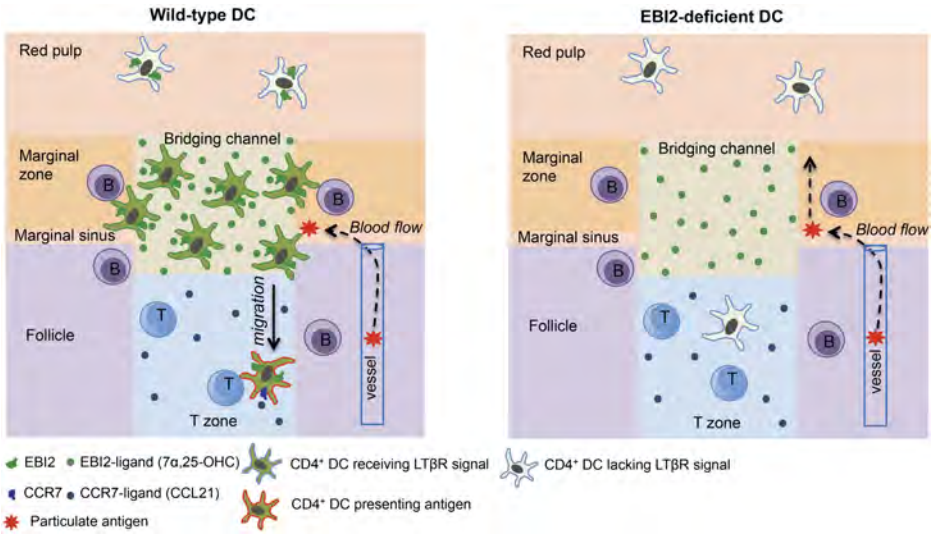

Figure 11: Positioning of CD4$^+$ DCs at bridging channels

Bridging channels represent the border of the lymphoid follicle to the surrounding red pulp. Concentrations of the EBI2 ligand are high at these sites. Among splenic dendritic cells (DCs), CD4$^+$ DCs have been shown to express high levels of EBI2 and thus localize at bridging channels where they sample blood born antigens. Therefore CD4$^+$ DCs deficient for EBI2 fail to position properly and do not receive appropriate survival signals, resulting in strong reduction of this subset. (Tangsheng Y. *et al.*; eLife; 2013)

Plasmacytoid dendritic cells (pDCs) represent another subset of DCs. They have been shown to be important during viral infections as they express high levels of TLR7 and TLR9 to detect ssRNA and dsDNA. Additionally they are capable to mount type I interferon responses upon TLR engagement in an IRF7 dependent manner. Chiang *et al.* could recently show, that EBI2 expression in pDCs negatively regulates expression of type I interferons, indicating regulation of gene expression via EBI2 signaling in addition to chemotactic functions (Chiang et al., 2013). This finding is interesting as other data indicate that CH25H expression in macrophages is induced by type I interferons to suppress secretion of the pro-inflammatory cytokine IL-1β and thereby limits immune responses in a sepsis model (Reboldi et al., 2014). Therefore it is possible that the EBI2:7α,25-OHC axis does not only influence lymphocyte migration, but also regulates immune responses by modifying cytokine expression.

2.5.3 Role of EBI2 in T cells

Recently, it was shown, that murine and human helper T cells express EBI2 and show migration to 7α,25-OHC. Mice deficient in EBI2 have been reported to show no overt differences in the T cell compartment. Additionally Suan *et al.* could demonstrate that EBI2 expression defines two different subpopulations of follicular helper T cells. They distinguished two subsets of primary Tfh cells according to their localization in either germinal centers (GC) or the follicular mantel (FM). Interestingly, they found EBI2 to be downregulated in GC Tfh cells, possibly in a Bcl-6 dependent manner. The low expression of EBI2 in this subset is similar to low EBI2 expression of B cells in the GC and correlates with low concentrations of the EBI2 ligand ath these sites. However further experiments revealed no differences in EBI2 and other chemokine receptors in secondary GC or FM Tfh cells after antigen re-challenge (Suan et al., 2015). Interestingly Chalmin *et al.* found that mice lacking CH25H show delayed onset of EAE after active immunization. Using a bone marrow chimeric approach they reported CH25H expression from hematopoietic cells to be essential for normal EAE development. Furthermore, immunization of mixed bone marrow chimeras reconstituted with 50% WT and 50 % EBI2$^{-/-}$ BM revealed less migration of EBI2 deficient T_H17 cells to the inflamed CNS, while numbers of T_H1 cells remained comparable to WT cells (Chalmin et al., 2015). This was in sharp contrast to the results of Reboldi *et al.* as they found severe disease development in CH25H$^{-/-}$ mice after active immunization (Reboldi et al., 2014). They claimed, that type I interferons induce expression of CH25H in macrophages in an INSIGN dependent manner, resulting in elevated 25-OHC levels which inhibit expression and maturation of IL-1β

from these cells, thereby limiting inflammation. Down-modulation of EBI2 expression is important for GC positioning of primary Tfh cells, consistent with positioning of B cells. However it seems that deficiency for EBI2 affects mainly specific subpopulations of T cells, e.g. Tfh or T_H17 cells rather than the complete T cell compartment. The exact reason for the controversial results regarding CH25H expression in EAE remain to be clarified and reproduced to determine the impact on EAE development.

2.5.4 Role of EBI2 in astrocytes and microglia

Previous studies have provided insight in the expression and function of EBI2 in various immune cells. Interestingly, Rutkowska et *al.* found expression of EBI2 in human astrocyte cultures from fetal cerebral cortex (Rutkowska et al., 2015). Additionally, EBI2 mRNA was also detectable in murine astrocytes although less abundant compared to human astrocytes. Moreover, mouse astrocytes express all enzymes necessary for synthesis of 7α,25-OHC in contrast to human astrocytes which lack expression of CH25H. Analysis of EBI2 signaling revealed increased phosphorylation of ERK, as well as Ca^{2+} mobilization upon stimulation with 7α,25-OHC in a dose dependent manner. Although they found EBI2 dependent migration of murine astrocytes towards 7α,25-OHC, no major differences in astrocytes *in vivo* were observed (Rutkowska et al., 2015). As astrocytes have been shown to be implicated in several neuronal disease, e.g. multiple sclerosis, Parkinson's and Alzheimer disease, further studies are needed to clarify the exact role of the

EBI2:7α,25-OHC system in these cells in naïve mice and under inflammatory conditions.

2.6 Aim of this work

Epstein Barr virus induced gene 2 (EBI2) and its ligand 7α,25-dihydroxycholesterol (7α,25-OHC) have been shown to play an important role in migration and positioning of B cells and dendritic cells in the lymphoid organs (Gatto et al., 2013; Hannedouche et al., 2011; Yi and Cyster, 2013; Yi et al., 2012). Additionally, in B cells the EBI2:7α,25-OHC axis is involved in mounting T cell dependent antigen responses. However, up to now only little is know about the function of this system in T cells in immunity.

In this work we will analyze the role of EBI2 in T cells in the context of inflammation. In particular we are interested to study the functional relevance of EBI2 expression in a murine model for MS termed EAE, especially in regard to pathogenic T_H1 and T_H17 subsets. To this aim we will use a novel reporter-knockin/EBI2-knockout (KO) mouse, which includes an EGFP reporter to monitor expression of EBI2. Furthermore, we will apply an adoptive transfer model of EAE to analyze T_H17 specific effects of EBI2 deficiency. Moreover we will investigate the expression of the enzymes synthesizing the EBI2 ligand in the course of EAE in different tissues. To analyze expression of EBI2 in human T cells we obtained a monoclonal antibody and will study expression of EBI2 in PBMCs of healthy donors and MS patients. Finally, we will visualize EBI2 expressing cells in MS lesions in autopsies from MS patients.

3. Material and Methods

3.1 Chemicals and buffers

Following chemicals and reagents were used:

Chemical/Reagent	Supplier	Catalogue #
7α,25 dihydroxycholesterol (7α,25-OHC)	Novartis	----------
Agarose	Biozym	840004
Brefeldin A	Sigma	B6542
Bovine serumalbumin (BSA)	Sigma	A7906
Collagenase II	Gibco	17101-015
Dimethylsufoxide (DMSO)	Sigma	D2650
DNase I	Roche	10104159001
dNTPset	Metabion	mi-N1006L
DPBS	Sigma	D8537
Ethylenediaminetetraacetic acid (EDTA)	Sigma	51128400
Ethanol	Roth	T913.7
Fetal calf serum (FCS)	Gibco	10270
Gene ruler 100 Bp DNA ladder	Thermo	SMQ241
Glycin	Roth	3790.2
HBSS	Invitrogen	14025-50
HEPES	Gibco	31330-038
Histopaque	Sigma	10771
Ionomycin	Invitrogen	I24222
L-Glutamin 200 mM	Gibco	
MEM	Gibco	11140-035
Methanol	Roth	4627.2
Natriumazid (NaN_3)	Applichem	A1430.0100
Normal goat serum	Gibco	16210064
Penecillin/Streptomycin (P/S)	Gibco	15140-122
Percoll	Sigma-Aldrich	P1644
Pertussis toxin	Biotrend	180
PMA	PromoCell	PK-CA577-1544-5
Proteinase K	Roche	03115852001
Red Taq Ready Mix PCR	Sigma	R2523-100RXN
Roti-Histofix 4%	Roth	P087.5
RPMI 1640	Lifetechnologies	21875-034
Sodiumchloride (NaCl)	Unimed	53439860
Sodiumdodecylsulfate (SDS)	Serva	20765.02
Sodiumpyruvate	Gibco	11360-039
X-VIVO 15	Lonza	BE04-418F

Table 1: Chemicals and reagents

Follwing buffers were used:

Buffer		Final concentration
FACS I	PBS	500 ml
	BSA	0.5 % (w/v)
	NaN_3	0.2 % (v/v)
FACS II	PBS	500 ml
	BSA	0.5 % (w/v)
	NaN_3	0.2 % (v/v)
	EDTA	2 mM
Freezing Medium A	RPMI 1640	40 % (v/v)
	FCS	60 % (v/v)
Freezing Medium B	FCS	80% (v/v)
	DMSO	20% (v/v)
Lysis buffer	Tris-HCl pH 8.0	50 mM
	NaCl	100 mM
	SDS	1 % (v/v)
	EDTA	100 mM
Staining buffer (Human cells)	PBS	500 ml
	Normal goat serum	5 % (v/v)
	NaN_3	0.2 % (v/v)
MACS Buffer	PBS	500 ml
	BSA	0.5 % (w/v)
	EDTA	2 mM
T cell Medium (TCM)	PBS	500 ml
	FCS	10 % (v/v)
	P/S	100 units/ml P; 100 µg/ml S
	L-Glutamine	2 mM
	MEM	1% (v/v)
	Sodium pyruvate	1 mM
	HEPES	10 mM
	ß-mercaptoethanol	50 µM

Table 2: Buffers

3.2 Cytokines and antibodies for cell culture

Following cytokines and antibodies were used for cell culture:

Cytokine/Antibody	Stock concentration	Supplier	Catalogue #
α-CD3	1 mg/ml	BioXCell	BE0001-1
α-CD28	6 µg/ml	BioXCell	BE0015-1
α-IFNγ	---------	BioXCell	BE0054
CCL19	50 µg/ml	R&D	587802
CCL21	50 µg/ml	R&D	586402
IL-1β	100 µg/ml	R&D	401-ML-005
IL-2	10 µg/ml	Promocell	D-61220
IL-4	10 µg/ml	R&D	404-ML-010
IL-6	10 µg/ml	Promocell	D-61632
IL-7	10 µg/ml	Promocell	D-61710
IL-9	----------	----------	----------
IL-12	10 µg/ml	Promocell	D-62210
IL-18	10 µg/ml	R&D	B001-5
IL-21	10 µg/ml	Promocell	D-62921
IL-23	10 µg/ml	Miltenyi	130096676
TGF-β1	2 µg/ml	R&D	240-B-002

Table 3: Cytokines and antibodies for cell culture

3.3 Mouse strains

Conditional EBI2$^{fl\text{-}EGFP}$ mice were made by Stefano Casola (Milan, Italy) and generated from 129/Ola-derived targeted ES cells (IB10) injected into C57BL/6J blastocytsts. Germline transmitted EBI2$^{fl\text{-}EGFP}$ mice were crossed to the Cre deleter strain to generate EBI2-deficient, *EBI2*^{EGFP} mice. In the latter animals the GFP reporter gene replaces the single coding exon of *EBI2*, placing the reporter gene under the transcriptional control of the *EBI2* locus. *EBI2*^{EGFP} mice used in this study were backcrossed at least 8 times onto the C57BL/6J genetic background. EBI2$^{-/-}$ mice without an EGFP reporter cassette were provided by Novartis/Basel.

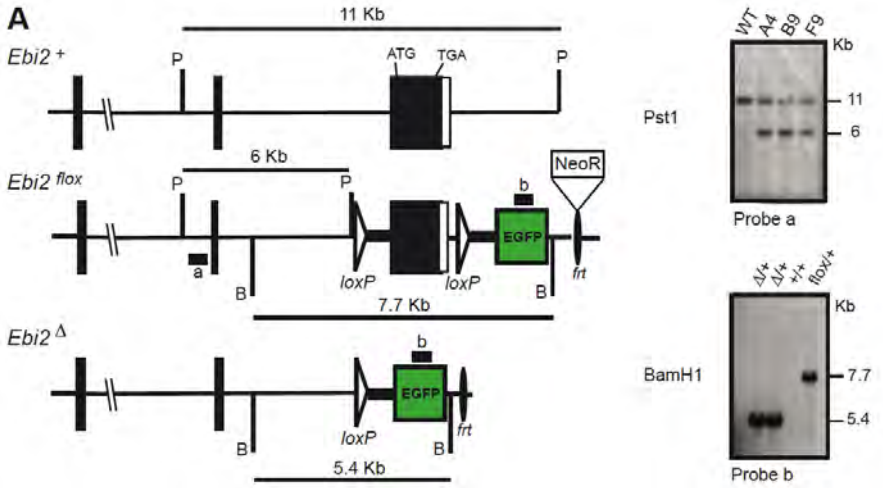

Figure 12: Generation of EBI2-EGFP knock-in/knock-out mouse

A) Conditional gene targeting strategy to generate conditional EBI2 knock-out (EBI2^{fl}) mice. The single EBI2 coding exon is flanked by loxP sites. The intronic region that precedes the EBI2 coding exon, and belonging to the floxed segment, was cloned upstream of an EGFP reporter cassette, which was finally placed downstream of the *EBI2*^{fl} DNA segment. Upon Cre-mediated recombination, the coding exon of *EBI2* is replaced by the EGFP minigene, generating a chimeric *EBI2-EGFP* allele that fails to express EBI2 (Ebi2$^{\Delta}$). The neomycin resistance gene flanked by FRT sites was eliminated *in vivo*, crossing EBI2^{fl} mice to the FLPe deleter strain. Correct targeting of ES clones was revealed by Southern blotting analysis using probes indicated (a and b). *In vivo* Cre-mediated recombination of the Ebi2^{fl} allele was confirmed by Southern blotting.

C57BL/6 mice were bought from Janvier, France. 2D2 x Thy1.1 and RAG1 deficient mice were obtained rom the general animal facility of the Johannes Gutenberg-University in Mainz. IL-17F-RFP mice were made available to us by the group of Chen Dong (Department of Immunology, M.D. Anderson Cancer Center, Houston, TX 77030, USA).

3.3 Genotyping of mice

Genomic DNA of individual mice was isolated by proteinase K digestion of tailpieces, followed by isopropanol precipitation. Polymerase chain reaction (PCR) was used to determine the genotype of each mouse. Reactions were performed using Red Taq Ready Mix PCR (Sigma) according to the manufacturer's protocol.

Following primers were used:

PCR	Primer Sequences	PCR Product
EBI2 delta	5'- AGT CTA ACG CCT GTC TAG AAT GT -3' (Forward) 5'- CTC CTG GAC GTA GCC TTC GG -3' (Reverse)	700 Bp
EBI2 wild type	5'- CTCTTCAGGACTGCCAAGCAG -3' (Forward) 5'- GCTGTGCTGTGAAGTCCCAAG -3' (Reverse)	450 Bp
IL-17F-RFP	5'- ACATTGCCCACCACCAGGGCTC -3' (Forward) 5'- CCCATGGGGAACTGGAGCGGTTC -3' (Reverse 1) 5'- CGGCTTCGGCCAGTAACGTTAGG -3' (Reverse 2)	WT: 250 Bp RFP: 400 Bp
Actin	5'- TGTTACCAACTGGGACGACA -3' (Forward) 5'- GACATGCAAGGAGTGCAAGA -3' (Reverse)	510 Bp

Table 4: Primer Sequences for PCRs

EBI2-EGFP and 2D2-Thy1.1 mice were additionally genotyped by flow cytometry. Therefore mice were bled and isolated PBMCs were stained for CD4 and the transgenic TCR (Vβ11) and analyzed on a BD FACS Scan.

3.4 Organ preparation

Mice were sacrificed with isofluorane. Organs were prepared and placed in PBS-FCS (2% FCS). Single cell suspensions were obtained by homogenization of the organs using a 40 µM cell strainer. Erythrocytes were removed by hypertonic lysis with ACK buffer. For preparation of the central nervous system (CNS), mice were perfused with isotonic NaCl dilution prior to preparation of the brain and spinal cord. Cut CNS was digested in PBS (with $MgCl_2/Ca^{2+}$) containing 1 µg/ml collagenase II and 100 µg/ml DNase I for 20 min at 37 °C and homogenized by using needle and syringe. Lymphocyes were isolated by centrifugation in a percoll gradient.

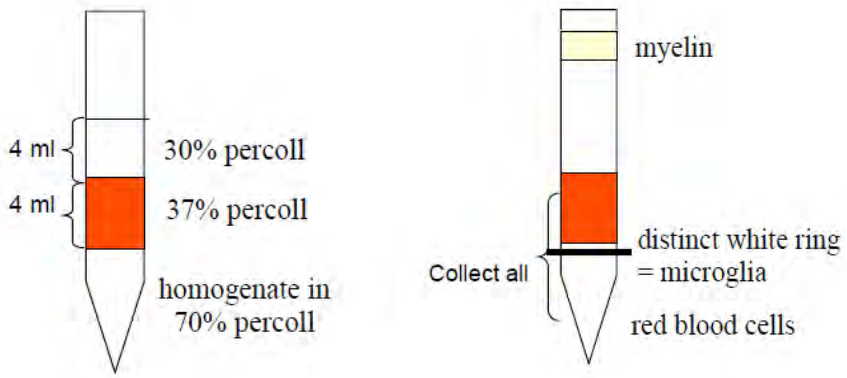

Figure 13: Percoll gradient for lymphocyte isolation
CNS homogenates were resuspended in 70 % Percoll and overlayed with the 37% Percoll (red color) and 30% Percoll fraction. After centrifugation for 30 min at 500 g the lymphocyte ring was collected and washed.
(Applied from the homepage of the Institute for Moleculare Medicine, Mainz, Germany)

Cells were counted by trypan blue staining using a Neubauer chamber.

3.5 Flow cytometry

Single cell suspensions were prepared as described above. Prior to staining of cells, Fc receptors were blocked. Cells were stained in FACS I buffer. For staining of cytokines, cells were activated in TCM with PMA, Ionomycin and monensin for four hours at 37 °C. After surface staining, cells were fixed with 2% formaldehyde and permeabilized with 1x Perm buffer (BD). Intracellular staining was done in 1x Perm buffer (BD) according to the manufacturers protocol. Staining of transcription factors was done as published by our group before (Heinen et al., 2014).

Following antibodies / reagents were used for stainings of murine cells:

Antigen	Fluorochrome	Dilution	Supplier	Catalogue #
Fc-Block	---------	1/100	BioXCell	BE0144
CD4	BV421	1/200	BioLegend	100438
CD8	PerCp	1/200	BioLegend	100732
CD11b	PeCy7 Biotin	1/1000 1/500	eBioscience eBioscience	25-0112-82 13-0112-82
CD11c	APC	1/200	BD	550261
CD44	Pe	1/400	eBioscience	12-0441-82
CD62L	APC	1/1000	eBioscience	17-0621-82
CD90.1	PeCy7	1/3000	eBioscience	25-0900
CD90.2	PerCp APC-Cy7	1/1000 1/1000	BioLegend eBioscience	140316 17-0902-82
IFNγ	PeCy7	1/1000	eBioscience	25-7311-82
GM-CSF	Pe	1/200	eBioscience	12-7331-82
IL-17A	APC	1/200	eBioscience	17-7177-81
TCR Vβ11	Pe	1/200	eBioscience	65-0865-18
Viability Dye	APCeF780	1/1000	BD	553198
7AAD	PerCp	1/100	eBioscience	00-6993-50

Table 5: Antibodies for staining of murine cells

Following antibodies were used for stainings of human cells:

Antigen	Fluorochrome	Dilution	Supplier	Catalogue #
Fc-Block	---------	---------	BioLegend	422302
CD3	APC	1/100	BioLegend	300311
CD4	Pe	1/100	BioLegend	357403
CD8	BV510	1/100	BioLegend	301047
CD14	PerCp	1/100	BioLegend	325631
CD19	PeCy7	1/100	BioLegend	302215
CD45RA	APC-Cy7	1/100	BioLegend	304127
EBI2	---------	1/100	Novartis	---------
IFNγ	PeCy7	1/20	BioLegend	502527
IL-17A	BV421	1/20	BioLegend	512321
GM-CSF	APC	1/20	BioLegend	502309
Goat-α-mouse-IgG	Biotin	1/200	Jackson	115-066-068

Table 6: Antibodies for staining of human cells

Samples were acquired on a BD FACS Canto II and FlowJo 9.7.5 was used for analysis.

For FACS sorting, Fc receptors were blocked for 10 min on ice and cells were stained

in sterile filtered PBS + 0.5% BSA + 2 mM EDTA. Cells were sorted on a FACS Canto II or BD Aria.

3.6 RNA Preparation

Suspension cells were resuspended in lysis buffer (Qiagen RLT buffer / Peqlab Lysis buffer T) and stored at -20 °C. RNA was extracted by using RNeasy Micro Kit (Qiagen) for cell numbers ≤ 1 x 10^6 cells or Total RNA Gold Kit (Peqlab) for higher cell numbers, according to the manufacturer's protocol. For preparation of total RNA from whole tissue, lysing matrix D (MP) was used. Snap frozen tissue was incubated with 800 µl Trizol (Invitrogen) for 10 minutes on ice prior to homogenization in MP FastPrep. Homogenates were incubated for additional 10 minutes on ice and cell debris was pelleted by centrifugation. RNA was extracted with phenol: chloroform and precipitated with isopropanol. Ethanol washed RNA was then resuspended in RNAse free water, incubated for 10 min at 55 °C and stored at -80°C. RNA concentration was measured in duplicates using NanoQuant 16 well flat back plates (Tecan) and respective reader (Tecan Infinite).

3.7 Reverse transcription

Preparation of cDNA from total RNA was done by using SuperscriptII Reverse Transcription Kit (Invitrogen) with random primers according to the manufacturer's protocol. Depending on the amount of RNA used for the reaction, resulting cDNA was diluted with ddH_2O and stored at

-20°C.

Step	Temperature	Duration
Denaturation	65 °C	5 min
Annealing	25 °C	10 min
Transcription	40 °C	40 min
Inactivation	72 °C	15 min
Hold	4 °C	-

Table 7: Program for reverse transcription

3.8 Quantitative Real-Time PCR (qRT-PCR)

For quantification of mRNA expression of different genes, cDNA was analyzed by qRT-PCR. Primers were bought from Qiagen (Quantitect Primer assays) with HPRT as reference gene. Reactions were performed using a SYBR green assay (Invitrogen) according to the manufacturer's protocol and carried out on a respective reader (Applied Biosystems). Expression of mRNA of analyzed genes was calculated relative to expression of HPRT using the $\Delta\Delta C_t$ method.

3.9 *In vitro* migration assay

Splenocytes or CD4 and CD8 purified T cells were stimulated over night in T cell medium with 1 µg/ml α-CD3 and 6 µg/ml α-CD28 antibodies. For some experiments 1 µM 7α,25-OHC was added during activation. Migration assays were performed by using 96-well transwell plates with 5 µm pore size. Chemokines in TCM were added to the lower chamber and 1 x 10^5 cells were loaded in the upper chamber. Plates were incubated at 37 °C and 5% CO_2. Input cells were cultured in parallel. After two hours, cells in the lower chamber and input cells were analyzed by flow cytometry.

Flow cytometric quantification was done using counting beads (Spherotech). Some *in vitro* migration assays were performed in collaboration with Dr. Denise Tischner and Prof. Dr. Nina Wettschureck (Max Planck Institute for Heart and Lung Research, Bad Nauheim, Germany)

3.10 *In vitro* T cell differentiation

T cells were isolated from spleen and lymph nodes by MACS purification using either CD4 microbeads or the naïve T cell isolation kit (Miltenyi). Cells were plated at 1 x 10^5 cells/well in 96-well culture plates in 200 µl/well T cell medium (TCM).

Following culture conditions were used for T cell differentiation:

Differentiation	Stimulus	Final concentration
T_H1	α-CD3	1 µg/ml
	α-CD28	6 ng /ml
	IL-12	4 ng/ml
T_{reg}	α-CD3	1 µg/ml
	TGF-β1	4 ng /ml
	IL-2	10 ng/ml
	α-IFNγ	10 µg/ml
T_H17	α-CD3	1 µg/ml
	α-CD28	6 ng /ml
	TGF-β1	4 ng /ml
	IL-6	5 ng/ml
	IL-23	20 ng/ml
	α-IFNγ	10 µg/ml
T_H17	α-CD3	1 µg/ml
	α-CD28	6 ng /ml
	IL-1β	40 ng /ml
	IL-6	5 ng/ml
	IL-23	20 ng/ml
	α-IFNγ	10 µg/ml

Table 8: T cell differentiation conditions

For T_H1 and T_{reg} differentiation cells were cultured at 37 °C and 5% CO_2 for three days, or for five days for T_H17 differentiation.

3.11 *In vivo* migration of T cells

T helper and cytotoxic T cells were isolated from spleen and lymph nodes of EBI2 deficient mice or wildtype littermates via MACS purification. Cells (5 x 10^6) were transferred i.v. into congenic Thy1.1 mice (express CD90.1 allele). After four hours, mice were sacrificed and spleen and lymph node cells were analyzed via flow cytometry and transferred cells were quantified.

3.12 *In vivo* T cell priming

T helper cells were isolated from spleen and lymph nodes of 2D2 x Thy1.1 mice by MACS purification and labeled with CFSE according to the manufacturers protocol. Cells (5 x 10^6) in PBS were transferred i.v. into either EBI2 deficient mice or wildtype littermates. One day after transfer, mice were immunized by subcuteanous injection of 100 µg MOG_{35-55} emulsified in complete Freund adjuvant (CFA), or left untreated. Five days after immunization, mice were sacrificed and spleen and lymph node cells were analyzed by flow cytometry.

3.13 Induction of EAE and scoring of disease severity

Active EAE was induced by subcuteanous administration of 100 µg MOG_{35-55} emulsified in CFA. Along with immunization and on day two, mice were injected i.p. with 200 ng Pertusis toxin (PTX) in PBS (-/-).

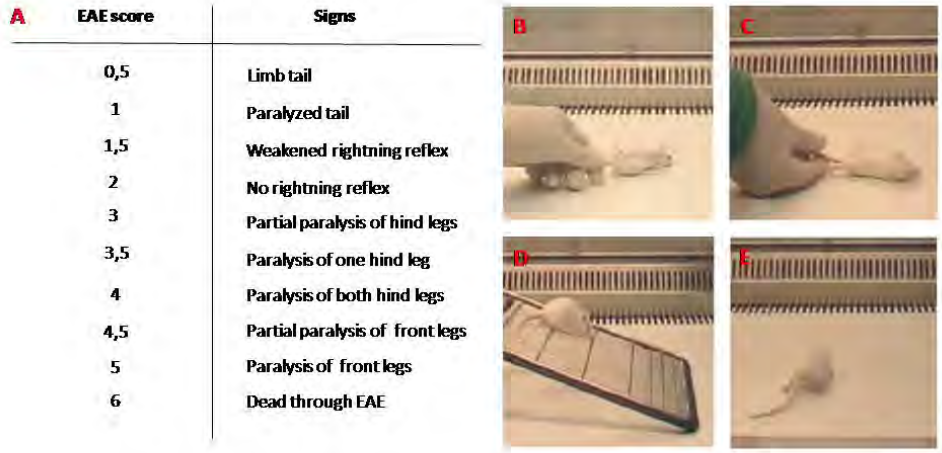

Figure 14: Scoring system for EAE

Mice were scored for onset of EAE starting from day seven post immunization or transfer of pathogenic T cells. A) Severity of EAE was quantified according to indicated signs. B)-C) Rightning reflex was tested by turning mice on the back. D)-E) Paralysis of legs was assessed by observing movement on a grid and on the ground.
(Applied from the homepage of the Institute for Moleculare Medicine, Mainz, Germany)

3.14 T_H17 Adoptive transfer EAE

Mice were immunized with MOG_{35-55} in CFA as described without injection of PTX. On day ten after immunization, spleen and lymph node cells were prepared and cultured *in vitro* in TCM.

Cells were cultured at 2.5 x 10^6 cells/ml TCM with 50 µg/ml MOG_{35-55}, 10 ng/ml IL-23 and 10 µg/ml α-IFNγ at 37 °C and 5% CO_2 for four days. Cells were harvested and analyzed by flow cytometry. Afterwards 2 x 10^5 blasting T_H17 cells were transferred i.v. into $RAG1^{-/-}$ mice. Pertussis toxin (200 ng) was administered i.p. along with transfer and on day two.

3.15 Transfer colitis

Naïve helper T cells from $EBI2^{EGFP/EGFP}$ mice or litter mate controls were isolated by using Naïve T cell Kit (Miltenyi) according to the manufacturers protocol. Afterwards 5 x 10^5 cells in PBS were transferred i.p. into $RAG1^{-/-}$ mice. Mice were scored for onset and severity of colitis weekly via mini-endoscopy. Quantification of disease severity was done by determining translucency, granularity of the gut, as well as fibrin levels, stool and body weight. Each parameter except for body weight was ranked from 0-3 according to severity, resulting in a maximum total score of 15.

3.16 Preparation of human PBMCs

Buffy coats were obtained from the blood donation facility of the University Medical Center in Mainz. Blood samples from MS patients were provided by Dr. Vinzenz Fleischer and Monika Firros (Department for Neurology, University Medical Center,

Mainz, Germany). PBMCs were prepared by centrifugation in a Histopaque gradient. In brief, one part blood was diluted with two parts PBS and underlayed with Histopaque. Cells were centrifuged for 30 minutes at 300g. Afterwards the lymphocyte ring was collected and washed extensively. For some experiments plasma was collected and stored at -80 °C.

3.17 Freezing / thawing of human PBMCs

Human PBMCs were resuspended at 10 x 10^6 cells/ml in freezing medium A and an equal part of freezing medium B was added while swirling. Cells were aliquoted in cryotubes and frozen in pre-chilled racks at -80 °C and later stored in liquid nitrogen. Cells were thawed quickly and warm FCS was added dropwise while swirling. Cells were then immediately transferred into 37 °C RPMI 1640 + 10 % FCS. Cells were washed twice and rested for 4h at 37 °C prior to further processing.

3.18 Staining of human EBI2

For staining of EBI2 on human PBMCs, 2x 10^6 cells were plated in 96-well V-bottom plates. Fc-receptors were blocked for ten minutes. Surface staining for EBI2 was done using a monoclonal antibody, made available to us by Novartis, Basel (mouse anti-human EBI2 Clone: 57C). Staining was performed in PBS (5% normal goat serum / 0.2% NaN_3). Therefore cells were incubated with mouse anti-human EBI2 antibody and washed intensively. Afterwards goat anti-mouse IgG-biotin antibody was added as secondary antibody and cells were intensively washed after incubation time. Cells were then stained with Streptavidin-Fitc and antibodies for surface staining and

washed again. For staining of cytokines, cells were stimulated in X-VIVO 15 medium (Lonza) with PMA, Ionomycin and Monensin for five hours prior to surface staining. Cells were fixed with 2% formaldehyde. Intracellular stainings were done in 1x Perm buffer (BD).

3.19 Human tissue samples and immunohistochemistry

We retrospectively investigated 5 brain biopsies from 5 MS patients. None of the study authors was involved in decision-making with respect to biopsy. All lesions fulfilled the generally accepted criteria for the diagnosis of multiple sclerosis (Prineas, 1985; Allen, 1991; Lassmann et al., 1998). The study was approved by the Ethics Committee of the University of Münster. Tissue specimens were fixed in 4 % paraformaldehyde and embedded in paraffin. Tissue samples were cut in 4 µm thick sections that were stained with haematoxylin and eosin and Luxol-fast blue. Immunohistochemical staining was performed with an avidin-biotin technique using an automated staining device (DakoLink 48). The primary antibodies were rabbit anti-myelin basic protein (1:1000) (Boehringer Mannheim, Mannheim, Germany), mouse anti-KiM1P (1:5000) (H.-J. Radzun, Department of Pathology, University of Göttingen, Germany), rabbit anti-Olig2 (1:300) (IBL, Spring Lake Park, Minnesota), rabbit anti-Nogo-A (1:750) (Chemicon International, Temecula, CA) and mouse anti-Nogo-A (1:15.000) (11c7, a generous gift from M.E. Schwab, Brain Research Institute, University of Zürich and Department of Biology, Swiss Federal Institute of Technology Zürich, Switzerland), mouse anti-CD68 (1: 200) (Dako), mouse anti-CD45 (1: 800) (Dako), rabbit anti-CD3 1: 100) (Dako), mouse anti-EBI2 (1: 500) (Novartis),

mouse anti-neurofilament (1: 1000) (Dako). For doublestainings sections were incubated with the appropriate primary antibodies followed by secondary antibodies conjugated to Cy3 (1: 200; Jackson Immunoresearch Laboratories) or Alexa488 (1: 200, Jackson ImmunoResearch Laboratories) conjugated antibodies and counterstained with DAPI (1: 5000, Invitrogen). All images were taken on an Olympus fluorescent microscope. These experiments were done by the group of Prof. Dr. Tanja Kuhlmann (Institute of Neuropathology, University Hospital Münster, Münster, Germany)

4. Results

4.1 EBI2 is highly expressed by naïve helper T cells but does not affect homing to peripheral lymphoid organs

It was previously shown that EBI2 is expressed on B cells and dendritic cells (DCs). Using our EBI2-EGFP reporter mice (Fig. 12), we were able to study its expression in T cells via flow cytometry. Thereby, we could show that EBI2 expression is highly regulated during thymic development of T cells (Fig. 15A). We observed that only 8% of T cell progenitors in the double negative stage ($CD4^-$, $CD8^-$) expressed EBI2. However further analysis of these cells according to CD44 and CD25 expression revealed that more than 50% of the progenitors in the DN1 stage express EBI2, but down-regulated it during further maturation. After positive and negative selection in the double positive state, EBI2 is expressed by 40% of $CD4^+$ T cells but only by few $CD8^+$ T cells in the thymus (Fig. 15A). Analysis of T cells in the spleen and lymphnodes of naïve mice revealed that EBI2 is expressed by the majority of $CD4^+$ T cells in contrast to $CD8^+$ T cells expressing EBI2 in a heterogeneous fashion (Fig. 16A and Fig. 17A).

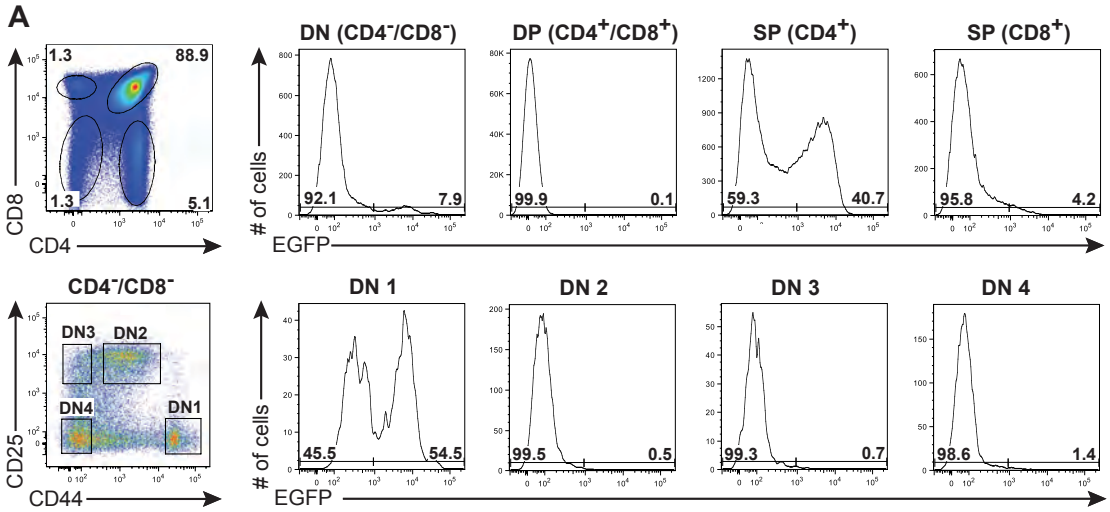

Figure 15: EBI2 expression in thymic T cells

A) Flow cytometric analysis of EBI2 expression of thymic T cells in EBI2$^{+/EGFP}$ animals. Cells were gated as CD19$^-$ / CD11c$^-$ live cells and divided by CD4 and CD8 expression. CD4$^-$ / CD8$^-$ T cells were further classified into DN1-4 stages according to CD25 and CD44 expression profile. Histograms show EBI2-EGFP expression in indicated populations. Plots are representative of at least two independent experiments (n=3)

Analysis of memory subsets (Fig. 17A) showed that EBI2 was expressed by about 80 % of naïve (CD62L$^+$CD44$^-$) T helper cells and by 70% of central memory cells (CD62L$^+$CD44$^+$), whereas only about half of the effector memory T cells (CD62L$^-$ CD44$^+$) expressed the reporter protein. Among CD8$^+$ T cells, strongest expression of EBI2 was found on central memory T cells with more than 60% being EBI2-EGFP$^+$. In contrast to helper T cells, only a few naïve (30%) and effector memeory CD8$^+$ T cells (30%) showed EBI2 expression. Moreover in line with our reporter data, CD4$^+$ T cells (Fig. 16B) as well as sorted EGFP positive effector T cells from EBI2$^{+/EGFP}$ mice (Fig. 18A) expressed EBI2 mRNA to high levels. In contrast EBI2 mRNA was hardly detectable in CD8$^+$ T cells (Fig. 16B) or sorted EGFP$^-$ effector T cells (Fig. 18A). This results indicate that our EBI2-EGFP reporter mouse faithfully reflects actual EBI2 expression.

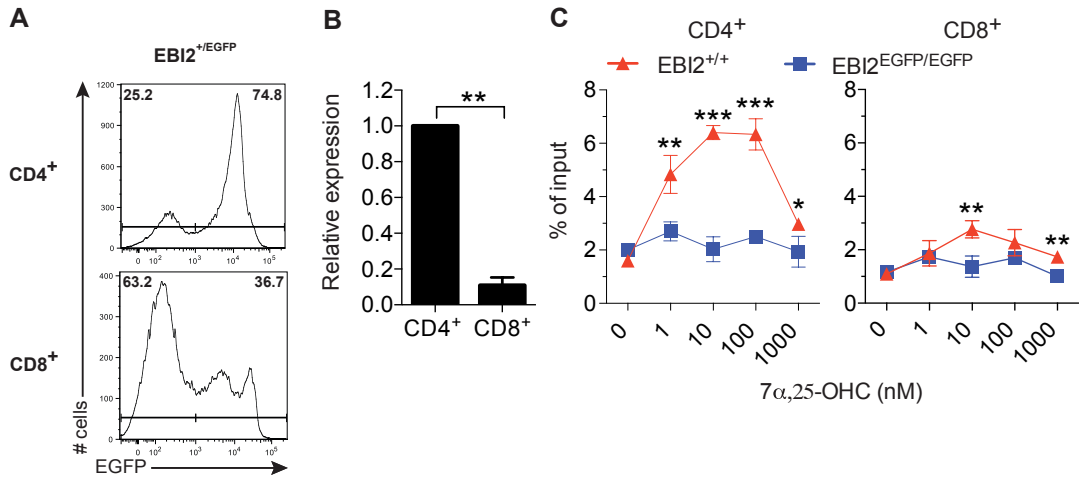

Figure 16: EBI2 expression T cells

A) Flow cytometric analysis of EBI2 expression in $CD4^+$ and $CD8^+$ T cells in the lymphnodes of $EBI2^{+/EGFP}$ reporter mice. Cells were pre-gated as $CD90.2^+$ living cells. Histograms are representative of at least three independent experiments (n=3) B) Relative expression of *ebi2* mRNA in $CD4^+$ or $CD8^+$ T cells from wild type mice. Cells were MACS purified from spleen and lymph nodes and mRNA expression was determined via qRT-PCR using hprt as housekeeping gene. Graph represents two independent experiments with n=3. C) In vitro migration assay of activated splenocytes form $EBI2^{EGFP/EGFP}$ mice and littermate controls towards indicated concentrations of 7α,25-OHC. Data is representative of three independent experiments (n=1)

As expression of the reporter protein as well as mRNA levels do not necessarily correlate with EBI2 surface distribution, we performed *in vitro* migration assays of activated splenocytes or purified T cells to 7α,25-OHC (Fig. 16C and Fig. 18B, respectively). Although migration to 7α,25-OHC was relatively low compared to migration towards CCL19/CCL21 (Fig. 18C), we found that $CD4^+$ T helper cells migrate stronger towards the ligand than $CD8^+$ T cells.

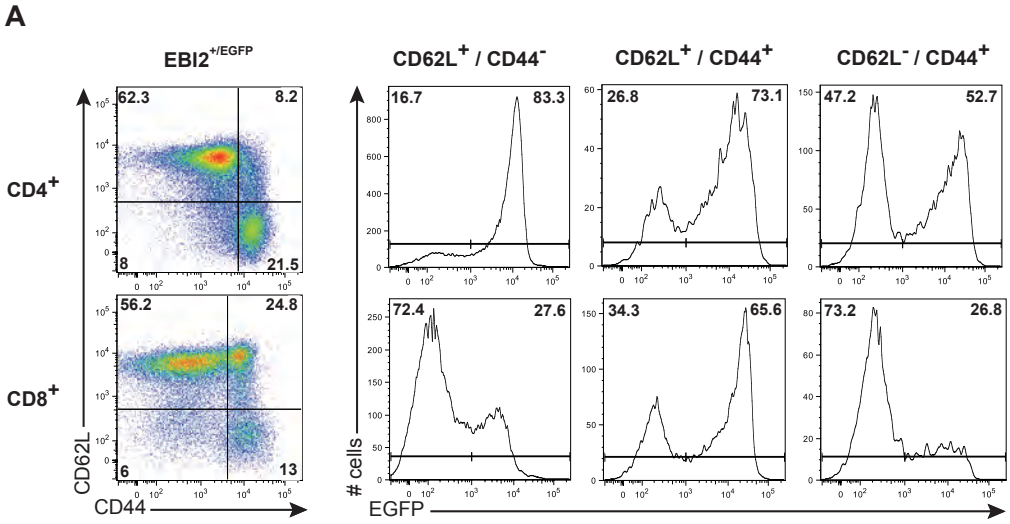

Figure 17: EBI2 expression on T cell subsets

A) Flow cytometric analysis of EBI2 expression in T cell subsets in the lymph nodes of EBI2$^{+/EGFP}$ mice. CD4^{+} and CD8^{+} T cells were gated as living CD90.2^{+} cells and further divided by expression of CD62L and CD44. Histograms show EGFP expression in indicated subsets: Naïve (CD62L^{+}/CD44^{-}), central memory (CD62L^{+}/CD44^{+}) and effector memory (CD62L^{-}/CD44^{+}) T cells. Data is representative of at least three independent experiments (n=3)

EBI2 deficient T cells from EBI2$^{EGFP/EGFP}$ mice did not migrate at all in these assays, excluding EBI2 independent chemotaxis to 7α,25-OHC (Fig. 16C and Fig. 18B). As shown before, increasing concentrations of 7α,25-OHC inhibited migration of wildtype (WT) T cells. We further analyzed the migratory behavior of EBI2-deficient T cells towards CCR7 ligands CCL19/CCL21 and found it comparable to WT T cells (Fig. 18C). Interestingly, pre-treatment of cells with 1 µM of 7α,25-OHC also significantly decreased migration of T cells towards CCL19 and CCL21 (Fig. 18D)

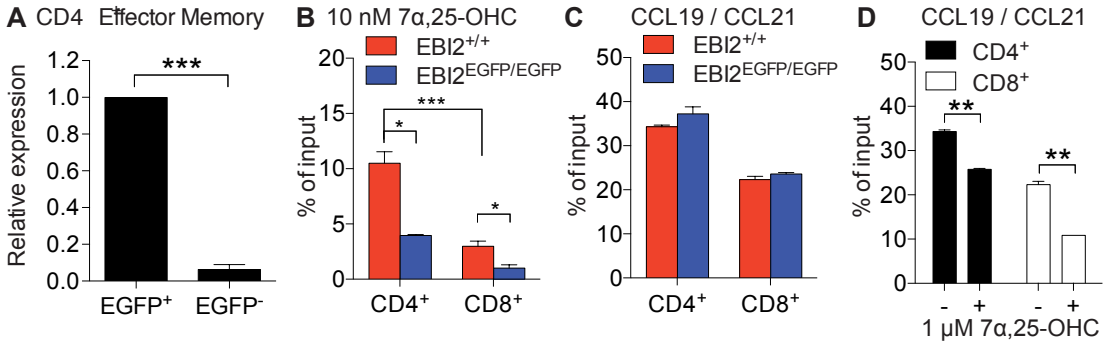

Figure 18: In vitro migration of T cells

A) Expression of *ebi2* mRNA in $CD4^+$ effector T cells, FACS sorted for EGFP expression from spleen and lymph nodes of $EBI2^{+/EGFP}$ mice. B) *In vitro* migration assay of purified and activated T cells from $EBI2^{EGFP/EGFP}$ mice or littermate controls towards 10 nM 7α,25-OHC. Data is representative of two independent experiments with n=1. C) *In vitro* migration assay of activated T cells form $EBI2^{EGFP/EGFP}$ mice and littermate controls towards 50 ng/ml CCL19/CCL21. Data is representative of three independent experiments with n=1. D) *In vitro* migration assay of purified and activated T cells from control animals towards 50 ng/ml CCL19/CCL21. T cells were either stimulated with 1 µM 7α,25-OHC or left untreated during activation. Data is representative of two independent experiments (n=1)

It was reported that EBI2 expression and generation of its ligand play an important role in positioning of B cells and dendritic cells within the lymphoid organs. Hence, we were curious to analyze its role in migration of T cells in an *in vivo* experiment. Therefore, we transferred purified $CD4^+$ and $CD8^+$ T cells from EBI2 deficient mice or control littermates intravenously (i.v.) into congenic hosts ($CD90.1^+$). Four hours later, transferred T cells were quantified in the spleen and lymph nodes (LNs) via flow cytometry (Fig. 19 AB and Fig. 19 CD, respectively).

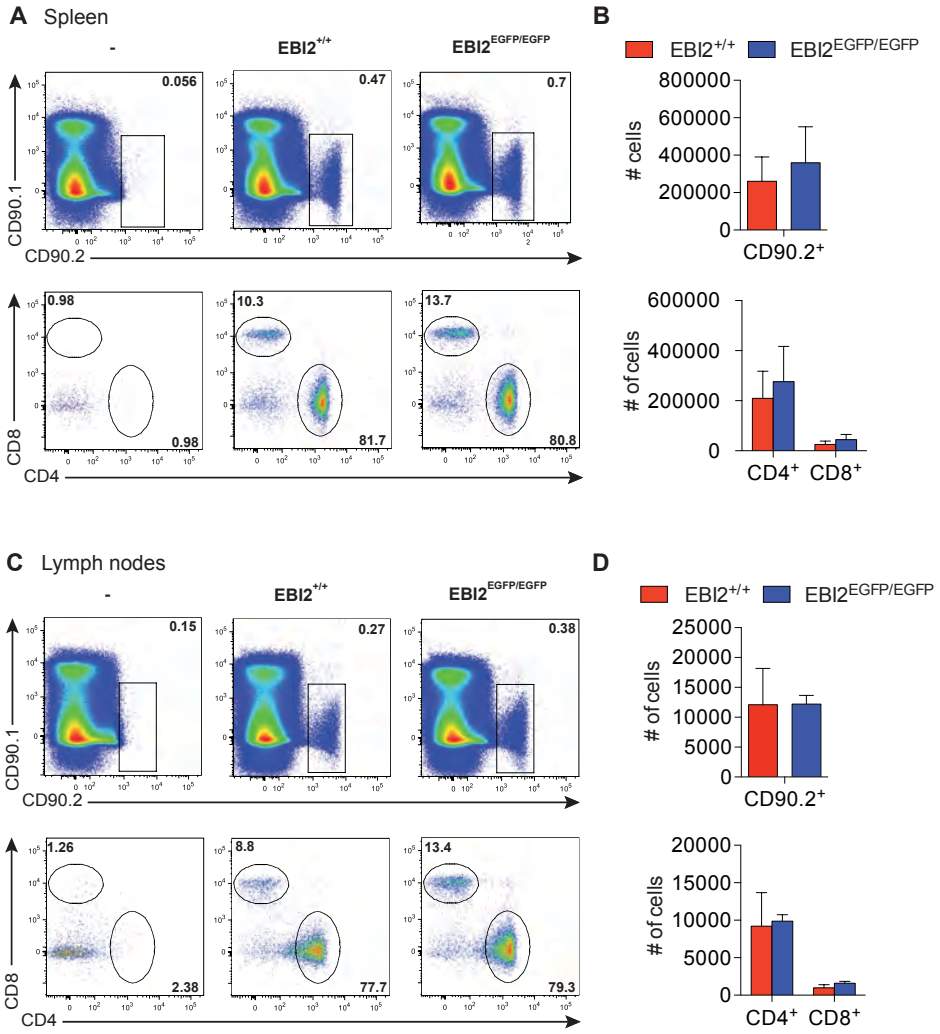

Figure 19: EBI2 deficient T cells show normal homing to peripheral lymphoid organs

T cells were purified from either EBI2$^{EGFP/EGFP}$ mice or littermate controls and transferred i.v. into congenic host mice. Four hours later cells in the spleen and lymph nodes were analyzed and quantified via flow cytometry. Transferred T cells were determined as living CD90.1$^-$ / CD90.2$^+$ T cells. A) Flow cytometric analysis of transferred T cells in the spleen of recipient mice. B) Quantification of transferred T cells from either EBI2$^{EGFP/EGFP}$ mice or littermate controls in the spleen of recipient mice. C) Flow cytometric analysis of transferred T cells in the lymph nodes of recipient mice. D) Quantification of transferred T cells from either EBI2$^{EGFP/EGFP}$ mice or littermate controls in the lymph nodes of recipient mice. Data is representative for two independent experiments (n=5).

However we did not detect significant differences in numbers of transferred CD4$^+$ and CD8$^+$ T cells in the secondary lymphoid organs between the two groups. This suggests that EBI2 expression does not influence homing of T cells to the spleen and lymphnodes.

4.2 EBI2 expression of helper T helper cell subsets

As we found strong EBI2 expression in most T helper cells, we further analyzed different T cell subsets in naïve mice. Therefore we studied the expression of EBI2 on IL-17A (T_H17) and IFNγ (T_H1) secreting T cells as well as on FoxP3$^+$ regulatory T cells (T_{regs}) in the lymph nodes and spleen of EBI2$^{+/EGFP}$ reporter mice via flow cytometry.

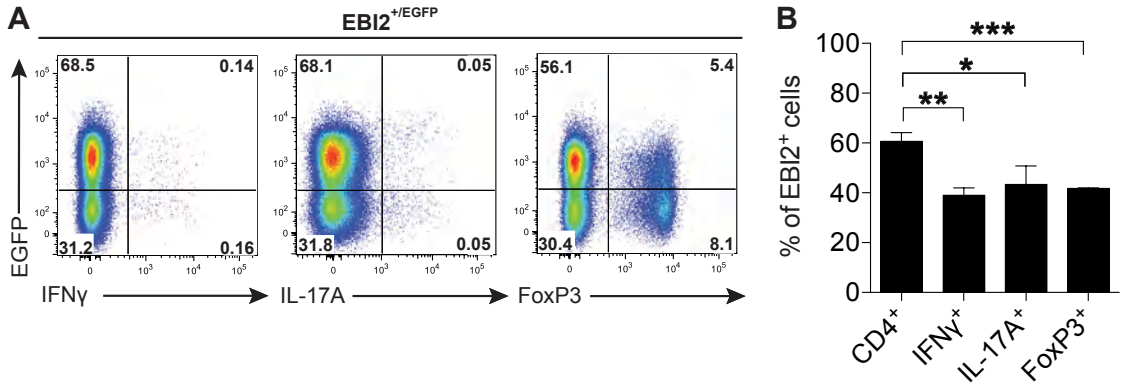

Figure 20: EBI2 expression in T cell subsets

A) Flow cytometric analysis of EBI2 expression in helper T cells expressing FoxP3, IFNγ or IL-17A in the lymphnodes of naïve EBI2$^{+/EGFP}$ mice. B) Statistical analysis of EBI2$^+$ helper T cell subsets in the lymph nodes of naïve EBI2$^{+/EGFP}$ mice. Data is representative of two independent experiments (n=3).

We recently developed a protocol, which maintains GFP fluorescence in cells intracellularly stained for FoxP3 (Heinen et al., 2014). We found FoxP3$^+$ regulatory T cells enriched in the EGFP$^-$ T cell fraction (Fig. 20AB). Similarly, only around 40% of T_H1 (IFN- γ$^+$) and T_H17 (IL-17A$^+$) cells from naïve mice expressed EBI2 (Fig. 20AB). Furthermore when we analyzed the different T cell subsets in the peripheral lymphoid organs, we could not detect differences in frequencies and numbers of T_H1, T_H17 and regulatory T cells (Fig. 21AB). These findings suggest, that EBI2 deficiency does not influence differentiation of these subsets under steady state conditions.

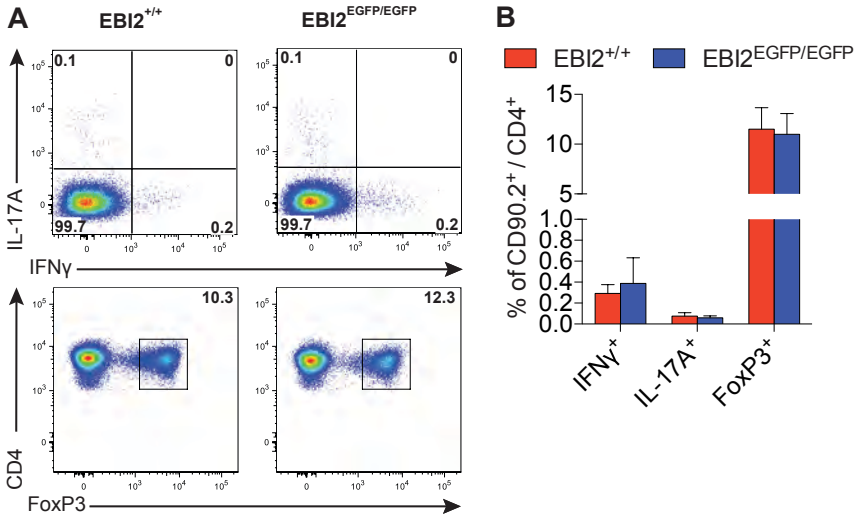

Figure 21: Normal T cell compartment in EBI2 deficient mice

A) Flow cytometric analysis of helper T cells in the lymphnodes of naive EBI2$^{EGFP/EGFP}$ mice or littermate controls. Cells were gated as living CD90.2^{+} / CD4^{+} cells and analyzed for expression of FoxP3, IFNγ and IL-17A. B) Quantification of helper T cells in the lymphnodes of naïve EBI2$^{EGFP/EGFP}$ mice or littermate controls. Data is representative for two independent experiments (n=3).

To further assess EBI2 expression by T cells differentiated *in vitro*, we polarized naïve T helper cells from EBI2$^{+/EGFP}$ mice under different conditions. In contrast to our *ex vivo* findings, most *in vitro* differentiated T_{regs} expressed EBI2, when polarized with TGF-β1 (Fig. 22ABC). However we observed that under the influence of IL-2, EBI2 expression was partially decreased (Fig. 22BC).

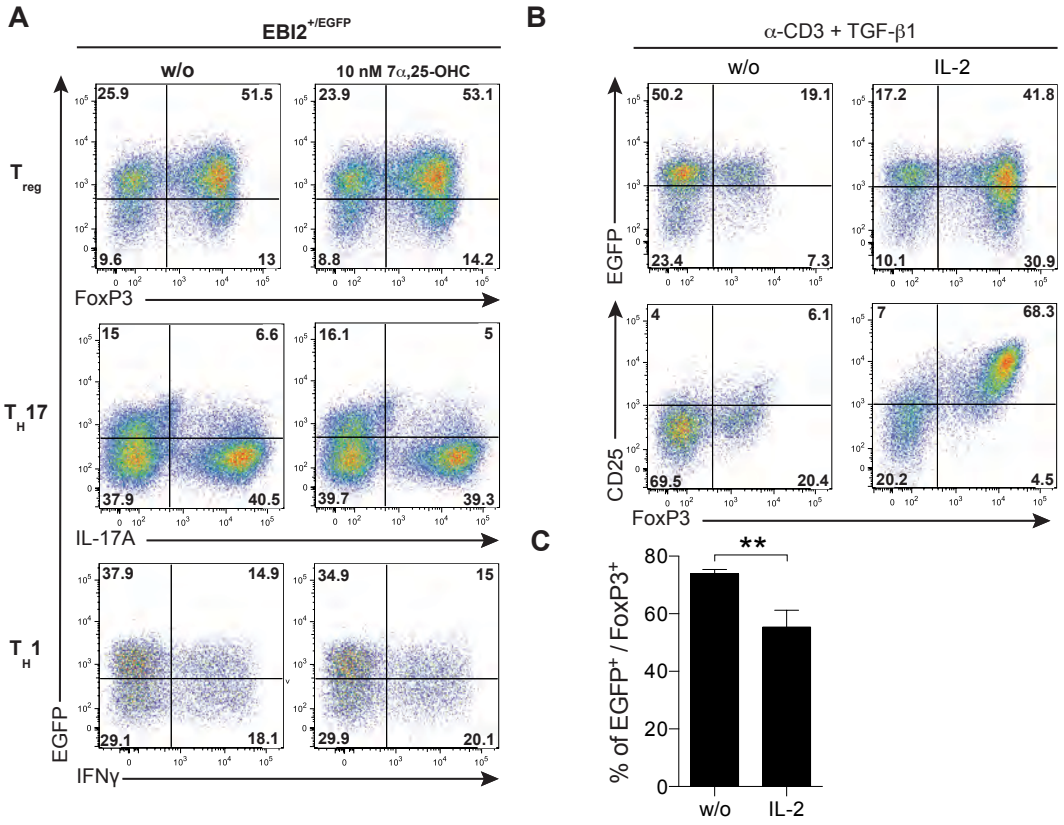

Figure 22: EBI2 expression of in vitro differentiated helper T cells

A) Flow cytometric analysis of helper T cells from EBI2$^{+/EGFP}$ mice differentiated *in vitro* in the presence or absence of 10 nM 7α,25-OHC. B) Flow cytometric analysis of EBI2 expression in helper T cells from EBI2$^{+/EGFP}$ mice differentiated *in vitro* under T_{reg} conditions with or with out addition of IL-2. C) Statistical analysis of EGFP^{+} / FoxP3^{+} T cells after *in vitro* differentiation under T_{reg} conditions with or without IL-2. Data is representative of at least two independent experiment (n=2).

Furthermore, T_H17 cells differentiated *in vitro* with TGF-β1 and IL-6 showed only weak expression of EBI2 (Fig. 22A) whereas T_H1 cells differentiated *in vitro*, were more comparable to *ex vivo* T_H1 cells (Fig. 20A), showing a bipartite expression of EBI2 (Fig. 20A and Fig. 22A). It was recently shown that signaling via S1P(1) receptor influences the balance between T_H1 and regulatory T cells (Liu et al., 2010). Therefore, we speculated that 7α,25-OHC might have an impact on T cell differentiation.

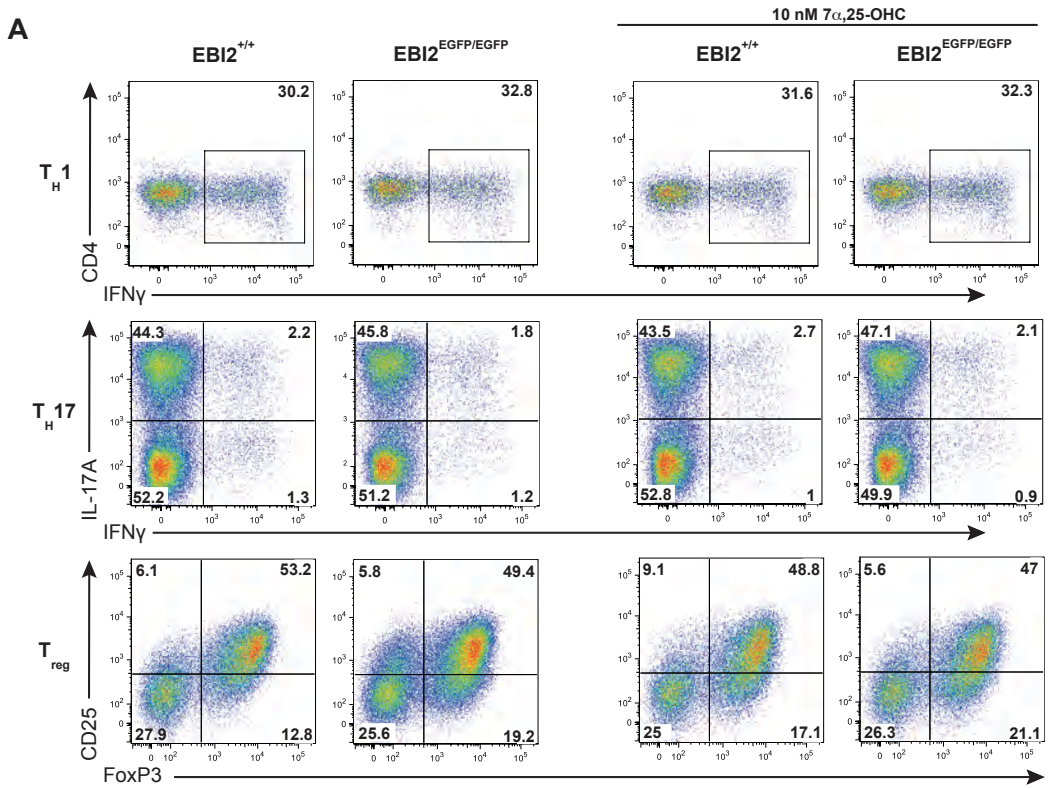

Figure 23: 7α,25-OHC does not influence in vitro T cell differentiation

A) Flow cytometric analysis of helper T cells from EBI2$^{EGFP/EGFP}$ mice or littermate controls differentiated *in vitro* in the presence or absence of 10 nM 7α,25-OHC. Data is representative for three independent experiments (n=1)

However, we did not find any effect on *in vitro* T cell differentiation to T_H1, T_H17 or induced T_{regs} when 7α,25-OHC was added (Fig. 23A). In line with this, EBI2 deficiency also did not impair *in vitro* T cell differentiation (Fig. 23A). To clarify whether the inhibitory effect we observed with addition of IL-2 on EBI2 expression was connected to proliferation and may be triggered also by other cytokines which signal via the common γ-chain we labeled $CD4^+$ T cells from EBI2$^{+/EGFP}$ mice with violet cell tracer (VCT) and analyzed proliferation together with reporter expression in dependence of the different cytokines.

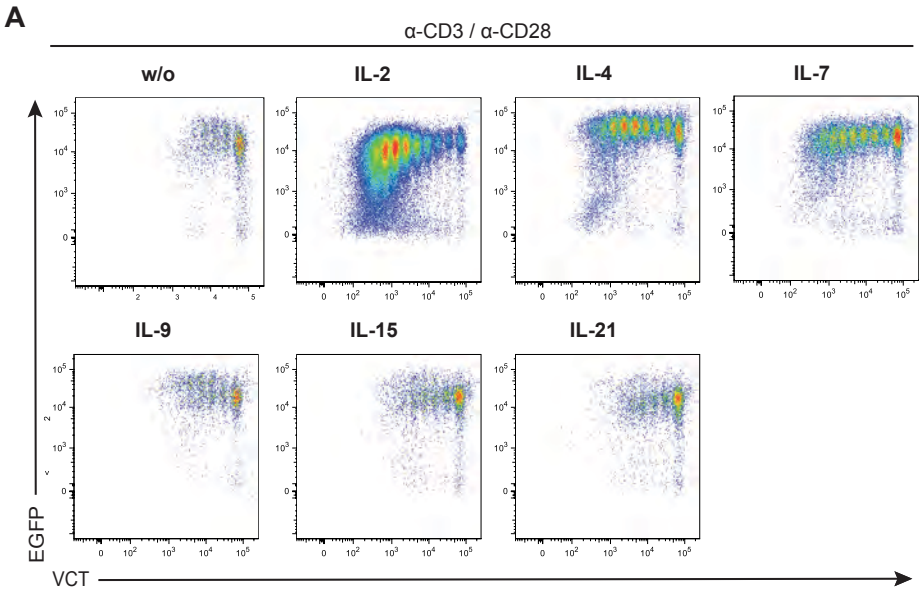

Figure 24: EBI2 expression of helper T cells during homeostatic proliferation

A) Flow cytometric analysis of helper T cells MACS purified from the spleen and lymph nodes of EBI2$^{+/EGFP}$ mice. Cells were labeld with violet cell tracer (VCT) and stimulated *in vitro* with α-CD3/α-CD28 antibodies and indicated cytokines for 5d. Data is representative of two independent experiments (n=2).

Proliferation of T cells was strongest when IL-2 was added, but also addition of IL-4 and IL-7 triggered strong T cell expansion, which was not the case for the other common γ-chain cytokines. More important, we found that only addition of IL-2 significantly decreased EBI2-EGFP expression during T cell proliferation (Fig. 24A), which was not the case for other cytokines signaling via the common γ-chain.

4.3 IL-1β and IL-23 strongly stabilize EBI2 expression of T_H17 cells

As we found T_H17 cells differentiated *in vitro* with TGF-β1 and IL-6 being negative for EBI2 expression we further characterized its expression under various differentiation conditions.

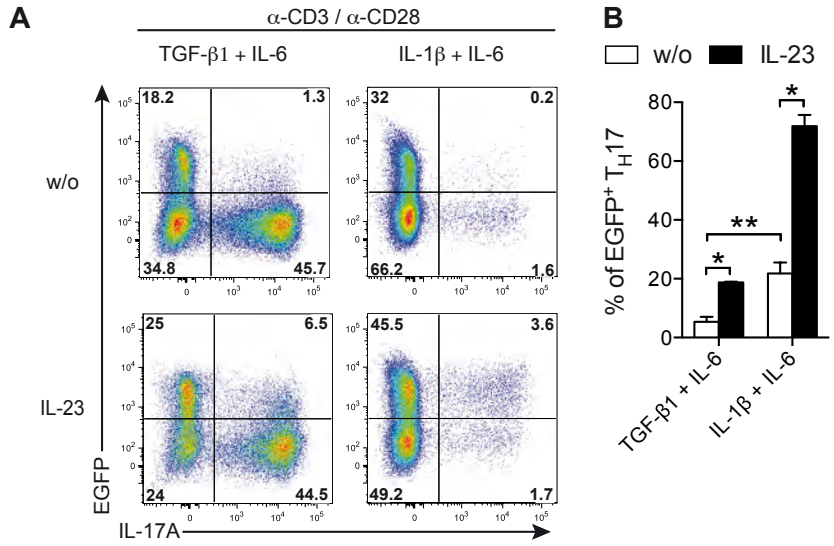

Figure 25: IL-1β and IL-23 stabilize EBI2 expression of T_H17 cells

A) Flow cytometric analysis of naïve helper T cells from EBI2$^{+/EGFP}$ mice were differentiated in vitro under indicated conditions. B) Statisical analysis of EGFP$^+$/IL-17A$^+$ T cells after in vitro differentiation under indicated conditions with or without addition of IL-23. Data is representative of at least two independent experiments (n=2)

Flow cytometric analysis of EBI2 expression on T_H17 cells revealed that during differentiation in the presence of TGF-β1 and IL-6, EBI2 expression was significantly elevated by addition of IL-23 (Fig. 25AB). It was shown that T_H17 cells might also be differentiated in the absence of TGF-β1 when IL-1β and IL-6 are used instead. However differentiation of T_H17 cells using these stimuli less IL-17A secreting T_H17 cells are generated compared to differentiation with TGF-β1 and IL-6. Interestingly, addition of IL-23 to this pathway led to stabilization of EBI2 expression with more than 70% of the resulting T_H17 cells being EBI2 positive (Fig. 25AB).

4.4 EBI2 deficient T cells transfer colitis to same extend than wild type T cells

Our analysis of EBI2 expression in different T cell subsets and of *in vitro* differentiated helper T cells revealed high expression in naïve CD4$^+$ T cells. To further

study the role of EBI2 in these cells *in vivo*, we made use of a transfer model of colitis which is induced by transfer of purified naïve helper T cells into immuno-deficient RAG1$^{-/-}$ mice. Disease development and severity was monitored weekly by mini-endoscopy.

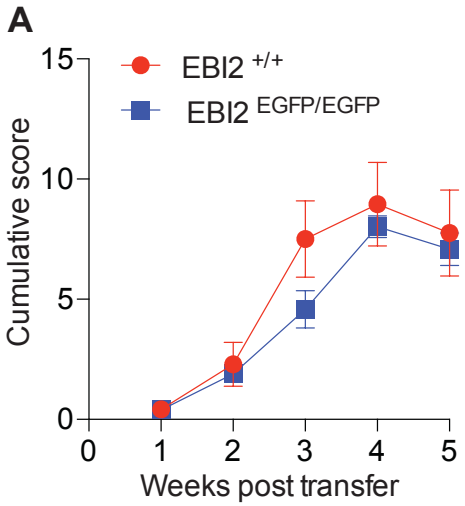

Figure 26: EBI2 deficient T cells transfer colitis comparable to wild type T cells
Naïve helper T cells were isolated from EBI2 deficient mice or littermate controls. Afterwards 5 x10^5 cells were transferred i.p. into RAG1$^{-/-}$ mice. Onset and severity of colitis were monitored weekly by mini-endoscopy. A) Colitis development and severity after T cell transfer. Mice were sacrificed five weeks post transfer. Graph shows mean with SD (n=6 for EBI2$^{+/+}$ and n=12 for EBI2$^{EGFP/EGFP}$)

We found most naïve helper T cells to express EBI2 indicating possible functional relevance for this subset. However when we performed transfer colitis with EBI2 deficient naïve helper T cells we could not detect differences in disease development compared to transfer of the same cells from littermate controls (Fig. 26A). By mini-endoscopy we could show that onset as well as severity of induced colitis was comparable between the two groups suggesting no functional relevance of EBI2 expression on naïve helper T cells in this model.

4.5 Enzymes involved in 7α,25-OHC generation are highly regulated in EAE

It was recently shown that mice deficient for CH25H show delayed onset of EAE upon active immunization with MOG/CFA (Chalmin et al., 2015). These findings suggest that expression of CH25H may contribute to migration of T cells into the inflamed CNS. However little is known about kinetics and expression levels of CH25H in the CNS during the course of EAE. Furthermore CYP7B1 and HSD3B7 expression have not been analyzed in the EAE model. Therefore we were curious to analyze the expression profile of the enzymes involved in the generation and inactivation of 7α,25-OHC in this model (Fig. 27A). Hence we analyzed mRNA expression of the different enzymes in tissue of naïve mice and of litter mate animals ten days after EAE induction.

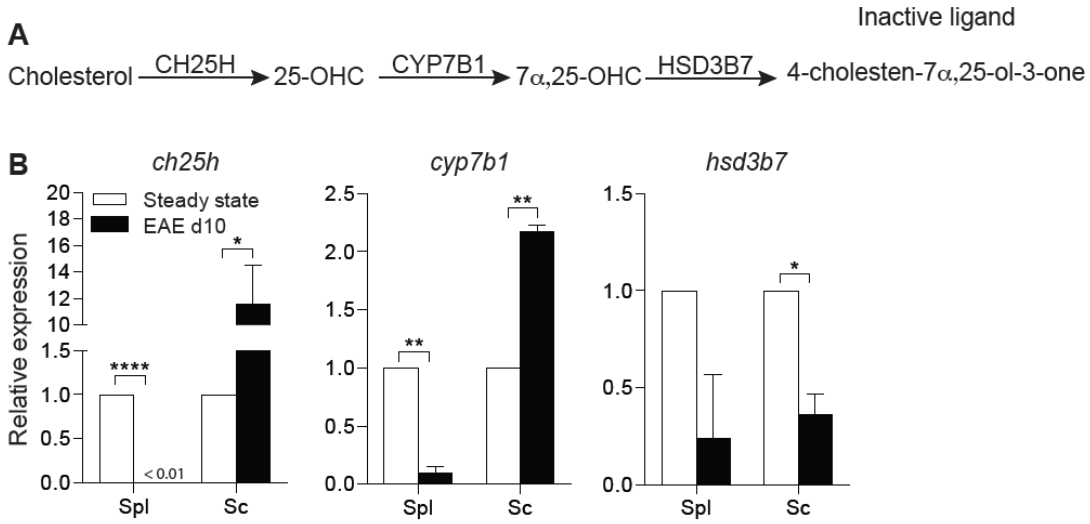

Figure 27: Expression of enzymes involved in generation of 7α-25,OHC during EAE

A) Synthesis and degradation of 7α,25-OHC: Cholesterol is converted in 25-OHC by CH25H and further converted into the active EBI2 ligand 7α,25-OHC by CYP7B1. Furthermore HSD3B7 may degrade the active ligand. B) Expression of *ch25h*, *cyp7b1* and *hsd3b7* mRNA in spleen and spinal cord of naïve mice or mice after EAE induction. qRT-PCR was carried out by using *hprt* as reference gen. Expression under steady state conditions was considered as the value of 1 and changes upon EAE induction was calculated in comparison. Data is representative of two independent experiments (n=3)

Strikingly we found that expression of *ch25h* and *cyp7b1* mRNA was significantly decreased in the spleen, but significantly up-regulated in the spinal cord after EAE induction. In contrast to the latter enzymes the EBI2 ligand degrading enzyme *hsd3b7* was found to be reduced in both the spleen and the CNS (Fig. 27B). This suggests that in naïve mice concentrations of 7α,25-OHC are high in the periphery and low in the CNS. However upon EAE induction, 7α,25-OHC synthesis becomes more abundant in the CNS than in the periphery.

4.6 Diminished $CD4^+$ DCs in EBI2 deficient mice do not affect priming of T cells

EBI2 has been shown to play a crucial role in positioning and homeostasis of $CD4^+$ dendritic cells and furthermore, EBI2 deficiency results in almost complete absence of this DC subset in the spleen (Gatto et al., 2013; Yi and Cyster, 2013).

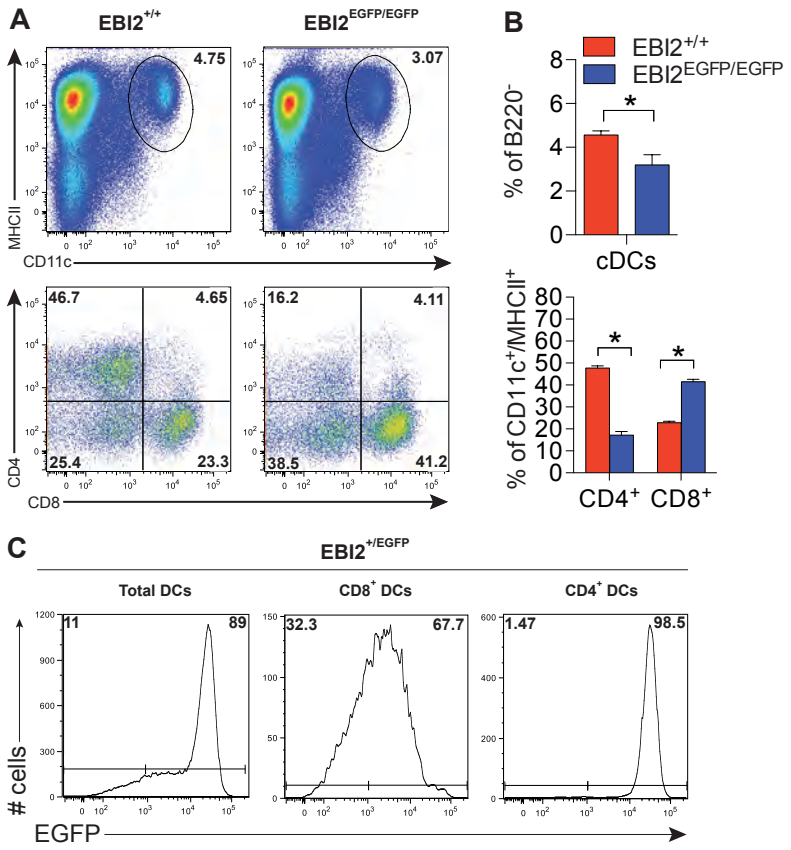

Figure 28: CD4$^+$ Dendritic cells express EBI2 and are diminished in EBI2 deficient mice

A) Flow cytometric analysis of dendritic cells in the spleen of EBI2$^{EGFP/EGFP}$ mice or litter mate controls. Dendritic cells were gated as CD90.2$^-$, B220$^-$, CD11c$^+$, MHCII$^+$ living cells and further analyzed for CD4$^+$ and CD8$^+$ DC subsets. B) Quantification of total and CD4$^+$ / CD8$^+$ dendritic cells in the spleen of naïve EBI2$^{EGFP/EGFP}$ mice or litter mate controls. Graphs show Mean with SD. C) Expression of EBI2 in indicated DCs from EBI2$^{+/EGFP}$ mice. Data is representative of at least three independent experiments (n=3).

Using our reporter mice we could also demonstrate that EBI2 expression is highest on CD4$^+$ DCs compared to CD8$^+$ DCs (Fig. 28C). Similarly we found the CD4$^+$ DC subset diminished in the spleen of EBI2 deficient mice (Fig. 28AB). As DCs represent a major subset of antigen presenting cells, we reasoned that the reduced numbers of this specific DC subset might influence the priming of T cells in the peripheral lymphoid organs upon EAE induction. To test this hypothesis we transferred CFSE labeled helper T cells from 2D2 mice (expressing CD90.1) i.v. into either EBI2 deficient mice or wt littermates (both expressing CD90.2). One day post transfer host mice were

immunized with MOG/CFA or left untreated and five days post immunization we analyzed and quantified transferred T cells in the spleen and lymphnodes via flow cytometry.

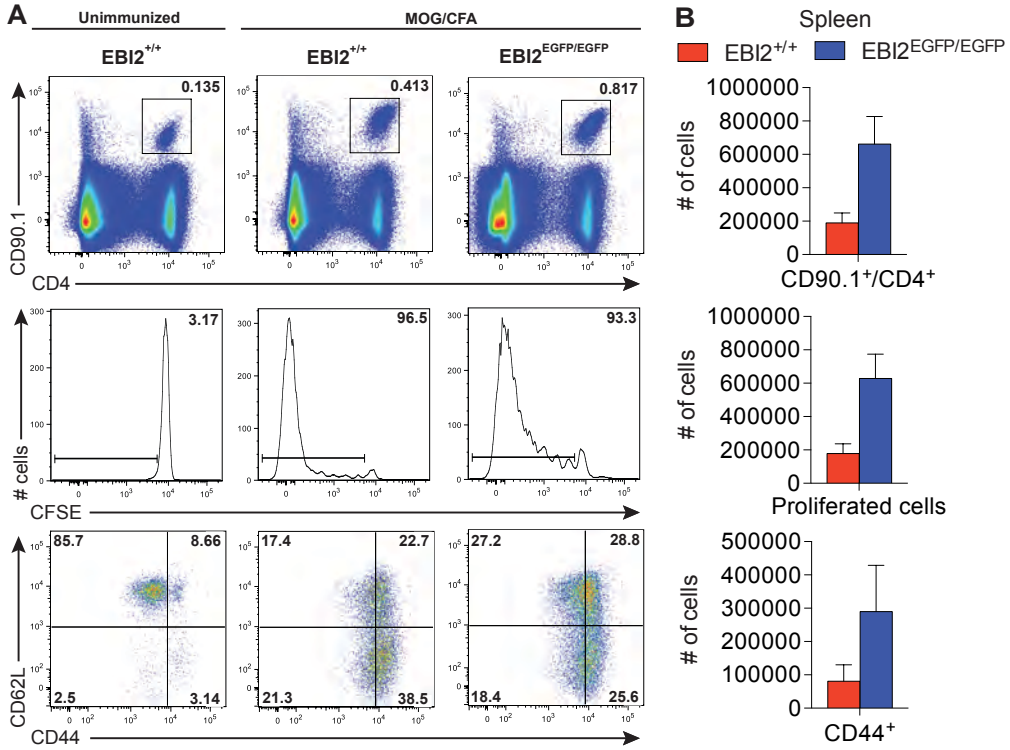

Figure 29: Normal priming of T cells in the spleen of EBI2 deficient mice

Purified helper T cells from 2D2 x Thy1.1 mice were labeled with CFSE and transferred into EBI2$^{EGFP/EGFP}$ mice or littermate controls. One day later, hosts were immunized with MOG/CFA or left untreated. Mice were sacrificed five days post transfer and analyzed by flow cytometry. Transferred T cells were gated as CD90.2^{-}, CD90.1^{+}, CD4^{+} living cells and further analyzed for CFSE dilution and expression of CD62L and CD44. A) Analysis of transferred T cells in the spleen of immunized or untreated hosts from indicated genotypes. B) Statistical analysis of transferred T cells in the spleen of indicated hosts. Graphs show mean with SD. Data is representative for two independent experiments (n=5).

We observed that transferred T cells did not proliferate and stayed in a naïve state, when host mice were left untreated (Fig. 29A). Upon immunization T cells proliferated and up-regulated expression of the activation marker CD44. However we did not detect differences in the number of proliferated or CD44^{+} T cells between the two groups in spleen (Fig. 29AB).

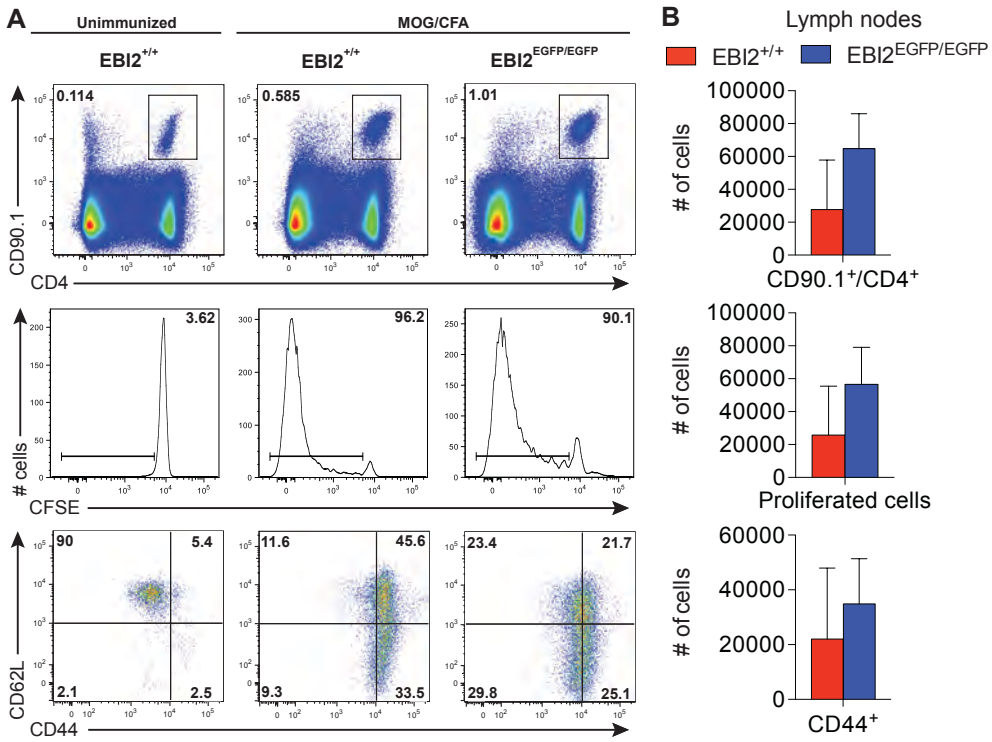

Figure 30: Normal priming of T cells in the lymph nodes of EBI2 deficient mice

Purified helper T cells from 2D2 x Thy1.1 mice were labeled with CFSE and transferred into EBI2$^{EGFP/EGFP}$ mice or littermate controls. One day later, hosts were immunized with MOG/CFA or left untreated. Mice were sacrificed five days post transfer and analyzed by flow cytometry. Transferred T cells were gated as CD90.2^{-}, CD90.1^{+}, CD4^{+} living cells and further analyzed for CFSE dilution and expression of CD62L and CD44. A) Analysis of transferred T cells in the lymph nodes of immunized or untreated hosts from indicated genotypes. B) Statistical analysis of transferred T cells in the lymph nodes of indicated hosts. Graphs show mean with SD. Data is representative for two independent experiments (n=5).

In the MOG induced EAE model pathogenic T cells are effectively primed in the lymph nodes, therefore we also analyzed priming of T cells in the lymph nodes of EBI2 deficient mice. Similar to our findings obtained by the analysis of T cells in the spleen, we could show that T cell proliferation and activation appeared to be normal in the lymph nodes of EBI2 deficient mice (Fig.30 AB). Hence reduced numbers of CD4^{+} DCs in EBI2 deficient animals seemed not to affect priming of T cells upon immunization with MOG/CFA.

4.7 EBI2 expression is high on pathogenic T_H17 cells but does not affect active EAE induction

As mentioned before CH25H deficient mice show a delayed onset of EAE (Chalmin et al., 2015). Therefore we reasoned that the increased 7α,25-OHC concentration in the CNS of immunized mice may influence the migration of pathogenic T helper cells from the periphery to the CNS in an EBI2 dependent manner.

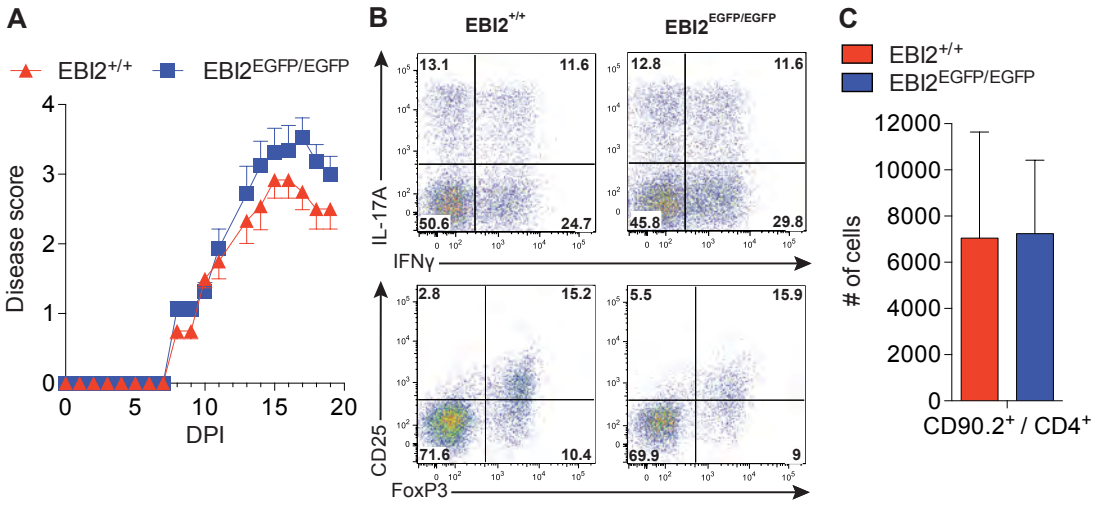

Figure 31: EBI2 deficiency does not affect active EAE induction

Active EAE was induced in $EBI2^{EGFP/EGFP}$ mice or littermate controls by immunization with MOG/CFA and administration of pertussis toxin. A) Clinical development of EAE of indicated genotypes. Graph shows mean with SEM (n=7). B) Flow cytometric analysis of cytokine and FoxP3 expressing helper T cells in the CNS of mice with EAE. Cells were gated as $CD11b^-$, $CD90.2^+$, $CD4^+$ living cells. C) Statistical analysis of infiltrating helper T cells in the CNS of mice with EAE. Graph shows mean with SD (n=7). Data is representative for three independent experiments (n=7).

When we immunized EBI2 deficient mice with MOG/CFA and pertussis toxin, we did not find differences in disease scores in active EAE in EBI2 deficient mice compared to control littermates (Fig. 31A and Table 9). Furthermore, onset of EAE was not delayed when EBI2 is absent.

	Genotype / mouse strain	Disease incidence	Mean Max.	Mean day of onset (p value)
Exp. 1	$EBI2^{+/+}$	100% (11/11)	3.3 ± 0.75	8.6 ± 0.5
	$EBI2^{+/EGFP}$	87.5% (7/8)	3.3 ± 1.5	10
	$EBI2^{EGFP/EGFP}$	88.9% (8/9)	3.2 ± 1.5	12.6 ± 1.4 (n.a.)
Exp. 2	$EBI2^{+/+}$	100% (8/8)	2.9 ± 0.6	8 (n.a.)
	$EBI2^{+/EGFP}$	87.5% (7/8)	3.4 ± 1.1	8 (n.a.)
	$EBI2^{EGFP/EGFP}$	100% (8/8)	3.5 ± 0.7	8 (n.a.)
Exp. 3	$EBI2^{+/+}$	100% (10/10)	2.9 ± 0.8	8 (n.a.)
	$EBI2^{-/-}$	87.5% (7/8)	3 ± 1.2	8.1 ± 0.4 (n.a.)

Table 9: Active EAE

Table shows results of three individual EAE experiments. Experiment 3 was performed using $EBI2^{-/-}$ mice, which lack the EGFP reporter. Data shows disease incidence as percent of total group size and mice numbers. Maximum disease score and mean day of onset (mice with EAE score ≥ 1) are shown with standard deviation. N.a.: not applicable.

When we analyzed CNS infiltrating cells via flow cytometry we did not observe differences in T cell numbers (Fig. 31C). Additionally we could not detect changes in the frequencies of cytokine expressing helper T cells as well as regulatory T cells (Fig. 31B).

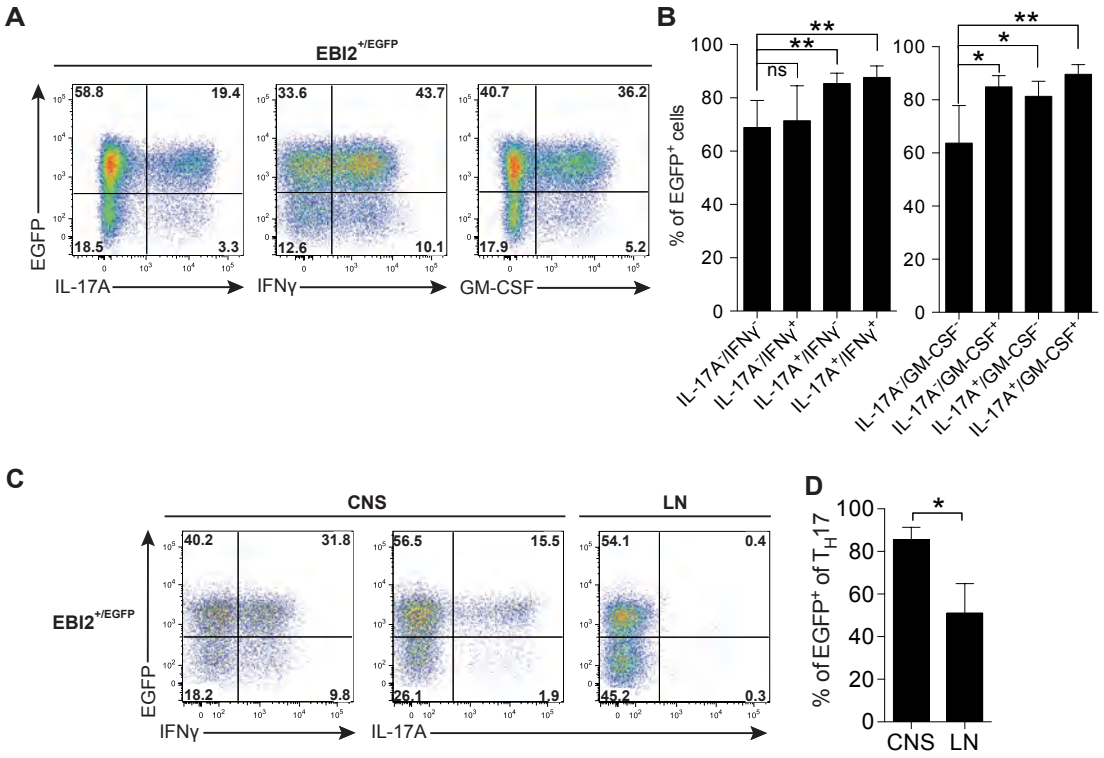

Figure 32: T_H17 cells in the inflammed CNS express high levels of EBI2

Active EAE was induced in EBI2$^{+/EGFP}$ mice or litter mate controls by immunization with MOG/CFA and administration of pertussis toxin. A) Flow cytometric analysis of EBI2 expression of T cells in the CNS of EBI2$^{+/EGFP}$ mice with EAE. Cells were gated as CD11b^{-}, CD90.2^{+}, CD4^{+} living cells. B) Statistical analysis of EGFP^{+} cytokine expressing helper T cells in the CNS of EBI2$^{+/EGFP}$ with EAE. Graph shows mean with SD (n=7). C) Flow cytometric analysis of EBI2 expression of T cells in the CNS and lymphnodes of EBI2$^{+/EGFP}$ mice with EAE. Cells were gated as CD11b^{-}, CD90.2^{+}, CD4^{+} living cells.D) Statistical analysis of EGFP^{+} T_H17 cells in the CNS and lymphnodes of EBI2$^{+/EGFP}$ with EAE. Graph shows mean with SD (n=7). Data is representative for three independent experiments (n=7).

However when we analyzed EBI2 expression by different effector T cells in the inflamed CNS we found that it correlated strongly with IL-17A and GM-CSF, but not IFN-γ expression (Fig. 32AB). In addition, the frequency of EBI2-expressing T_H17 cells was significantly higher in the CNS compared to spleen and lymphnodes (Fig. 32CD).

4.8 EBI2 expression confers pathogenicity to myelin specific T_H17 cells

Given our findings that T_H17 cells express EBI2 more uniformly than T_H1 cells, we reasoned that they might be influenced stronger by EBI2 mediated chemotaxis.

Therefore we analyzed the pathogenicity of EBI2 deficient T_H17 in an adoptive transfer model of EAE. Strikingly, when we transferred EBI2 deficient T_H17 cells into $RAG1^{-/-}$ hosts, onset of EAE was significantly delayed (Fig. 33A and Table 10). Analysis of CNS infiltrating T cells at this early time point revealed significantly reduced numbers of $CD4^+$ T cells and IL-17A expressing effector T cells when EBI2 deficient T_H17 cells were transferred (Fig. 33BC).

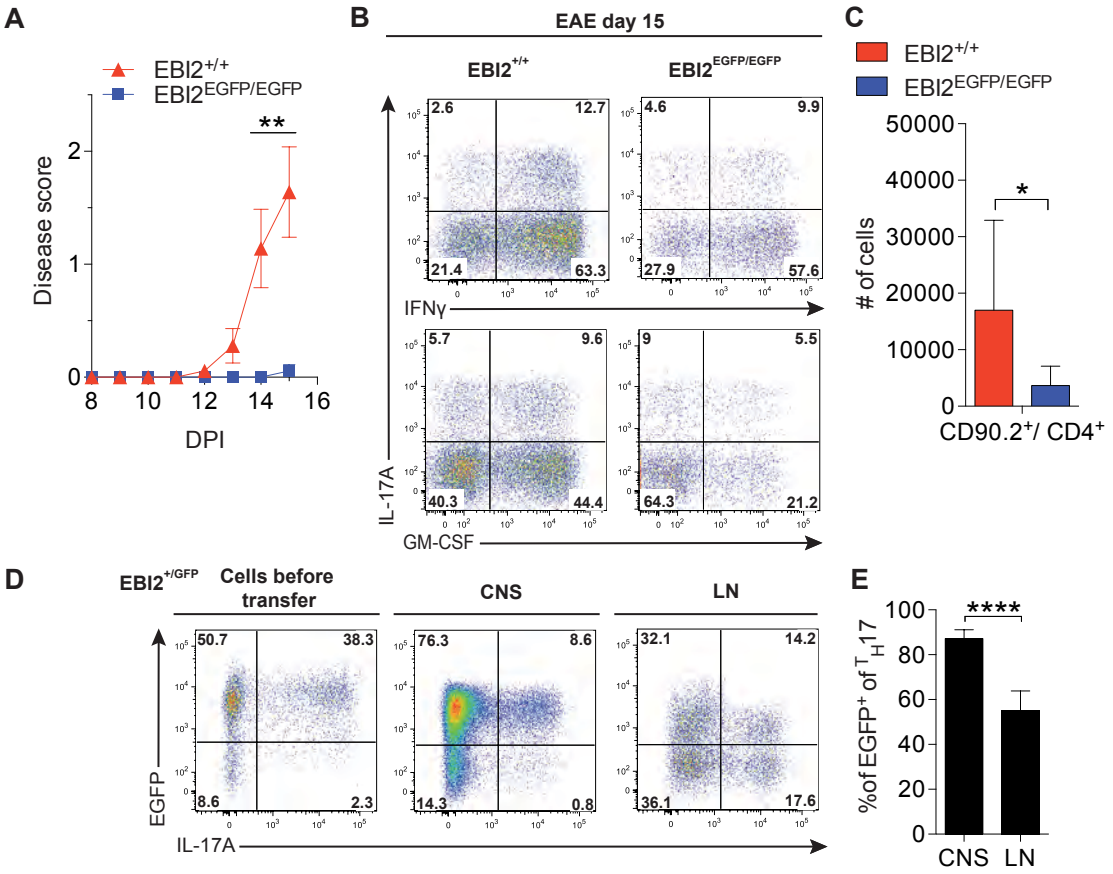

Figure 33: EBI2 deficient TH17 cells transfer EAE with delayed onset

T_H17 cells from $EBI2^{EGFP/EGFP}$ mice or littermate controls were generated as described and transferred into RAG1 deficient mice. Pertusis toxin was administred on d0 and d2. A) EAE development of RAG1 hosts transferred with T_H17 cells from indicated genotypes. Graph shows mean with SEM (n=8). B) Flow cytometric analysis of cytokine expressing helper T cells in the CNS of mice with EAE. Cells were gated as $CD11b^-$, $CD90.2^+$, $CD4^+$ living cells. C) Statistical analysis of infiltrating helper T cells in the CNS of mice with EAE. Graph shows mean with SD (n=8). D) Flow cytometric analysis of EBI2 expression of T_H17 cells in the CNS and lymphnodes after transfer of $EBI2^{+/EGFP}$ T_H17 cells. Cells were gated as $CD11b^-$, $CD90.2^+$, $CD4^+$ living cells. E) Statistical analysis of $EGFP^+$ T_H17 cells in the CNS and lymphnodes after transfer of $EBI2^{+/EGFP}$ T_H17 cells. Graph shows mean with SD (n=8). Data is representative for three independent experiments (n=8).

However at later time points severity of EAE and infiltration of T_H17 cells became comparable between the two groups (Fig. 34A-D and Table 10).

	Genotype / mouse strain	Disease incidence	Mean Max.	Mean day of onset (p value)
15 days: Exp. 1	$EBI2^{+/+}$	100% (10/10)	1.9 ± 1.2	n.a.
	$EBI2^{EGFP/EGFP}$	55.6 (5/9)	1.2 ± 0.5	n.a.
15 days: Exp.2	$EBI2^{+/+}$	88.9 % (8/9)	1.8 ± 1.1	n.a.
	$EBI2^{EGFP/EGFP}$	11.1% (1/9)	0.5	n.a.
28 days: Exp.1	$EBI2^{+/+}$	100% (4/4)	3.3 ± 1.2	19.2 ± 2.2 (0.021)
	$EBI2^{+/EGFP}$	100% (4/4)	3 ± 1.5	21 ± 0.7 (0.006)
	$EBI2^{EGFP/EGFP}$	75% (3/4)	4	24 ± 1 (n.a.)
28 days: Exp.2	$EBI2^{+/+}$	100 % (7/7)	3.9 ± 0.7	17.8 ± 3 (0.026)
	$EBI2^{+/EGFP}$	83.4% (5/6)	4.3 ± 0.3	15.8 ± 2.9 (0.002)
	$EBI2^{EGFP/EGFP}$	100% (11/11)	4	21.4 ± 2.4 (n.a.)

Table 10: T_H17 Transfer EAE

Table shows results of four individual transfer EAE experiments, which were either run for 15 or 28 days. Data shows disease incidence as percent of total group size and mice numbers. Maximum disease score and mean day of onset (mice with EAE score ≥ 1) are shown with standard deviation. P value was calculated for mean day of onset compared to $EBI2^{EGFP/EGFP}$ mice using unpaired two-tailed Students t-test.

Analysis of cytokine expression by infiltrating T cells showed, similar as previously reported (Kurschus et al., 2010) that many T cells had become IFN-γ positive (Fig.33B and Fig. 34C).

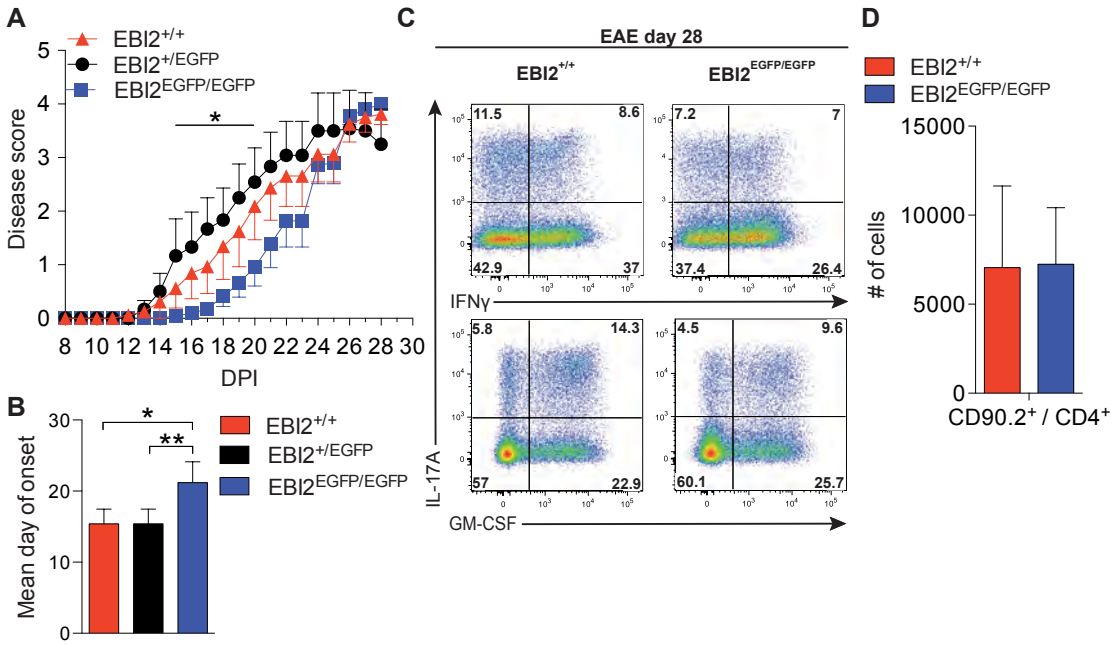

Figure 34: Transfer EAE severity is comparable at later time-points

T_H17 cells from indicated genotypes were generated as described and transferred into RAG1 deficient mice. Pertusis toxin was administred on d0 and d2. A) EAE development of RAG1 hosts transferred with T_H17 cells from indicated genotypes. Graph shows mean with SEM (n=8). B) Statistical analysis of mean day of EAE onset after transfer of T_H17 cells from indicated genotypes. Graph shows mean with SD (n=8) C) Flow cytometric analysis of cytokine expressing helper T cells in the CNS of mice with EAE. Cells were gated as $CD11b^-$, $CD90.2^+$, $CD4^+$ living cells. D) Statistical analysis of infiltrating helper T cells in the CNS of mice with EAE. Graph shows mean with SD (n=8). Data is representative for three independent experiments (n=8).

These cells probably induced EAE in an EBI2 independent fashion. Similar to our observations after active EAE induction, we also found in this model higher frequencies of EBI2-expressing T_H17 cells in the CNS compared to cells before transfer and in the lymphnodes (Fig. 33DE). These findings suggested that EBI2 expression may influence the migration of T_H17 cells from the periphery to the CNS.

4.9 Human T_H17 cells express EBI2

As we found implications of EBI2 in pathogenicity of murine T_H17 cells, we also analyzed its expression in human cells by using a monoclonal antibody specific for human EBI2.

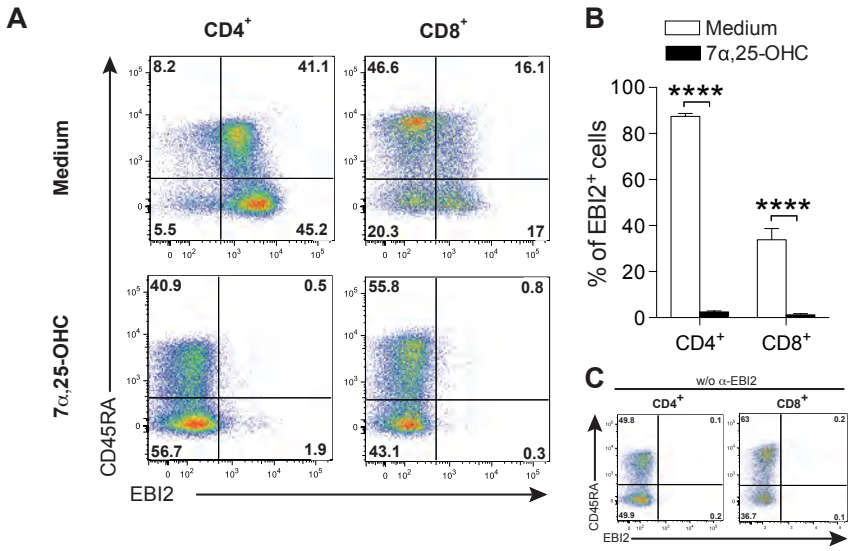

Figure 35: Human helper T cells express high levels of EBI2

A) Flow cytometric analysis of $CD4^+$ and $CD8^+$ T cells from PBMCs of healthy donors stimulated with 1 µM 7α,25-OHC for 4 h or left untreated. Cells were gated as $CD14^-$, $CD3^+$ cells. B) Statistical analysis of $EBI2^+$ T cells stimulated with 1 µM 7α,25 OHC or left untreated. Graph shows mean with SD (n=5). C) Flow cytometric analysis of human T cells stained without addition of mouse-α-EBI2 antibody, but other reagents. Data is representative of at least three independent experiments (n=5).

Staining of human PBMCs from healthy donors revealed that EGFP expression in our reporter mice reflected very well expression of EBI2 by human T cells. Indeed, the majority of T helper cells expressed EBI2 in contrast to $CD8^+$ T cells (Fig. 35A-C and Fig. 36A) However in contrast to the mouse data, EBI2 expression was higher on effector ($CD45RA^-$) than on naïve ($CD45RA^+$) T helper cells (Fig. 35A and Fig. 36B).

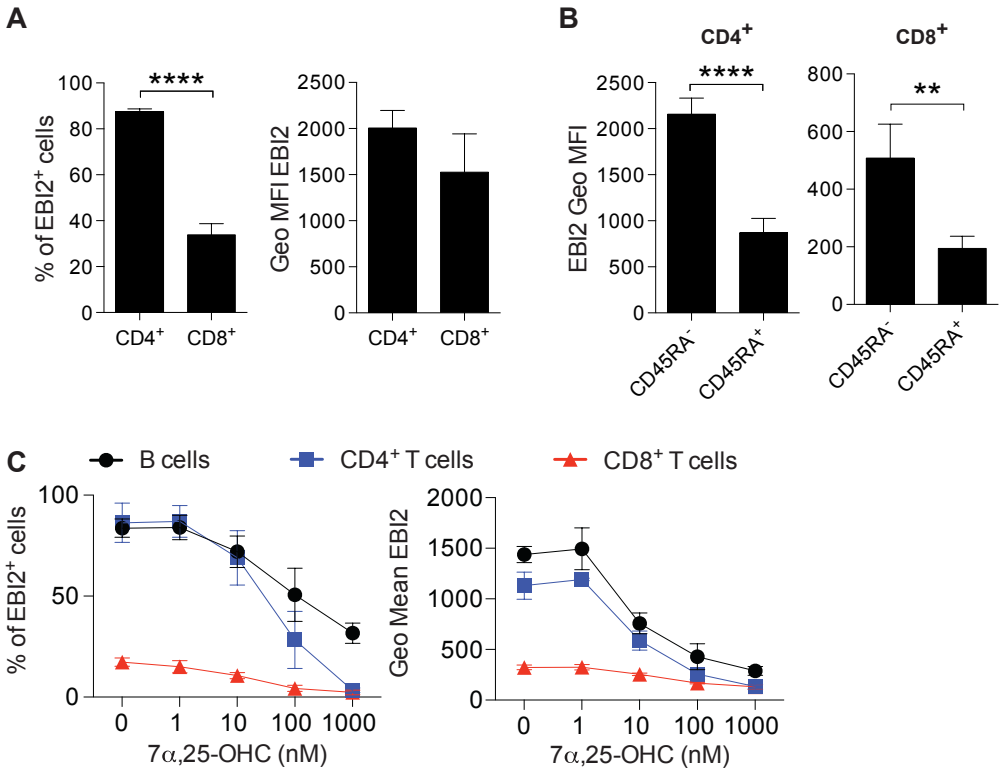

Figure 36: EBI2 expression in human PBMCs

A) Statistical analysis of EBI2$^+$ T cells and geometrical mean fluorescence intensity (Geo. MFI) of EBI2-Fitc on indicated T cell subsets. Graph represents mean with SD (n=5). B) Statistical analysis of geometrical mean fluorescence intensity (Geo. MFI) of EBI2-Fitc on indicated T cell subsets. Graph represents mean with SD (n=5). C) Statistical analysis of EBI2$^+$ cells and geometrical mean fluorescence intensity (Geo. MFI) in indicated cell types in relation to pre-treatment with increasing concentrations of 7α,25-OHC. Graph represents mean with SD (n=5). Data is representative for at least two independent experiments.

In line with the *in vitro* migration assay of murine T cells, increasing concentrations of 7α,25-OHC reduced EBI2 cell surface expression on human T cells (Fig. 35AB) in a dose dependent manner (Fig. 36C) near to background levels (Fig. 35C).

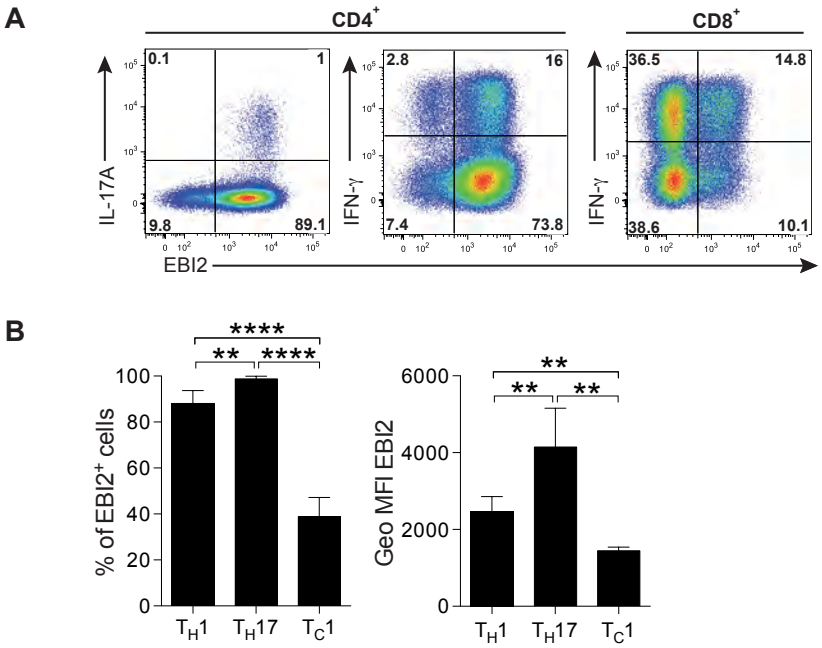

Figure 37: Human T_H17 cells express high levels of EBI2

A) Flow cytometric analysis of EBI2 expression in IL-17A or IFNγ secreting T cells of PBMCs from healthy donors. Cells were stimulated 5 h with PMA/Ionomycin/Monensin prior to staining and T cells were gated as $CD14^-$, $CD3^+$ cells. B) Statistical analysis of $EBI2^+$ T cells and geometrical mean fluorescence intensity (Geo. MFI) of EBI2-Fitc on indicated T cell subsets. Graph represents mean with SD (n=5). Data is representative for at least two independent experiments (n=5).

Strikingly, when we analyzed cytokine secreting cells, we found that T_H17 cells expressed higher levels of EBI2 than T_H1 or IFN-γ expressing $CD8^+$ T cells (Fig. 37AB) both in percentage as well as in fluorescence intensity.

4.10 T cells in the blood of MS patients show normal expression of EBI2

Using the monoclonal antibody to EBI2 we could show that human helper T cells express EBI2. Moreover EBI2 expression was highest on human T_H17 cells compared to other subsets analyzed. Using EAE as murine model for human MS we found that EBI2 expression is elevated in pathogenic T_H17 cells in the inflamed CNS. Therefore we were curious to analyze EBI2 expression on T cells from MS patients and obtained

PBMCs from untreated MS patients. However analysis of EBI2 expression revealed that it is comparable to healthy donors (Fig 38AB).

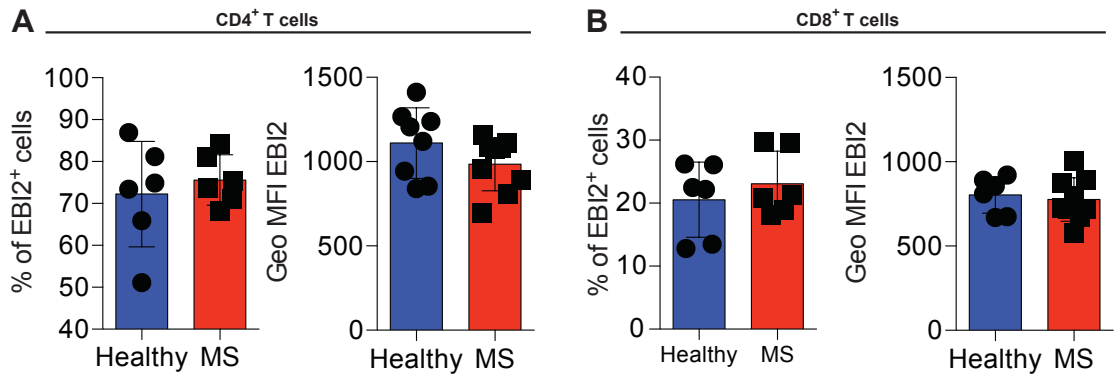

Figure 38: EBI2 expression in T cells from MS patients

PBMCs from Healthy donors and untreated MS patients were analyzed via flow cytometry. T cells were gated as $CD14^-$ / $CD3^+$ cells which were further devided based on CD4 and CD8 expression. Graph represents mean with SD (n=6) A) Statistical analysis of $EBI2^+$ cells on CD4 positive T cells. Geo MFI of EBI2-Fitc on $EBI2^+$ CD4 T cells cells. B) Statistical analysis of $EBI2^+$ cells on CD8 positive T cells. Geo MFI of EBI2-Fitc on $EBI2^+$ CD8 T cells cells.

We did not detect significant changes in the percentage of EBI2 positive T cells nor in the expression levels on T cells from MS patient PBMCs.

4.11 T cells in MS lesions express EBI2

We did not observe differences in EBI2 expression in T cells of healthy donors compared to MS patients. However we were still interested to analyze CNS infiltrating T cells in lesions of MS patients. Therefore we performed histology of MS tissue sections and stained them for EBI2.

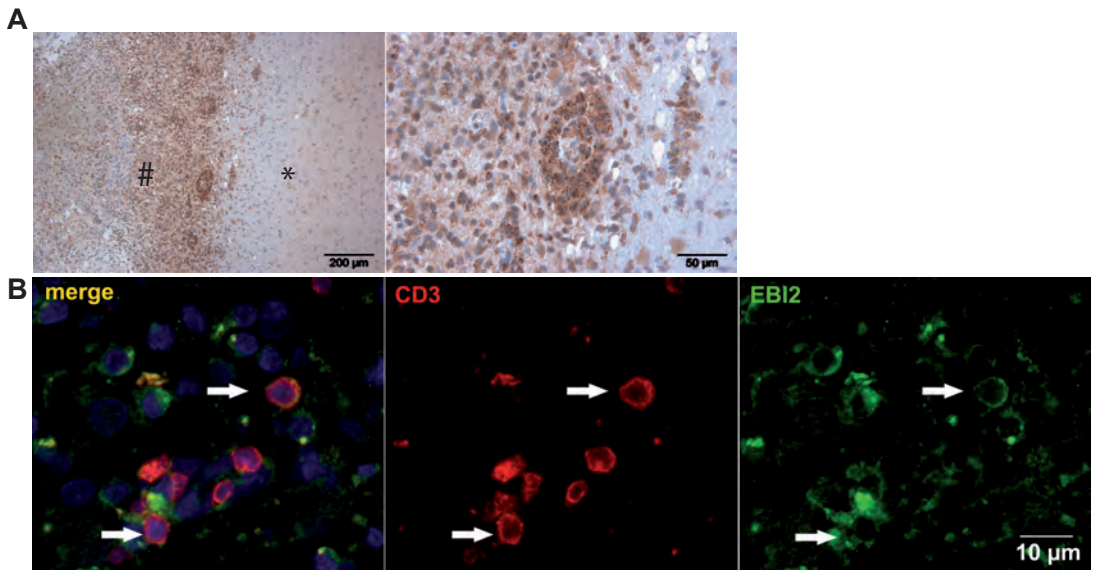

Figure 39: EBI2 is expressed in MS lesions

A) Human MS tissue sections were stained with anti-EBI2 (brown). In the left panel the infiltration of the lesion with numerous EBI2-positive cells (#), whereas in the adjacent periplaque white matter (*) only few positive cells were detected. The right panel shows in higher magnification a perivascular infiltrate with numerous EBI2-positive cells. B) Immunofluorescence picture of human MS tissue sections stained with anti-CD3 (red), anti-EBI2 (green) and DAPI (blue). In the left panel the overlay of all colors is shown. Arrows, $CD3^+$ T cells expressing EBI2.

Strikingly we could show, that localization of EBI2 expressing cells is almost completely restricted to the inflamed white matter (Fig. 39A). In contrast the non-affected white matter (NAWM) does not show high distribution of EBI2 expressing cells (Fig. 39A). Morphological appearance of EBI2 expressing cells points to a subset of macrophages, therefore we further stained for T cells to analyze EBI2 expression elusively on this cells. Indeed we found that $CD3^+$ T cells within the inflamed white matter express EBI2 (Fig. 39B).

5. Discussion

It was shown that B cells and DCs migrate towards 7α,25-OHC in an EBI2 dependent manner. B cells deficient for EBI2 show delayed T cell dependent antibody responses and plasma cell differentiation upon antigen contact. Furthermore EBI2 expression is crucial for the development and maintenance of $CD4^+$ DCs in the spleen of naïve mice. We have analyzed the role of EBI2 in T cells in naïve mice and upon induction of EAE and could demonstrate that EBI2 is most uniformly expressed by encephalitogenic T_H17 cells and crucial for their early CNS transmigration. Moreover, we analyzed the expression of the enzymes involved in synthesis of 7α,25-OHC in the context of EAE. Our findings therefore indicate that concentrations of the EBI2 ligand are highly regulated in the course of EAE and increase in the inflamed spinal cord. In parallel with our data of studies in mice, we found human T_H17 cells to express EBI2 and furthermore could show that EBI2 expressing cells are highly abundant in the inflamed white matter of MS patient autopsies.

5.1 T cell development and migration in EBI2 deficient mice

Up to know a role for EBI2 in immune cells was only shown for localization of B cells during germinal center reaction and for a certain subpopulations of DCs in the spleen (Gatto et al., 2013; Hannedouche et al., 2011; Liu et al., 2011; Pereira et al., 2009; Yi and Cyster, 2013; Yi et al., 2012). Using our novel $EBI2^{+/EGFP}$ reporter mice we found that expression of EBI2 in T cells is strongly regulated also during thymic development. We found expression of EBI2 in the early DN1 stage of double negative

($CD4^-$ / $CD8^-$) precursor cells. In later stages (DN2-4), as well as in double positive ($CD4^+$ / $CD8^+$) T cells EBI2 expression was absent. Although we found this strong regulation of EBI2 expression during thymic maturation of T cells, we did not detect significant differences in thymic T cells in EBI2 deficient animals compared to littermate controls. Up to now we only analyzed T cells using flow cytometry. Therefore, it is possible that localization of EBI2 deficient T cells in the thymus is different, although its deficiency does not lead to significant changes in the developmental process. As we are working with mice completely deficient for EBI2 another possibility could be that the lack of EBI2 expression was compensated by other pathways triggered to a greater extent in our EBI2 deficient mice. Therefore, it would be interesting in this context to work with T cell specific knock-out mice (e.g. $EBI2^{Fl/Fl}$ animals crossed to CD4-Cre or Lck-Cre mice) to further analyze the role of EBI2 expression in T cells in the thymus. It is also be possible that EBI2 is regulated together with other genes, which are more relevant for the different maturation steps and that EBI2 does not play a role during thymocyte maturation. When we analyzed EBI2 expression in T cells from the peripheral lymphoid organs, we found the most uniform expression in naïve T helper cells compared to $CD8^+$ T cells. RT-PCR analysis of sorted EGFP positive and EGFP negative helper T cells showed that expression of the EGFP reporter corresponded with actual *ebi2* mRNA levels. We performed *in vitro* migration assays towards 7α,25-OHC and we indeed found stronger chemotaxis of $CD4^+$ T cells towards the EBI2 ligand compared to $CD8^+$ T cells. In addition EBI2 deficient T cells did not migrate at all to 7α,25-OHC, ruling out the possibility of EBI2 independent migration towards 7α,25-OHC. However the

migration of T cells towards 7α,25-OHC was relatively low compared to chemokines such as CCL19/CCL21, and T cells migrated towards the EBI2 ligand only after pre-activation with α-CD3 and α-CD28 antibodies. This is similar to the behaviour of B cells as they need to be pre-activated by engagement of the B cell receptor and CD40. Furthermore, it was demonstrated that EBI2-mediated chemotaxis is rather involved in specific steps during positioning and migration of B cells within the lymphoid follicles (Hannedouche et al., 2011; Liu et al., 2011; Yi et al., 2012) rather than in homing of B cells to the secondary lymphoid organs. In line with this we did not detect differences in homing of EBI2 deficient T cells to the spleen and lymphnodes when adoptively transferred into congenic wt hosts. Interestingly, we also found that high concentrations of 7α,25-OHC did not only impair EBI2-mediated chemotaxis but also reduced migration towards CCL19/CCL21. This effect has also been shown for migration of B cells and is called heterologous desensitization. However, it remains to be elucidated whether levels of the EBI2 ligand may reach these values *in vivo* to limit cell migration. The fact that we did not detect differences in T cells from naïve mice deficient for EBI2 although this subset highly expressed EBI2 is in line with previous publications analyzing the role of EBI2 in other immune cells. Thus far, EBI2 expression under steady state conditions was only found to be crucial for $CD4^+$ DCs in the spleen (Gatto et al., 2013; Yi and Cyster, 2013). In contrast, EBI2 deficient B cell development, maturation and appearance is normal. However, EBI2 knockout mice show delayed antibody responses and reduced numbers of plasma cells after immunization. Therefore EBI2 might be involved in T cell responses during inflammation, but is not needed for normal T cell

development in naïve mice. We did not analyze positioning of T cells in the spleen and lymphnodes via histology, which could reveal differences in EBI2 deficient mice and would be interesting to analyze upon EAE induction.

5.2 EBI2 expression in different T cells subsets

We found that only around 50% of effector memory ($CD44^+$, $CD62L^-$) helper T cells in naïve mice express EBI2. When we further analyzed different subsets, we could show that expression of EBI2 was not specific to T_H1, T_H17 or T_{reg} cells in the spleen and the lymphnodes. Additionally, cell numbers of these subsets were comparable to wt littermates, suggesting that EBI2 expression did not affect T cell differentiation in naïve mice. However, analysis of EBI2 expression after *in vitro* T cell differentiation showed different results as we found T_H17 cells differentiated with TGF-ß and IL-6 were negative for EBI2. MOG-specific T_H17 cells differentiated *in vitro* with TGF-ß and IL-6 have been shown to be poorly encephalitogenic in transfer models of EAE (Lee et al., 2012). In contrast, differentiation of T cells using IL-1ß and IL-6 increased pathogenicity of these cells, with IL-23 acting synergistically in both pathways. Strikingly, we could show that *in vitro* differentiation with IL-1ß, IL-6 and IL-23 resulted in the majority of T_H17 cells expressing EBI2. Therefore we propose that EBI2 might be a marker for pathogenic T_H17 cells differentiated *in vitro*. Whether this is indeed the case, remains to be clarified. To this aim we already crossed our EBI2 reporter mice to IL-17F-RFP animals (Yang et al., 2008a) on the 2D2 background. Using these mice we will be able to sort MOG specific EBI2 positive and EBI2 negative T_H17 cells after *in vitro* differentiation with IL-1ß, IL-6 and IL-23 and transfer them

into RAG1 deficient mice to monitor encephalitogenicity. When we differentiated regulatory T cells (iT_{regs}) we found them to be mainly EBI2 positive in contrast to *ex vivo* regulatory T cells. However, when IL-2 was added to the culture, EBI2 expression was significantly reduced. This effect was specific for IL-2 signaling and not a result of homeostatic proliferation, as stimulation with other common-γ chain cytokines did not lead to downregulation of EBI2 expression. It might be that the differences in EBI2 expression we observed when analyzing *in vitro* differentiated and *ex vivo* regulatory T cells might reflect two populations with naturally occurring T_{regs} (nT_{regs}) being $EBI2^-$ and inducible T_{regs} (iT_{regs}) expressing it. However up to now no reliable marker is known to distinguish these different subsets of regulatory T cells. Therefore, it would be interesting to compare EBI2 positive and EBI2 negative T_{regs} by RNA sequencing to clarify if they indeed represent two different subsets. Interestingly, we found T_H1 cells differentiated *in vitro* to express similar levels of EBI2 as *ex vivo* T_H1 cells. Therefore, it could be that the conditions used to differentiate these cells *in vitro* are more similar to the stimuli present *in vivo* compared to conditions used to differentiate T_{reg} and T_H17 cells. We also speculated that 7α,25-OHC might influence the differentiation of T cells, as oxysterols have been shown to be agonistic ligands for RORγT and drive T_H17 differentiation (Soroosh et al., 2014). Furthermore, spingosine-1-phosphate has been shown to balance differentiation of T_{reg} and T_H1 cells (Liu et al., 2010). However, we did not observe differences in T cell differentiation in general when different concentrations of the EBI2 ligand were added. In addition, we found similar numbers of these T cell subsets *in vivo* in EBI2 deficient mice compared to littermate controls, suggesting

that signaling via EBI2 does not influence T cell differentiation. This is in line with previous findings analyzing the T cell compartment in CH25H deficient mice, which also did not show altered numbers of the different subsets (Chalmin et al., 2015). Moreover, proliferation and cell viability was not influenced when 7α,25-OHC was added to the culture and we did not detect differences in T cell numbers in naïve EBI2 deficient mice, although EBI2 signaling was previously found to increase phosphorylation of ERK as well as Ca^{2+} mobilization (Rutkowska et al., 2015). We also did not detect differences in severity and onset of colitis when we transferred naïve helper T cells from EBI2 deficient mice into $RAG1^{-/-}$ hosts. Therefore, the EBI2: 7α,25-OHC axis seems not to influence the *in vivo* proliferation and differentiation of helper T cells, although it would still be interesting to characterize the effect of sorted EBI2-$EGFP^{+}$ versus EBI2-$EGFP^{-}$ naïve helper T cells in this model. This experiment may clarify if EBI2 positive and negative cells represent different subsets or progenitors to specific effector cells. Furthermore, the functionality of EBI2 deficient regulatory T cells could be determined in the future in the before mentioned transfer model of colitis by co-transfer with naïve helper T cells.

5.3 Role of EBI2 for T cell priming

Two groups have demonstrated that EBI2 is highly expressed by CD4 positive DCs in the spleen and found that enzymes generating 7α,25-OHC are abundant at marginal zone bridging channels (Gatto et al., 2013; Yi and Cyster, 2013). When we analyzed dendritic cells in our EBI2 deficient mice, we also found this DC subset to be reduced in the spleen and were curious to analyze the relevance of this phenotype on

priming of T cells in the EAE model. Therefore we performed transfer of MOG-specific T cells from congenic wt mice into EBI2 deficient hosts or control littermates. After immunization of the host mice with MOG/CFA, we did not detect significant differences in proliferation or activation of the transferred T cells in the spleen and lymphnodes between the two groups. These results indicated that either the CD4 positive DC subset is not involved in the priming of T cells in this model or that the remaining cells in EBI2 deficient mice are sufficient for accurate priming of T cells. However DCs at marginal zone bridging channels are mainly involved in sampling antigen from the blood stream. As mice are immunized subcutaneously for EAE induction, it might be possible that this DC subset is not involved in T cell priming in this model. It would be still interesting to analyze the effect of EBI2 deficiency on DCs when models are used where the antigen is mainly present in the blood, e.g. viral models. Finally we did not analyze the fate of the transferred T cells in our *in vivo* priming experiments and therefore could not exclude that they differ in cytokine or transcription factor expression.

5.4 Regulation of EBI2 ligand synthesis in EAE

Chalmin *et al.* have shown that deficiency for CH25H results in delayed onset of EAE when mice are actively immunized. They found that monocytes highly express CH25H whereas CYP7B1 expression was higher in cDCs. Furthermore, using bone marrow chimeric mice they verified that CH25H needs to be expressed by hematopoietic cells for normal EAE development (Chalmin et al., 2015). When we analyzed mRNA expression of both enzymes in whole spinal cord and spleen tissue,

we found that they are highly regulated upon EAE induction. In naïve mice expression of CH25H is abundant in the spleen, but only low in the CNS. In contrast upon EAE induction, expression changes to an almost complete absence in the spleen with strong up-regulation in the spinal cord; CYP7B1 expression also showed the same regulation pattern. The mRNA levels of HSD3B7 were not affected as strong, but were reduced in the spinal cord after immunization. These findings suggest that levels of the EBI2 ligand are high in the periphery and low in the CNS in naïve mice. However upon EAE induction they change to be elevated in the CNS and low in the periphery. These changes in 7α,25-OHC distribution may influence migration of EBI2 expressing T cells to the CNS, although other oxysterols may be elevated as well, thereby attracting T cells into the inflamed CNS via other receptors mediating chemotaxis. Which cells express these enzymes in the inflamed spinal cord and in which regions they are present still needs to be clarified. Furthermore, it might be that attracting cells from the periphery to the CNS is not the only mechanism by which elevated 7α,25-OHC levels contribute to EAE pathogenesis. It might also influence cellular processes at the blood brain barrier by regulating cell adhesion via either direct mechanisms or through induction of other molecules involved in CNS transmigration.

5.5 Role of EBI2 in EAE

As our findings suggested elevated levels of 7α,25-OHC in the spinal cord of mice with EAE, we speculated that T cells would need EBI2 for migration to the inflamed CNS. However we did not detect differences in active EAE in the absence of EBI2.

Accordingly, numbers and composition of infiltrating T cells were similar to wild type littermates at the time point of analysis. Furthermore the turnover of IL-17A expressing cells to GM-CSF and IFNγ production was the same. However, we found that more T_H17 cells expressed EBI2 in the CNS compared to the same subset in the lymphnodes and to T_H1 cells. Our analysis was only focused on the peak of the disease, therefore it might be possible that during onset of the disease the composition of infiltrating cells in EBI2 deficient animals was different or that recovery from EAE might differ. Moreover, we only analyzed T cells in the inflamed CNS and did not dissect the influence of EBI2 deficiency on myeloid cells and brain resident cells. To analyze T cell specific effects of EBI2 deficiency we used an adaptive transfer model of EAE. Using EBI2 reporter animals in active EAE we found highest and most uniform expression of EBI2 in T_H17 cells in the inflamed CNS and therefore transferred pathogenic T_H17 cells into RAG1 deficient mice. Using this model we found that EBI2 deficiency resulted in a delayed onset of EAE. Accordingly, the number of T cells in the CNS was reduced at early time points but no changes in the composition of infiltrating cells were detected compared to transfer of wild type T_H17 cells. However, further disease development was not different between the two groups and reached the same severity with comparable CNS infiltration of T cells. As with active EAE induction, we found EBI2 expression to be highest in T_H17 cells within the CNS compared to the same subset in the lymphnodes. We therefore suggest that EBI2 expression plays a role in early migration of T_H17 cells from the lymphnodes to the CNS and might not be important for the migration of pathogenic T_H1 cells. The fact that EBI2 deficient mice show comparable disease development

after active EAE induction might be due to GM-CSF and IFNγ production from T_H1 cells which could compensate for the delayed CNS infiltration of T_H17 cells. Furthermore, it is unclear if the delayed CNS infiltration of EBI2 deficient T_H17 cells is due to reduced egress from the lymphnodes or defects in transmigration into the CNS (Odoardi et al., 2012).

5.6 EBI2 expression on human T cells and in MS lesions

We obtained a monoclonal antibody to stain human EBI2 from Novartis, Basel with which we analyzed EBI2 expression in human T cells. We found EBI2 expression to be comparable to the expression pattern of murine T cells, with the majority of $CD4^+$ T cells but only few $CD8^+$ T cells showing surface expression. However unlike in mice, human effector T cells showed higher expression of EBI2 than naïve cells. This is in line with previously published data showing the same expression patterns of EBI2 on human T cells (Chalmin et al., 2015), but the functionality of EBI2 on human T cells remains to be clarified. It is still not known if human T cells and other lymphocytes migrate towards 7α,25-OHC and if the intensity of chemotaxis is comparable to that of murine T cells. However we already have preliminary data which show that indeed human T cells migrate towards 7α,25-OHC even to greater extend than murine T cells. Furthermore pre-treatment with the EBI2 antagonist NIBR189 (Novartis, Basel) significantly inhibited migration, indicating that EBI2 is the sole receptor directing migration of human T cells towards 7α,25-OHC. Furthermore we found murine T cells to migrate towards 7α,25-OHC only when pre-activated with α-CD3 and α-CD28 antibodies, which suggests a role of EBI2 mediated chemotaxis in response to

inflammation. Therefore, it would be interesting to compare migration of non-activated and pre-activated human T cells in relation to other chemokines. Application of EBI2 antagonists is of particular interest as antagonistic blockade of EBI2 may be used for treatment of autoimmune disease. This is supported by our histological analysis of EBI2 expression in lesions in the inflamed CNS of MS patients showing that EBI2 expressing cells are mainly localized within the inflamed white matter compared to non-affected regions. However we did not analyze EBI2 expression in the inflamed CNS of patients with other disease than MS, e.g. Encephalitis or Neurovasculitis. Therefore we could not exclude the possibility that accumulation of EBI2 expressing cells in the inflamed white matter is a result of the inflammation itself, rather than being specific to MS pathology. Moreover, morphological analysis of EBI2 positive cells suggested that they are mainly macrophages. By staining EBI2 together with CD3 we could show that indeed a part of the T cells within the lesion expressed EBI2. Flow cytometric analysis of T cells in the blood of healthy donors revealed that only few $CD8^+$ T cells expressed EBI2 and it has been shown that many CD8 positive T cells are present in MS lesions (Babbe et al., 2000). Therefore it should be further verified by costaining for CD4 and CD8 if indeed the EBI2 negative T cells in the inflamed white matter are $CD8^+$ T cells. If this would be the case, it would suggest that EBI2 is involved in migration of $CD4^+$ T cells rather than $CD8^+$ T cells into the CNS. As we found EBI2 expressing T cells in MS lesions, we also analyzed PBMCs from MS patients and compared them to healthy donors. However, we did not detect differences in the number of T cells expressing EBI2 nor in the expression levels indicated by similar mean fluorescence intensity.

Hence it might be possible that, as in mice, expression of the enzymes involved in the generation of 7α,25-OHC are increased in the CNS of MS patients and therefore lymphocyte trafficking to the CNS may be enhanced irrespective of changes in EBI2 expression on responding T cells. It would be interesting to quantify the levels of the EBI2 ligand in the serum and cerebrospinal fluid of MS patients and compare it to levels in healthy individuals. This could be done in parallel with histological analysis of CNS sections from MS patients to analyze expression of the EBI2 ligand generating enzymes. Furthermore we did not analyze the effect of MS therapy on the expression of EBI2. It was shown that CH25H is induced by type I interferon signaling (Reboldi et al., 2014) and it might be that medication with IFN-ß (e.g. Rebif) upregulates the expression of this enzyme in human cells and triggers increased 7α,25-OHC concentrations. If the induction of CH25H expression would be sufficient to increase 7α,25-OHC levels to concentrations triggering internalization of EBI2, it might affect the migratory behavior of pathogenic cells to the CNS.

5.7 Outlook

We crossed our EBI2 reporter / knock-out mice to IL-17F-RFP reporter animals on the 2D2 background. This strain will allow for more detailed analysis of the function of EBI2 on T_H17 cells and comparison of EBI2 positive versus EBI2 negative T_H17 cells. We will be able to sort these two subsets and perform RNA sequencing to reveal if they differ in their expression profile, which might possibly lead to the identification of other genes being involved in pathogenicity of T_H17 cells. Moreover these cells may be transferred separately into RAG1 deficient mice to monitor

encephalitogenicity, which could further foster our hypothesis that EBI2 is a marker for pathogenic T_H17 cells in EAE. Additionally this strain will allow us to sort for MOG specific EBI2 positive and EBI2 negative T_H17 cells after *in vitro* differentiation with IL-1ß, IL-6 and IL-23 and transfer them into RAG1 deficient mice. As we found a correlation between numbers of EBI2 expressing T_H17 cells with their published ability to transfer EAE (Lee et al., 2012), this experiment combined with RNA sequencing might lead to a better understanding of the pathogenic signature of *in vitro* differentiated T_H17 cells. However, it is still not clear if the delayed CNS infiltration of EBI2 deficient T_H17 cells is due to reduced egress from the lymphnodes or defects in transmigration into the CNS. To clarify this point we will use EBI2 sufficient and deficient IL-17 reporter mice on the 2D2 background and perform *in vivo* imaging of T_H17 cells after EAE induction. This will allow us to analyze the behavior of these cells in the lymphnodes and at the blood brain barrier or in the spinal cord. As we are working with full knockout mice, other mechanisms may compensate for the deficiency of EBI2, therefore EAE induction using conditional EBI2-floxed mice bred to T cell specific Cre mouse strains would be highly desirable. Furthermore, treatment of wt mice during EAE with an EBI2 antagonist (NIBR189) can be envisaged and might ameliorate disease in the case that in full knockout mice other redundant systems compensate for the lack of EBI2. This is of particular interest as we could show that human T_H17 cells express high levels of EBI2 and we found high numbers of EBI2 positive cells within MS lesions, which suggests a role in the pathogenesis of MS. Due to the lack of antibodies to stain CH25H in different tissues and cells, making a CH25H-RFP reporter / knock-out mouse to enable the

detection of its expression in naïve mice and under inflammatory conditions would be a great tool. Together with our EBI2 reporter mice, this would allow *in vivo* imaging to track migration of EBI2 expressing cells in comparison to CH25H expression. Furthermore, we believe that up-regulation of CH25H and CYP7B1 expression upon inflammation might be a general mechanism of immunity to attract lymphocytes to the site of inflammation in an EBI2 dependent manner. Therefore, it will be interesting to study the role of the EBI2 : 7α,25-OHC axis in other models, e.g. viral infections and tumor models. Furthermore, our findings are of great interest as it is now clear that IL-17 expressing γδ T cells are involved in the pathogenesis of Imiquimod-induced psoriasis (Gray et al., 2013; Hartwig et al., 2015; Ramírez-Valle et al., 2015). We found a strong correlation of EBI2 expression and IL-17 secretion in helper T cells and this correlation might also be found in γδ T cells expressing IL-17. As induction and developments of psoriasis has been shown to be highly dependent on IL-17 (Krueger et al., 2012), EBI2 expression might have a great impact on the pathogenicity of psoriasis. Furthermore, recent publications have identified another type of lymphoid cells termed innate lymphoid cells (ILCs), which are also able to secrete IL-17. By analyzing these cells in our EBI2 reporter mice, we will gain deeper insight, whether EBI2 expression indeed correlates with IL-17 expression in various cell types in general, or is only found for helper T cells. This is of particular interest as IL-17 has been implicated with a broad variety of autoimmune diseases and could expand possible implications for treatment with EBI2 antagonists.

5.13 Zusammenfassung

Epstein-Barr virus-induced gene 2 (EBI2), auch bekannt als GPR183 sowie dessen Ligand 7α,25-OHC spielen eine wichtige Rolle bei der Migration von Leukozyten und deren Positionierung in den sekundären lymphatischen Organen. Wir verwendeten ein neues Reporter-knockin / knockout (KO) Mausmodell und konnten zeigen, dass IL-1β und IL-23 die Expression von EBI2 in T_H17 Zellen induzieren. Des Weiteren war die Expression von EBI2 in Myelin Oligodendrozyten Glykoprotein (MOG)-spezifischen T_H17 Zellen involviert in der Induktion von ZNS Entzündung in einem Transfermodell der Experimentellen Autoimmmunen Enzephalomyelitis (EAE). Zudem konnten wir zeigen, dass die Expression der Enzyme CH25H und CYP7B1, welche 7α,25-OHC ausgehend von Cholesterol synthetisieren, sich stark ändert in der Milz und dem ZNS nach Induktion von EAE. Die Expression war reduziert in der Milz, jedoch stark erhöht im ZNS nach Immunisierung. Unsere Ergebnisse legen nahe, dass sich die Verteilung von 7α,25-OHC während EAE von der Peripherie zum ZNS verlagert und dadurch die Migration von pathogenen T_H17 Zellen in das entzündete ZNS unterstützt.

6. Figure index

7. Table index

8. Curriculum vitae

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Sprachen

- Englisch: fließend
- Französisch: fließend

Referenzen

Mainz, im März 2016

9. Publications

1. Heinen, A.P., **Wanke, F.**, Moos, S., Attig, S., Luche, H., Pal, P.P., Budisa, N., Fehling, H.J., Waisman, A., and Kurschus, F.C. (2014). Improved method to retain cytosolic reporter protein fluorescence while staining for nuclear proteins. Cytometry A *85*, 621–627.

2. Zayoud, M., Malki, El, K., Frauenknecht, K., Trinschek, B., Kloos, L., Karram, K., **Wanke, F.**, Georgescu, J., Hartwig, U.F., Sommer, C., et al. (2013). Subclinical CNS inflammation as response to a myelin antigen in humanized mice. J Neuroimmune Pharmacol *8*, 1037–1047.

10. Versicherung

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Mainz, 22. November 2016

Florian Wanke

11. Abbreviations

7α,25-OHC	7α,25-dihydroxycholesterol
25-HC	25-hydroxycholesterol
Akt	Protein kinase B (a.k.a. Akt)
APC	Antigen presenting cells
ATP	Adenosinetriphosphate
CAMK	Ca^{2+}/calmodulin-dependent kinases
cAMP	Cyclic Adenosinemonophosphate
CD	Cluster of differentiation
CFS	Complete Freunds Adjuvant
CIS	Clinically Iiolated syndrome
CNS	Central nervous system
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
CYP7B1	25-hydroxycholesterol 7-alpha-hydroxylase
DC	Dendritic cell
DAG	Diacetylglycerol
dsDNA	Double stranded desoxyribonucleic acid
EAE	Experimental autoimmune encephalomyelitis
EBV	Epstein-Barr-Virus
EBI2	Epstein-Barr-Virus induced Gene 2
ERK1/2	Extracellular-signal regulated kinase 1/2
FDC	Follicular Dendritic Cell
Flt3	FMS-related tyrosine kinase 3
FoxP3	Forkhead-Box-Protein P3
GATA3	GATA binding protein 3
GC	Germinal center
GDI	Guanine nucleotide dissociation inhibitor
GDP	Guanine diphosphat
GEF	Guanine exchange factor
GIRK	G protein-regulated inward-rectifier K^+ channels
GM-CSF	Granulocyte/Monocyte-Colony Stimulating Factor
GPCR	G Protein Coupled Receptor
GRK	GPCR kinases
GTP	Guanine triphosphate
GTPase	Guanine triphosphatase GATA Binding protein 4
HIV	Human immunodeficiency virus
HSD3B7	3β-hydroxy-Δ^5-C_{27} steroid oxidoreductase
IBD	Inflammatory bowel disease
IDO	Indoleamine-2,3-dioxygenase
IFN-ß	Interferone beta
IFNγ	Interferone gamma
IgG	Immunglobulin G
IL	Interleukine

IL-12rb2	Interleukine-12 receptor beta 2
ILC	Innate lymphoid cell
INAD	Inactivation-no-afterpotential D protein (INAD)
INSIGN	Insulin-induced gene
IP3	Inositol (1,4,5) trisphosphate
IRF4	Interferon regulatory factor 4
IRF7	Interferon regulatory factor 7
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
MAPK	p38 mitogen activated protein kinases
MBP	Myelin basic protein
MHC	Major histocompatibility complex
MOG	Myelin oligodendrocyte glycoprotein
MS	Multiple sclerosis
pDC	Plasmacytoid Dendritic Cells
PI3K	Phosphoinositid 3-kinase
PIP2	Phosphatidylinositol-4,5-biphosphate
PLCβ	Phospholipasce C beta
PLP	Proteolipid protein
PKA	Protein kinase A
RGS	Regulators of G protein signaling
RORγt	RAR-related orphan receptor gamma t
S1PR	Sphingosine-1-phosphate receptor
ssRNA	Single stranded ribonucleic acid
SSTR 2	Somatostatin receptor type 2
STAT4	Signal transducer and activator of transcription
TCR	T cell receptor
TGF-ß	Transformin growth factor beta
Tfh	T follicular helper cells
T_H1	T helper cell 1
T_H17	T helper cell 17
TLR	Toll like receptor
T_R1	T regulatory cell 1
T_{reg}	Regulatory T cell
VLA-4	Very late antigen 4
WT	Wild type

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