Autoimmune T cell – B cell interaction in experimental autoimmune encephalomyelitis

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Declaration

I hereby declare that I have written the PhD thesis entitled “Autoimmune T cell – B cell interaction in experimental autoimmune encephalomyelitis” on my own with no other sources and aids than quoted. The thesis has not been submitted elsewhere for any academic degree.

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Abbreviations

APC	antigen-presenting cell
ALCAM	activated leukocyte cell adhesion molecule
BBB	blood-brain barrier
BCR	B cell receptor
CCR	CC chemokine receptor
CCL	CC chemokine ligand
CFA	complete freund's adjuvant
CFSE	carboxyfluorescein succinimidyl ester
CNS	central nervous system
Cre	Cre recombinase
CSF	cerebrospinal fluid
CXCR	CXC chemokine receptor
CXCL	CXC chemokine ligand
d p.i.	days post immunization
EAE	Experimental autoimmune encephalomyelitis
ELISA	Enzyme linked immunosorbent assay
GFP	green fluorescent protein
GM-CSF	granulocyte/macrophage- colony stimulating factor
h	hour
HPRT	hypoxanthine phosphoribosyltransferase 1
ICAM-1	intercellular adhesion molecule 1
IFNγ	interferon γ
Ig	immunoglobulin
IL-	interleukin-
i.p.	intraperitoneal
i.v.	intravenously
LFA-1	lymphocyte function-associated antigen-1; $\alpha L\beta 2$
MHC	major histocompatibility complex
min	minute
ml	milliliter
MOG	myelin oligodendrocyte glycoprotein

MOG_{p35-55}	myelin oligodendrocyte glycoprotein aa 35-55, 2D2 TCR epiptope
rhMOG	recombinant human myelin oligodendrocyte glycoprotein
rrMOG	recombinant rat myelin oligodendrocyte glycoprotein
MS	Multiple sclerosis
NP	4-Hydroxy-3-nitrophenylacetyl hapten
NP-OVAL	4-Hydroxy-3-nitrophenylacetyl hapten fused to OVA protein
OVA	Ovalbumin
RFP	red fluorescent protein
RT-PCR	real-time polymerase chain reaction
s.c.	subcutanously
TCR	T cell receptor
TH cells	helper T cells
VCAM-1	vascular cell adhesion molecule 1
VLA-4	very late antigen 4; $\alpha 4\beta 1$
XBP-1	X-box binding protein 1

Abstract

Although Multiple sclerosis (MS) and its animal model experimental autoimmune encephalomyelitis (EAE) are known as T cell-mediated autoimmune diseases, there is increasing evidence that B cells also play a critical role in their pathogenesis. However, there are conflicting data about the exact role of B cells in the pathogenic process. To study the interaction of MOG-specific T and B cells in the course of actively induced EAE, we developed a model enabling us to track and analyze the cells *ex vivo* during disease progression. We induced EAE with the encephalitogenic MOG_{p35-55} peptide and found that the transfer of MOG-specific B cells had a disease promoting effect with an accelerated onset. When we focused on the underlying mechanism, we could show that MOG-specific B cells did not enhance MOG-specific T cell proliferation, activation or differentiation during the activation phase or immediately before EAE onset. Moreover, MOG-specific B cells did not enhance T cell activation in the reactivation phase within the CNS in our experimental model. The presence of MOG-specific B cells had no impact on the number of circulating inflammatory myeloid cells. Furthermore, endothelial cells of spinal cord microvessels displayed similar adhesion molecule and chemokine expression levels in the presence of MOG-specific B cells. The accelerated onset was accompanied by an increased number of CNS infiltrated T cells, leading to the speculation that MOG-specific B cells might influence T cell trafficking. The latter is supported by our finding that peripheral MOG-specific T cells showed an enhanced expression of CXCR4 and CCR6 in the presence of MOG-specific B cells. Our clinical data revealed that the presence of activated MOG-specific B cells is critically required for the accelerated disease onset, as activated MOG-unspecific B cells had no effect on the disease onset. When we focused on the mechanism utilized by MOG-specific B cells to promote disease initiation, we found that B cells with an impaired development into antibody secreting plasma cells did not accelerate the disease onset. This led to the conclusion that, even in EAE induced by MOG peptide, B cells promote the initiation of EAE by their secretion of MOG-specific antibodies.

1. Introduction

1.1. The immune system

The immune system protects the organism against pathogens and can be divided into an innate and an adaptive immune system. The innate immune response occurs immediately after recognition of the pathogen by macrophages, dendritic cells, natural killer cells and the complement system. The defence mechanisms of the adaptive immune system are mediated by lymphocytes and are characterized by a specific recognition of the pathogen due to an antigen-specific surface receptor and the development of an immunological memory. Under normal circumstances, the processes of an immune response are optimally coordinated and regulated by specialized cells of the immune system to avoid overshooting reactions or to mediate tolerance against non-pathogenic antigens (e.g. self antigens). However, a dysfunction of any factor involved in this process can have fatal consequences for the organism, leading for example to the development of an autoimmune disease like diabetes type I, inflammatory bowel disease or multiple sclerosis.

1.1.1. Innate immune system

The innate immune response provides the first line of defence against an invading pathogen. Cells of the innate immune system detect molecular patterns found in most microorganisms by pattern recognition receptors (PRRs) [1]. These microbial structures are referred to as pathogen-associated molecular patterns (PAMPs), whereas molecular patterns from dead or damaged cells are defined as damage-associated molecular patterns (DAMPS) [2]. The most prominent PRRs are the Toll-like receptors (TLRs) [3]. Recognition of the pathogen by cells of the innate immune system either results in its phagocytosis and successful clearing or in the stimulation of the adaptive immune system. Macrophages and dendritic cells act as antigen-presenting cells (APCs). They present antigens, loaded on major histocompatibility complex (MHC) molecules to either $CD8^+$ T cells (MHC-I) or $CD4^+$ T cells (MHC-II), which recognize the MHC-antigen complex due to their specific antigen receptor. Macrophages and dendritic cells derive from a common myeloid precursor, the monocyte. Monocytes originate in the bone marrow and are then released into the

peripheral blood where they appear predominantly as two distinct populations [4]. The short-lived monocyte subset was identified as $CCR2^{+}Ly6C^{+}CX_3CR_1^{-}$. These are commonly referred to as 'inflammatory' monocytes as upon inflammation they were found to be immediately recruited into the tissue in a CCR2-dependent manner [5, 6]. In contrast, the $CCR2^{-}Ly6C^{lo}CX_3CR_1^{+}$ monocyte population have a longer half-life and are considered as 'resident' monocytes. They migrate into various tissues under non-inflammatory conditions in a CX_3CR_1-dependent manner [4]. A third very small monocyte population was identified as $CCR7^{+}CCR8^{+}$ and it was shown that they emigrate to lymph nodes where they appear as monocyte-derived dendritic cells [7].

1.1.2. Adaptive immune System

T and B lymphocytes are the main players of the adaptive immune system, which can be divided into two functional parts: the cellular and the humoral system. B cells mediate humoral immunity by their production and secretion of high-affinity antibodies and are part of the cellular system due to their antigen-presenting capacity and their secretion of cytokines. One main characteristic of cells from the adaptive immune system is their specific antigen receptor. The antigen receptor specificity is determined by the antigen-binding site of the T cell receptor (TCR) and the B cell receptor (BCR). The enormous range of specificity is achieved by somatic recombination of the DNA which encodes the different segments of the receptor.

T cells can be further divided into $CD4^{+}$ (co-receptor) or $CD8^{+}$ (co-receptor) T cells. Both T cell subtypes share the feature that they only recognize an antigen when it is bound to a MHC molecule on the surface of an antigen-presenting cell. Due to the different co-receptors, that interact with MHC molecules on the APC, $CD4^{+}$ T cells recognize the antigen in the context of MHC-II and $CD8^{+}$ in the context of MHC-I. The major function of $CD8^{+}$ T cells is the elimination of intracellular pathogens by cytolysis of the infected cell and they are therefore called cytotoxic T cells. $CD4^{+}$ T cells are also known as helper T cells (TH cells). They show a high phenotype plasticity and, depending on the surrounding cytokine milieu, they develop into different helper T cells subtypes as schematically depicted in Figure 1.

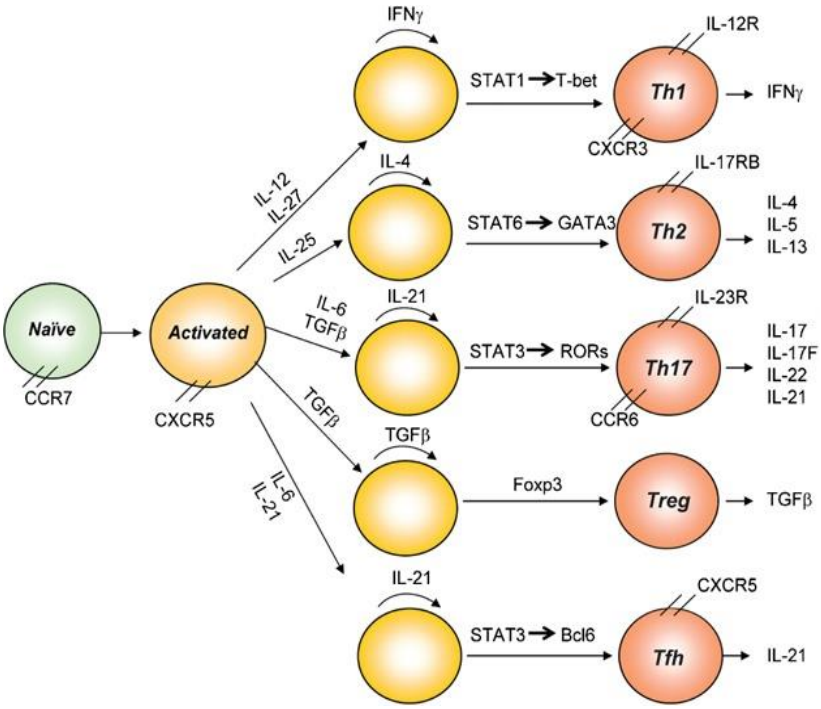

Figure 1: Schematic overview of helper T cell differentiation. Upon naïve T cell (blue) activation by antigen encounter the T cells develop either into TH1, TH2, TH17, induced regulatory T cells (iTreg cells) or T follicular helper cells (Tfh) depending on the cytokine milieu. The different $CD4^+$ T cell subsets are characterized by their main transcription factors and their secreted cytokine(s). Figure modified from [8].

Together with antigen presentation, the production of interleukin-12 (IL-12) by activated macrophages and dendritic cells induces the differentiation of naïve T cells into TH1 cells [9, 10]. IL-12 binds to the IL-12 receptor on the T cell surface which activates a signal transducer and activator 4 (STAT4) signaling pathway resulting in the transcription of the transcription factor T-box expressed in T cells (T- bet) and IFNγ [11]. In a positive feedback loop, IFNγ binds to its receptor on the T cells surface, thereby activates the signal transducer and activator 1 (STAT1) signaling pathway and enhance T-bet and its own expression [12]. In contrast, the cytokine IL-25 triggers the differentiation of naïve T cells into IL-4, IL-5 and IL-13 producing TH2 cells [13]. IL-4 then leads to the activation of the STAT6 pathway [14] resulting in an upregulation of the transcription factor GATA-3 enabling the expansion of TH2 cells [15]. The differentiation of naïve T cells into TH17 cells is triggered by either the cytokines IL-6 or IL-21 in combination with transforming growth factor beta (TGFβ), and the transcription factor orphan nuclear receptor RORγt is critical for TH17 cell development [16-18]. The cytokines IL-1β and IL-23 are crucial for the maintenance

of the TH17 phenotype [19, 20]. TH17 cells produce a variety of effector cytokines like IL-17A, IL-17F, IL-21, IL-22, TNFα and granulocyte/macrophage-colony stimulating factor (GM-CSF) [21]. The cytokines TGFβ, IL-2 and IL-10 induce the differentiation of naïve $CD4^+$ T cells into induced regulatory T cells (iTreg cells) by the induction of their critical transcription factor FOXP3 [22-24]. In contrast, natural regulatory T cells (nTreg cells) develop in the thymus during the process of negative selection [25]. Regulatory T cells play an important role in mediating tolerance to self-antigens and establishing immune homeostasis by e.g. suppression of T cell proliferation or their differentiation into effector T cells [26, 27]. T follicular helper cells (Tfh cells) provide help for B cells to induce germinal center formation and are mainly characterized by their surface expression of CXCR5 and ICOS. The transcriptional repressor Bcl6 was shown to be critical for the differentiation of naïve T cells into Tfh cells upon IL-21 and IL-6 stimulation [28-30].

1.2. Multiple sclerosis

Multiple sclerosis is an autoimmune disease affecting the central nervous system (CNS). An autoimmune disease is defined by the presence of an immune response directed against self- antigens leading to the activation and amplification of self-reactive cells which results in organ-specific inflammation and tissue disruption. Multiple sclerosis is considered as a T- cell mediated autoimmune disease, where effector mechanisms of autoreactive myelin-specific T cells are directed against myelin antigens leading to myelin sheaths disruption (demyelination) and axonal damage.

Multiple sclerosis is the most common inflammatory disease of the central nervous system (CNS) affecting more than 2 million people worldwide, but it is more common among Caucasians. First clinical signs typically appear between 20 to 40 years of age and females show a higher susceptibility than males. Typical clinical symptoms are paralysis and impaired vision and cognitive function [31]. There are different forms of MS regarding the clinical course of disease. The most common form is the relapse-remitting MS one (RR-MS). Patients of this MS form suffer from periods of acute attacks alternating with periods of recovery to near normal neurological function. In some cases, the RR-MS form turns into the secondary-progressive MS

(SP-MS) form, which is accompanied by irreversible progression of clinical disability after a relapse. Patients of primary-progressive MS (PP-MS) and progressive-relapsing MS (PR-MS) suffer a clinical progression from the initiation of the disease. The PP-MS course shows a gradual increase of disease symptoms, whereas PR-MS disease course is accompanied by acute relapses [32].

The etiology of MS is a central question of research. A high number of factors are associated with MS susceptibility. Environmental triggers, genetic risk factors and infectious diseases are those predominantly discussed. Environmental risk factors are for example reduced Vitamin D levels, smoking or special diets (association of high salt intake and its effects on the disease have only been investigated in the MS animal model EAE until now) [33-38]. In addition, infectious agents like the Epstein-Barr virus (EBV) and, related to this, the hygiene hypothesis, are also considered to increase the susceptibility for MS [39]. Observations from population-based studies of twins and an elevated frequency of the disease in relatives from affected patients argue for a genetic predisposition for MS [40]. Genome-wide association studies revealed several risk loci associated with MS and almost all of them are related to the immune system. The most prominent of these is the HLA (Human Leukocyte Antigen) allele *DRB*11501*- it was shown that single nucleotide polymorphism (SNP) in this allele has the strongest association with MS [41]. Moreover, there were risk loci of genes identified encoding cytokine receptors (IL-2RA, IL-7RA) or co-stimulatory molecules (CD80, CD86) [41], suggesting an immune dysfunction as a potential cause for MS.

In MS, tissue damage occurs predominantly in the white matter of the brain and the spinal cord. Focal inflammatory demyelinated lesions are characterized by immune cell infiltrates and results in axonal damage and gliosis. In the progressive stages of the disease (SP-, PP-MS), also cortical demyelination can be detected [42]. Histopathological studies reveal a high heterogeneity in lesional profiles. However, by investigating actively demyelinating lesions of biopsy and autopsy cases, four different patterns of demyelination were defined. The presence of infiltrating T cells and macrophages is common in all cases, but they differ for example in the localization of the demyelinated plaques or the presence of immunglobulins and complement [43]. However, the reason for the different patterns is not clear and it is not a direct evidence for the pathogenic mechanism.

Several cellular dysfunctions have been uncovered in MS patients. Interestingly, myelin- specific T cells are not exclusively detected in the blood of MS patients, but also in healthy individuals. However, it was shown that myelin-specific T cells isolated from the blood and especially from the CSF of MS patients persist in a different activation status than T cells from healthy controls leading to a higher sensitivity upon antigen encounter [44-47]. Also regulatory cells from MS patients reveal an impaired cellular function. The suppressive capacity of regulatory T cells from MS patients is decreased leading to a loss of regulatory function [48, 49]. There is evidence that also pro-inflammatory and regulatory B cells are critically involved in the progression of MS. Ectopic lymphoid follicle-like structures containing a high number of B cells and plasma cells were found in the meninges of SP-MS patients [50, 51] and the presence of oligoclonal bands in the CSF of almost all MS patients can be used as a supportive diagnostic criteria [52]. In addition, autoantibodies against MOG are directly associated with myelin damage [53, 54]. Peripheral B cells isolated from blood of RR-MS patients exhibit an augmented pro-inflammatory cytokine response in comparison to B cells from healthy controls [55, 56]. Clinical studies with Rituximab, a monoclonal antibody which selectively targets and depletes $CD20^+$ B cells, reveal beneficial effects in the treatment of MS. In a phase 2 study, relapse-remitting multiple sclerosis patients show reduced lesions compared to placebo treated patients and the proportion of Rituximab treated patients with relapses was reduced [57]. The treatment of RR-MS patients results in a reduced T and B cell number in the cerebrospinal fluid at six months post treatment and an accompanied reduction of serum antibodies against myelin oligodendrocyte glycoprotein and myelin basic protein in some patients [58]. However, the beneficial effects observed with Rituximab therapies are in contrast to clinical trials with another B cell-depleting drug called Atacicept. Atacicept is a humanized recombinant fusion protein containing the extracellular ligand- binding portion of the human transmembrane activator and calcium modulator and cyclophilin-ligand interactor (TACI) receptor fused to a recombinant Fc domain of human IgG. The receptor binds the cytokines B-lymphocyte stimulator (BLyS) and a proliferation-inducing ligand (APRIL) –cytokines involved in B cell differentiation, maturation, and survival, and thus inhibits their action on B cells [59, 60] Clinical studies with Atacicept reveal increased clinical disease activity [61]. The underlying reasons for the observed worsening of clinical symptoms are not yet clarified in detail, but it is considered that Atacicept, in contrast

to Rituximab, also targets and affects plasma cells [62], which were recently shown to play a regulatory role in the pathogenesis of MS. Regulatory B cells are a recently discovered B cell subpopulation and their contribution to CNS autoimmune disease was until now mainly investigated in the MS mouse model EAE (regulatory B cells are described in detail in chapter 1.3.4.1.4.). However, in mice as well as in humans, they exert their regulatory function mainly by the production of IL-10 and IL-35 and it was shown that B cells from MS patients exhibit a reduced production of IL-10 [63].

1.3. Experimental autoimmune encephalomyelitis

The animal model experimental autoimmune encephalomyelitis (EAE) mimics several aspects of the human autoimmune disease Multiple sclerosis. Most of the EAE studies were performed in rodents like mice and rats but it can also be induced in other animals like non-human primates [64, 65]. In susceptible mouse strains, EAE can be induced in two ways– either by adoptive transfer of myelin antigen-primed encephalitogenic T cells (transfer EAE) or by immunization with a myelin antigen emulsified in adjuvant (active EAE). For transfer EAE, antigen-specific T cells are obtained by immunization with a respective antigen following by in vitro T cell re-stimulation with antigen and cytokines pushing the T cells into T helper cell differentiation. It was shown that TH1 and TH17 cells, but not TH2 cells, can efficiently induce EAE in different mouse strains [21, 66-70]. The critical cytokine of TH1 cells in the context of EAE is IFNγ [66, 67, 69]. TH17 cells produce a wide range of cytokines [21], whereas only IL-17A and GM-CSF seem to play a critical role in EAE [69, 71, 72]. The myelin proteins predominantly used to induce EAE are the myelin oligodendrocyte glycoprotein (MOG), proteolipid protein (PLP) and the myelin basic protein (MBP) and their respective T cell relevant peptide epitopes (e.g. MOG_{p35-55}, $PLP_{139-151}$) [73-75]. An overview of their location within the myelin sheath is depicted in Figure 2.

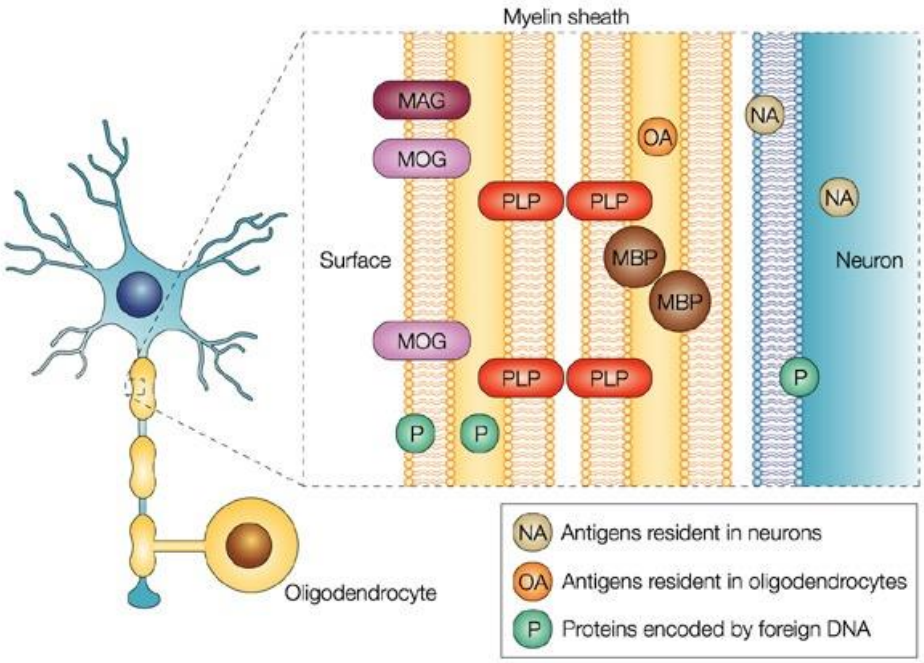

Figure 2: Localization of myelin proteins in the myelin sheath surrounding the neuron. Myelin-associated protein (MAG) and myelin oligodendrocyte glycoprotein (MOG) are located on the outer surface of the myelin sheaths, proteolipid protein (PLP) and myelin basic protein (MBP) are located in the inner layers of the myelin sheaths [76].

1.3.1. Different EAE models in the mouse

EAE and MS are both autoimmune in nature. The physical disabilities are the consequence of mononuclear cell infiltration into the central nervous system leading to demyelination, axonal damage and tissue destruction. However, the animal model does not resemble the full spectrum of the human disease. To overcome this problem several EAE models have been established with each mimicking different characteristics of the human disease. Differences of the clinical phenotypes are due to the different genetic repertoire of each susceptible mouse strain, especially influenced by different MHC-haplotypes. Immunization of C57Bl/6 mice with either the MOG protein or with the T cell epitope MOG_{p35-55} as well as the adoptive transfer of MOG-specific TH1 or TH17 cells leads to a chronic form of EAE with inflammatory foci predominantly present within the spinal cord rather than within the brain [69, 75]. MOG-specific T cell receptor transgenic mice (2D2 mice) can develop EAE spontaneously but with a very low incidence (4%) [77], but the cooperation of MOG-specific T and B cells in a double-transgenic mouse model led to the spontaneous development of EAE with an incidence of 59% [78, 79]. The relapse-remitting course

of MS, the most common form of the disease, can be resembled by the adoptive transfer of $PLP_{139-151}$ primed T cells or by immunization of SJL/J mice with PLP. These mice develop EAE with a relapse-remitting course [74, 80]. Additionally, a T cell receptor transgenic mouse model on the SJL/J background spontaneously develop EAE with a relapse-remitting disease course [81]. In most EAE models the infiltration of cells into the CNS is restricted to the spinal cord rather than to the brain. However, there are some rodent models where immunization leads to brain inflammation mimicking the predominant MS CNS histopathology [82, 83].

1.3.2. Immune reactions within the central nervous system

The central nervous system is often considered as an immune privileged organ e.g. due to its special microvessel endothelial cells restricting cell entry or the absence of lymphatic vessels. However, with increasing investigation it became clear, that the CNS is not completely devoid of immune reactions. CNS resident cells like microglia and astrocytes express MHC-II as well as co-stimulatory molecules, but also a low number of perivascular and meningeal macrophages [84] could be found under healthy physiological conditions. Activated T cells injected into the periphery were also found to be able to cross the blood brain barrier [85-87]. Additionally, the rejection of tissue transplants into the brain of immunologically primed recipients in contrast to immune deficient recipients emphasize the potential for immune reactions within the CNS [88].

The CNS is surrounded by a special membrane structure termed meninges. This structure is composed of three different membrane layers- the dura mata, which is in direct contact to the skull and the arachnoid mater which together with the pia mater define the subarachnoidal space comprising the cerebrospinal fluid (CSF). The CSF is produced within the ventricles of the brain by the choroid plexus and circulates through the brainstem around the outer surface of the spinal cord and is crucial for CNS metabolism and homeostasis. The subarachnoidal space is crossed by an artery network whose capillaries penetrate into the CNS parenchyma. An endothelial basement membrane on the vessel wall side and the glia limitans enclose the perivascular space. The CNS microvessels are lined by special endothelial cells also defined as blood-brain barrier (BBB). The endothelial cells are connected by tight

junctions limiting paracellular entry of soluble factors. The glia limitans surrounding the endothelial cells play an important role in maintaining the BBB integrity [89, 90].

1.3.3. T cell migration in EAE

Under physiological conditions, the frequency of cell migration into the CNS is very low. However, under some circumstances as in the autoimmune disease MS or its animal model EAE, the infiltration of e.g. T cells and mononuclear phagocytes is augmented causing disruption of the BBB integrity, further cell invasion and manifestation of inflammation within the CNS. Generally, there are some possible sites for T cells to enter the CNS- across the choroid plexus into the CSF, from the blood into the subarachnoidal space or into the perivascular space. Even though, the exact molecular mechanisms utilized by T cells to interact and finally to overcome the BBB does not seem to be clarified in detail, it is clear that the extravasation of T cells is a multi-step process, as depicted in Figure 3

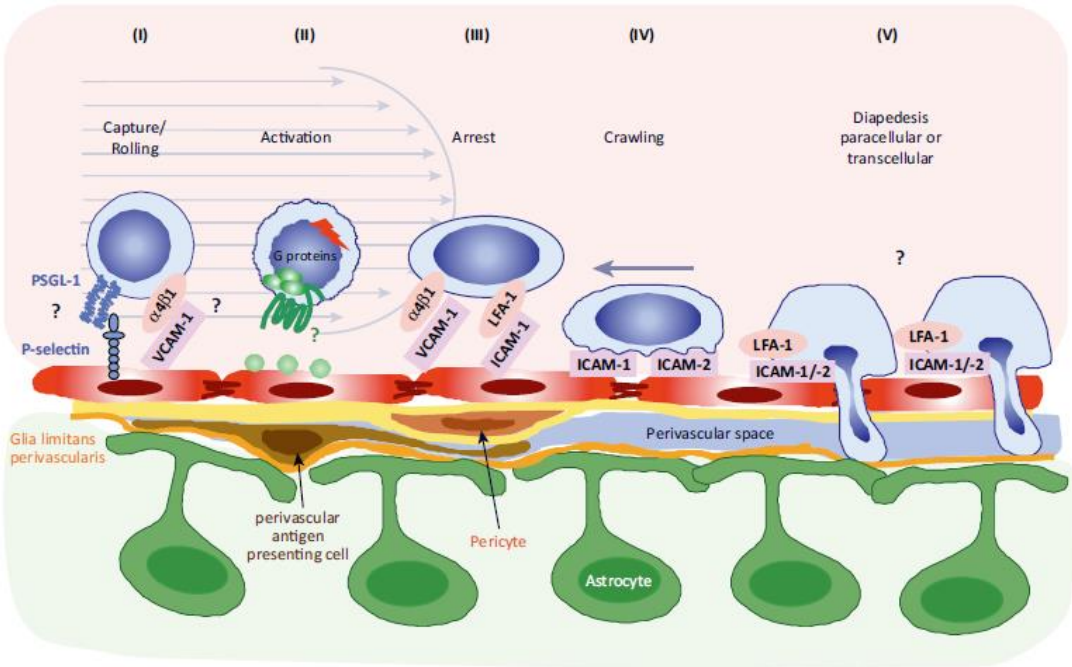

Figure 3: T cell–endothelial cell interaction steps in the process of T cell extravasation. T cell invasion can be divided into different steps each underlying different molecular mechanisms: 1) capture/ rolling, 2) activation, 3) firm adhesion (arrest), 4) crawling and 5) transmigration [91].

The initial interaction of T cells with CNS microvessel endothelial cells is mediated by selectins and integrins and their respective counterparts expressed by endothelial

cells. In EAE, the role of P-selectin glycoprotein ligand-1 (PSGL-1) expressed by T cells is controversial. Whereas PSGL-1 expression play a critical role for T cells to home into inflamed skin, it rather seems to be dispensable for EAE development [92-95]. The interaction of alpha 4 beta 7 integrin (α4β1; very late antigen 4 (VLA-4)) expressed by T cells with vascular cell adhesion molecule 1 (VCAM-1), expressed by endothelial cells, is one of the most critical factors involved in EAE development as functional interference results in complete absence of EAE symptoms [70, 96, 97]. VLA-4 expression by T cells mediate the initial capture as well as the firm adhesion of T cells to endothelial cells [98, 99]. Due to its critical involvement, the VLA-4–VCAM-1 interplay arose as a promising target for therapeutical treatment of MS. The humanized monoclonal antibody natalizumab binds to α4β1 and the treatment of RR-MS patients shows beneficial effects [100, 101]. The initial capture brings the chemokine receptor expressing T cells in close proximity to chemokines secreted by endothelial cells (chemokine receptors and chemokines are described in detail in chapter 1.3.3.1.). The chemokine receptor signaling in turn leads to the activation of integrins resulting in its conformational changes and clustering which increases their affinity and avidity [102, 103]. Consequently, the initial capture changes into a firm arrest enabling the T cell to further interact with the endothelial cells. T cells express the alpha L beta 2 integrin (αLβ2; lymphocyte function-associated antigen-1 (LFA-1)) which interacts with intercellular adhesion molecule 1 (ICAM-1) on endothelial cells [104]. *In vitro* and *in vivo* studies under healthy conditions reveal that the LFA-1–ICAM-1 interaction play a role in the transmigration step [105, 106]. However, under inflammatory conditions its role is controversial and difficult to interpret because LFA-1 also plays a role in T cell activation [107]. Therefore, the results of immunization of LFA-1-deficient mice range from a higher EAE susceptibility to milder course of disease [108, 109]. Similar results were obtained by ICAM-1 neutralization in the rat system [110, 111]. Activated leukocyte cell adhesion molecule (ALCAM) is an adhesion molecule which is upregulated on CNS vessels of active MS lesions and by spinal cord cells during active EAE. Blockade of ALCAM ameliorate EAE symptoms and is accompanied by a reduced number of infiltrating cells but does not affect T cell activation in the periphery. Therefore, it presumably plays a role in T cell trafficking into the CNS rather than in interfering with T cell activation [112].

Once T cells cross the BBB, they localize within the leptomeningeal space or the perivascular space, where they experience a second auto-antigen encounter by

resident APCs leading to their reactivation [113, 114]. The local reactivation is a crucial step to enable T cell infiltration into the CNS parenchyma and to cause disease. It was shown, that also activated myelin-unspecific T cells can enter the CNS tissue, even in equal numbers to that observed with myelin-specific T cells, but due to their failed reactivation they are not able to cause clinical symptoms [115, 116]. The reactivation of T cells is accompanied by an upregulation of various cytokines and chemokines leading to the attraction of not only further T cells but also other cells like macrophages and neutrophils [117].

1.3.3.1. Chemokines and Chemokine receptors in EAE

Several publications suggest an involvement of chemokines and their respective receptors during the course of EAE. Upon inflammatory conditions, TH1 cells upregulate the chemokine receptors CXCR3 and CCR5 [118, 119]. $CXCR3^+$ and $CCR5^+$ T cells have been found in the CNS of EAE animals or in MS patients suggesting a role for mediating CNS trafficking in CNS inflammation [120-122]. However, under experimental conditions, the roles of CXCR3 and CCR5 and their respective ligands are not clearly defined. Whereas active immunization of CXCR3 knockout mice results in a higher EAE severity, as well as in a more severe chronic phase, CXCR3 blockade in an adoptive transfer model leads to a milder disease course and a delayed onset [120, 121, 123, 124]. The CXCR3 ligand IFN-γ-Inducible Protein-10 (IP-10, CXCL10) becomes upregulated during the course of EAE, but similar to observations with CXCR3 deficiency, the neutralization of IP-10 in adoptive transfer EAE reduces clinical signs, whereas in actively induced EAE it rather exacerbates the disease, presumably due to an additional role in T cell activation [125-127]. Immunization of either CCR5- or macrophage-inflammatory protein-1α (MIP-1α) knockout mice, a CCR5 ligand which is upregulated during EAE, does not influence the clinical disease, but MIP-1α neutralization in an adoptive transfer EAE model prevents induction of acute and relapsing EAE without affecting T cell activation [128, 129]. Beside inflammatory chemokines, cells from the CNS also upregulate the expression of homeostatic lymphoid chemokines like CCL19, CCL21 and CXCL12 suggesting a role for chemoattraction of T cells during inflammation [103, 130]. In line with this, $CXCR4^+$ T cells are present within the inflamed CNS [120]. In healthy tissue, CXCL12 is expressed by spinal cord endothelial cells at the basolateral surface where it is suggested to retain CXCR4-expressing cells in the

perivascular space inhibiting them to migrate into the parenchyma. In this context, CXCR4 antagonization with AMD3100 during EAE leads to worsening of clinical signs, whereas CXCR4 antagonization with a CXCL12 mutant ($CXCL12_{P2G2}$) ameliorates EAE in another mouse model [120, 130]. In contrast to TH1 cells, TH17 cells predominantly express the chemokine receptor CCR6 and its ligand CCL20 was shown to attract TH17 cells to various sides of inflammation [131-134]. CCR6 knockout mice are resistant to actively-induced EAE, but this resistance is abrogated by transfer of CCR6-sufficient T cells, suggesting a role for CCR6 in TH17 cell traffic into the CNS. CCR6-sufficient T cells enter the CNS through CCL20-expressing epithelial cells of the choroid plexus, from where they initiate inflammation, which in turn recruits a second wave of T cells in a CCR6-independent manner [135].

1.3.4. Role of different lymphocyte populations in EAE

Although myelin-specific T cells have been identified as the crucial cells to initiate CNS autoimmunity in various animal models of MS, it is also considered that other cell types of the adaptive and of the innate immune systems are also critically involved in the initiation and progression of EAE and MS.

1.3.4.1. B cells

Although Multiple sclerosis and its animal model experimental autoimmune encephalomyelitis are T-cell mediated autoimmune diseases, the role of B cells in its pathologies have come into focus within the past decade. Beneficial effects of Rituximab therapy, the presence of oligoclonal bands in the cerebrospinal fluid of MS patients as well as beneficial effects of therapeutic plasma exchange support the hypothesis of B-cell contribution to MS [136].

There are many publications reporting on studies into the role of B cells in EAE. They differ in the model used and in the antigens used. C57bl/6 WT and B- cell deficient mice (µMT; [137]) are equally susceptible to MOG peptide-induced EAE, but when immunized with human recombinant MOG protein (rhMOG) B cell deficient mice are protected from EAE, which suggests a pronounced role for B cells in human MOG protein induced EAE [138, 139]. In addition, the ability of rhMOG to induce EAE in B cell deficient mice was restored by the injection of rhMOG-primed B cells (but not by injection of naïve WT or unspecifically activated B cells) prior to disease induction but

with a later onset and milder disease course compared to WT control group [140]. This observation was confirmed by others and it was additionally shown that the immunization with recombinant mouse MOG (rmMOG) is also B cell independent [139, 141]. However, the B cell depletion by an anti-CD20 depletion antibody at the onset of MOG peptide-induced EAE results in a faster recovery, suggesting also a role for B cells in MOG peptide induced EAE [56, 142]. The contribution of B cells to the development of EAE is also emphasized in spontaneous EAE models. Only 4% of the MOG-specific T cell receptor transgenic mice (2D2 mice, [77]) on the C57bl/6 background develop spontaneous EAE. However, when they are crossed with MOG-specific Ig heavy-chain knock-in mice (Th mice, [143]) 59% of the animals develop spontaneous EAE accompanied by a higher clinical score, earlier day of onset and a higher number of inflammatory foci [78, 79]. A second model using MOG-specific T cell receptor transgenic mice (1C6, [144]) on the NOD background shows a rare spontaneous EAE development, but when crossed with Th mice, 45% of male and 79% of female mice develop EAE spontaneously.

1.3.4.1.1. B cells and autoantibodies

The mechanism utilized by B cells to contribute to the pathogenesis of EAE is controversially discussed. The main characteristic of B cells is their ability to produce and secrete antibodies. Due to the occurrence of oligoclonal bands in the cerebrospinal fluid in almost all MS patients [145], beneficial plasma exchange therapies [136] and the contribution of autoantibodies to other autoimmune diseases [146-149], it has been suggested that autoantibodies against myelin antigens may play a role in MS and EAE pathology. Immunization of MOG-specific B cell receptor transgenic mice (Th, [144]) either on the C57Bl/6 or on the SJL background results in an accelerated and exacerbated disease course compared to non-transgenic littermates emphasizing a role for anti-MOG-specific antibodies [143]. Studies with MOG peptide-induced EAE of B cell deficient mice reveal a dispensable role for B cells and for autoantibodies against myelin antigens [140, 150]. Double-transgenic mice and also mice immunized with rhMOG or rrMOG have high anti-MOG IgG titers [78, 141, 151] and i.v. sera transfer into B cell deficient mice at the time point of immunization restores the susceptibility to EAE to similar levels observed in WT mice [140]. However, the transfer of anti-MOG 8-18C5 (m-monoclonal antibody against MOG) starting at the onset of EAE only partially restores EAE susceptibility in B cell

specific MHC-II knockout animals [139], whereas when administered into SJL/J mice after the animals recovered from an attack, it induces severe relapses [152]. In a transgenic model where B cells express the membrane MOG-specific immunoglobulin, but are incapable of secreting it, immunization either with MOG peptide, rmMOG or rhMOG results in the same onset and clinical severity, suggesting a dispensable role for α-MOG antibodies in this experimental setting [139]. The contribution of α-MOG antibodies to the pathogenesis of EAE in the SJL/J mouse strain seems to be more convincing. The EAE severity of WT SJL/L mice immunized with a low dose of $PLP_{139-151}$ could be increased by serum transfer and by direct administration of the anti-MOG monoclonal antibody 8.18C-5 [81]. Additionally, the housing of the TCR-transgenic mice under germ-free conditions results in a protection from spontaneous developed EAE, accompanied by low anti-MOG antibody titers. In turn, a recolonization of the gut reestablishes the spontaneous EAE susceptibility which is correlated with high anti-MOG antibody titers and a higher frequency of germinal center B cells within the lymph node [153].

1.3.4.1.2. B cells as antigen-presenting cells

B cells carry an antigen-specific B cell receptor enabling them to bind and process even small amounts of antigen and present it to T cells. Therefore, their contribution to EAE as antigen-presenting cells was investigated extensively. BM chimeric mice where MHC II deficiency is restricted to the B cell compartment, are resistant to rhMOG protein induction but not to MOG peptide or rmMOG protein induction, suggesting a role for B cells as antigen-presenting cells after immunization with rhMOG. The resistance is accompanied by reduced frequencies of IFNγ- and IL-17-producing $CD4^+$ T cells in the periphery and a reduced number of CNS infiltrating $CD4^+$ T cells. However, the ex vivo proliferation of splenocytes from either MHC II sufficient or MHC II deficient host animals is not affected [139]. The spontaneous double-transgenic models also reveal a role for B cells as antigen-presenting cells. MOG specific T cells isolated from double-transgenic mice show a stronger pro-inflammatory cytokine production and proliferation when co-cultured with MOG specific B cells instead of antigen-unspecific B cells and recombinant rat MOG protein (rrMOG) [78, 79]. Nevertheless, conditional MHC-II deletion in different APC subsets demonstrate that the antigen-presenting capacity of B cells alone is not sufficient to trigger EAE [38]. It is also suggested that B cells play a role in the initial

steps of the reactivation phase within the CNS, leading to an increased pro-inflammatory milieu [154].

1.3.4.1.3. B cells and cytokines

The cytokine interleukin-6 (IL-6) plays a critical role in the development of EAE as IL-6 deficient mice are completely resistant to MOG-peptide induced EAE [155]. B cells are the major source of IL-6 in secondary lymphoid tissues and abrogation of IL-6 producing B cells via anti-CD20 treatment ameliorates EAE symptoms [56]. The depletion of IL-6 producing B cells or the selective B cell IL-6 deficiency affects the frequency of $IL17^+$ $CD4^+$ T cells but had no effect on $IFN\gamma^+$ $CD4^+$ T cells [56, 139]. Peripheral blood B cells from MS patients produce more IL-6 before Rituximab treatment and in vitro depletion of B cells from PBMCs of MS patients resulted in reduced IL-17 levels but had no effect on IFNγ [139]. Additionally, ex vivo BCR and CD40-activated B cells isolated from PBMCs of RR-MS patients produced elevated levels of lymphotoxin and TNFα compared to B cells isolated from healthy controls, and the culture supernatant transfer triggered T cell proliferation in vitro [55]. B cells from the CNS of naïve C3HeB/Fej are the main producers of IL-12p35 and TNFα but their direct contribution to EAE development has not yet been investigated in detail [154].

1.3.4.1.4. Regulatory B cells

Besides the pro-inflammatory roles for B cells in the initiation and progression of EAE, it is also considered true that B cells can act as regulatory cells exerting anti-inflammatory functions. The regulatory B cell (Breg) subset was identified as $CD19^+CD1d^{hi}CD5^+$ cells representing 1-2% of spleen $B220^+$ cells in WT mice [156]. BM chimera experiments reveal interleukin-10 (IL-10) as one of the crucial cytokine for Breg cells to exert their regulatory function [157]. Regulatory B cells can be expanded antigen-unspecific by CD40- and TLR stimulation *in vitro* [158]. Whereas TLR signaling alone triggers the production of IL-10, the simultaneous activation of CD40 and TLR4 leads to the production of IL-35 which in turn reduce the antigen-presenting potency of B cells [159]. To exert their anti- inflammatory function in EAE, regulatory B cells require IL-21 and CD40 signaling, as well as cognate T cell interaction, shown by the fact that the transfer of IL-21 receptor knockout, CD40 knockout or MHC-II knockout regulatory B cells did not ameliorate EAE symptoms

[160]. Upon EAE induction, there is an increase in the total number of IL10-producing B cells as well as a higher frequency of $CD1d^{hi}CD5^{+}$ cells among total $CD19^{+}$ B cells [161]. Regulatory B cells influence T cell effector functions directly, but they also act on other antigen-presenting cells [160, 161].

1.3.4.2. Myeloid cells

In addition to cells of the adaptive immune system, cells from the innate immune system are also critically involved in the development and progression of EAE and MS. Macrophage depletion studies in EAE models of mice and rats reveal beneficial effects for the disease [162-164]. The chemokine ligand for CCR2, macrophage-chemoattractant protein-1 (MCP-1, CCL2), is upregulated in spinal cord tissue during EAE. Active immunization of MCP-1 knockout mice leads to an ameliorated disease course and MCP-1 knockout recipients who received MCP-1 sufficient T cells do not develop EAE [165, 166]. In line with this, CCR2 knockout mice are relatively resistant to EAE induction, whereas the transfer of CCR2-deficient T cells induce EAE like WT T cells [165, 167, 168]. On the cellular level, the EAE resistance is accompanied by a reduced number of CNS infiltrating mononuclear cells, especially macrophages, and no upregulation of inflammatory factors like CXCL10, CCL5 which caused the reduced T cell attraction to the CNS. In contrast, neither absence of CCR2 nor of its ligand has an effect on T cell activation within the periphery. Bone-marrow chimera experiments identified the ‘inflammatory’ $Ly6C^{hi}CCR2^{+}$ monocyte subset as the critical factor for EAE induction in CCR2 knockout animals [169].

These studies led to the conclusion that the solely presence of myelin-specific T cells is not sufficient for the development of an autoimmune disease, but the interaction of T cells with other cells types (like B cells or myeloid cells) is critically required for an autoimmune response.

1.4. Objective

There are several investigations studying the role of B cells in EAE and especially their impact on T cells during the course of disease. However, their exact role and the

critical time point when they contribute to the disease pathogenesis is not yet uncovered in detail.

In my PhD-thesis I resumed the investigations about T cell–B cell interaction in the MOG peptide active immunization EAE model. We have now developed a system where we are able to exclusively investigate the interplay between MOG-specific T cells and MOG-specific B cells in the different phases of EAE development. The main objectives of this study were:

1. To establish a model which makes it possible to track and analyze MOG-specific lymphocytes during the course of actively-induced EAE

2. To elaborate the critical time point and mechanism of the contribution of MOG-specific B cells to the development and progression of EAE (activation phase, onset, recovery)

2. Material and Methods

2.1. Material

2.1.1. Reagents and Kits

Immunization

Freund's Incomplete Adjuvant (IFA)	Difco Laboratories, US
Mycobacterium Tuberculosis H37 Ra	Difco Laboratories, US
MOG_{p35-55}	Charité Berlin, Inst. for med. Immunology
4-Hydroxy-3-nitrophenylacetyl hapten-ovalbumin protein (NP-OVAL)	Biosearch Technologies, US
Ovalbumin	Sigma Aldrich, D
Pertussis toxin (PTX)	List Biological Laboratories, US
Ketamine	Medistar
Xylariem	Ecuphar

ELISA

BD OptEIA™ mouse IFNγ ELISA Kit	BD, D
Mouse IL-17 ELISA reagents:	
rec. mouse IL17A	
mIL-17 biotinylated Det. Ab	
mIL-17 MAb (Clone 50101) Cap. Ab	R&D Systems, US
Mouse GM-CSF ELISA reagents:	
rec. mouse GM-CSF	
mGM-CSF biotinylated Det. Ab	
mGM-CSF MAb (Clone MP122E9) cap.Ab	R&D Systems, US
Anti-mouse IgM Peroxidase	Sigma Aldrich, D
Anti-mouse IgG Peroxidase	Sigma Aldrich, D
3,3', 5,5' tetramethylbenzidine (TMB)	BD, D

Cytometric Bead Array (CBA)

Mouse Th1/Th2/Th17 Cytokine Kit	BD, D

Cell isolation kits

EasySep™ Mouse T Cell Enrichment Kit	Stemcell Technologies, CA
EasySep™ Mouse B Cell Isolation Kit	Stemcell Technologies, CA

RNA purification, cDNA synthesis, RT-PCR

RNeasy Micro Kit	Qiagen, D
RNeasy Mini Kit	Qiagen, D
RevertAid First Strand cDNA Synthesis Kit	Thermo Scientific, D
qPCR Master Mix	Eurogentec, D
Primer and Probes	Sigma Aldrich, D

Others

Percoll	GE Healthcare, D
Trypan blue (0.4%)	Sigma Aldrich, D
Dextran 70000	Sigma Aldrich, D
0.9% NaCl	Braun, D
Lymphocyte separation medium (LSM)	Thermo Scientific, D
CFSE	Thermo Scientific, D
Calibrite APC beads	BD,D

2.1.2. Buffers and media

If not otherwise stated, chemicals were purchased from Carl Roth, D.

Standard buffers

10x PBS:

400g NaCl

10g KCl

71g Na_2HPO_4

69g NaH_2PO_4, in 5l a.dest, pH 7.4

EH-medium ("Erhaltungsmedium"):

13.38g/l Dulbeccos Modified Eagle Medium Powder (DMEM)
(Gibco, Life Technologies, D.)
3.7g/l $NaHCO_3$
25mM Hepes (Gibco, Life Technologies, D.) in a.dest

Erythrocytes lysis buffer

0.15M NH_4Cl
1mM $KHCO_3$
0.1mM EDTA, in a.dest pH 7.3

FACS buffer: PBS, 2% BSA, 2% NaN_3

Sorting buffer: EH+ 2mM EDTA

Sorting collection buffer: EH+10% FCS

rrMOG purification

Sonification buffer:	2x PBS 300mM NaCl, 25mM Na_2HPO_4, pH 7.4
Wash buffer:	Sonification buffer+ 0.5% N,N Dimethyldodalylamin-N-oxid (LDAO)
Solubilisation buffer:	6M Guanidinium chloride+ 10mM beta-mercaptoethanol
Column loading buffer:	1% NiCl in a.dest
Column Washbuffer:	6M Guanidinium chloride+ 40mM imidazole
Elution buffer:	6M Guanidinium chloride+ 0.5M imidazole
Dialysis buffer 1:	1x PBS+ 0.4M arginine+ 50mM glutathione, pH 8
Dialysis buffer 2:	1x PBS+ 0.4M arginine, pH 8
Inductor:	1mM IPTG (Thermo Scientific)

T and B cell negative selection

Isolation buffer : PBS, 5% FCS, 2% normal rat serum (provided by the manufacturer)

Cell isolation from brain and spinal cord

Resuspension/percoll dilution buffer: PBS, 1% Glucose, 0,1% BSA

***In vitro* co-culture/ *in vitro* restimulation**

(cell culture supplements were purchased from Gibco, Life Technologies, D.)

Restimulation medium (ReMed)

RPMI 1640 pH 7.4
1% non-essential amino acids
1% sodium-pyruvate
1% L-glutamine
1% penicillin/streptamycine (U)
5% FCS (GE Healthcare, D)
0.2% ß-MEtOH

for ELISA

coating buffer:	IFNγ:	0,1M Carbonate-Bicarbonate buffer
	IL-17:	phosphate buffer: 137mM NaCl, 2.7mM KCl, 8.1mM Na_2HPO_4, 1.5mM NaH_2PO_4, pH 7.2
	GM-CSF:	PBS
wash buffer:	PBS+ 0,05% Tween20	
blocking/dilution buffer:	IFNγ:	PBS, 10% FCS
	IL-17:	PBS, 1% BSA
	GM-CSF:	blocking: PBS, 5% sucrose, 1% BSA, 0.05% NaN_3
		dilution: TBS, 0,1% BSA, 0,05% Tween20
for serum antibody detection:		PBS, 5% BSA

2.1.3. Equipment and consumables

2.1.3.1. Plastic ware

Cell culture plates (6-well, 24-well, 96-well U-bottom/Flat-bottom)	Thermo Scientific, D
Small reagent tubes (0.2ml, 1,5ml, 2ml)	Sarstedt, D
Falcon tubes (15ml, 50ml)	Greiner Bio-One GmbH, D
T and B cell isolation tubes (13ml)	Sarstedt, D
Tips (1ml, 0.2ml, 0.01ml)	StarLab, D
Filter Tips (1ml, 0.2ml, 0.01ml)	StarLab, D

ELISA plate	Thermo Scientific Nunc, D
Parafilm	Picheney, US
Gloves	Kimberly-Clark, US
Combitips plus (5ml, 1ml, 0.5 ml, 0.25ml)	Eppendorf, D
Petri Dish	Greiner Bio-One GmbH, D
Syringes (5ml, 10ml)	Braun, D
Insulin syringes (1ml)	BD, D
Sterile needles (24G, 20G, 27G, 26G)	Braun, D
FACS tubes (+/- Filter)	BD, D

2.1.3.2. Glas ware

Glass pipettes (5ml, 10ml, 20ml)	HBG, D
Dounce tissue grinder + Pestle (0.071-0.119mm) (0.02-0.056mm)	Kimble (Sigma-Aldrich, D)
Neubauer cell chamber	Brand, D
Syringe for immunization (1ml, 2ml) Tuberculin glass/ metal syringes	Hartenstein, D

2.1.3.3. Equipment

Pipettes (0.5-10µl; 10-200µl; 100-1000µl)	Eppendorf, D
Pipettus	Hirschmann, D
“Big Easy” silver EasySep magnet	Stemcell Technologies, CA
Mesh (40µm pore size)	UMG factory
Multichannel pipette	StarLab, D

2.1.3.4. Machines

RT-PCR Cycler	StepOnePlus Real-Time PCR System (Applied Biosystems)
Flow Cytometer	BD FACSCalibur
Cell Sorter	BD FACSAria II cell sorter
Nanodrop	Peqlab, D
ELISA plate reader	Tecan, CH
Thermocycler	Mastercycler (Eppendorf, D)
Centrifuges:	
Multifuge 1 S-R	Heraeus, D
Centrifuge 5415 R	Eppendorf, D
Incubator	Heraeus, D
Laminar flow	Heraeus, D
Inverted bright field Microscope	Zeiss, D

2.1.3.5. Software

Microsoft Office	
FlowJo V10	Tree Star, US
StepOnePlus Software v2.0	Applied Biosystems
GraphPad Prism 5	GraphPad Software, US
BD FACSDiva Software	
BD CellQuestPro Software	
PrimerExpress v2.0	Applied Biosystems

FCAP Array v3.0.1 Software for BD Cytometric Bead Array (CBA) Analysis

2.1.3.6. Flow cytometry fluorochrome labeled antibodies

Table 1: Flow cytometry antibodies

Specificity	Label	Clone	Company
CD3e	Alexa-Fluor 647	145-2C11	BioLegend
	Biotin	145-2C11	BD
CD4	APC	RM4-5	BioLegend
	APC-Cy7	RM4-5	BioLegend
	PE	Gk1.5	BD
	PE-Cy5	H129.19	BioLegend
CD8	PerCP	53-6.7	BD
	FITC	53-6.7	BD
	PE-Cy7	53-6.7	BioLegend
CD25	APC	3C/	BiolLegend
	FITC	3C7	BioLegend
CD69	APC-Cy7	H1.2F3	BioLegend
	FITC	H1.2F3	BioLegend
CD44	APC	IM7	BD
	APC-Cy7	IM7	BioLegend
CD62L	Biotin	MEL-14	BD
	FITC	MEL-14	BioLegend
CD45R/B220	APC	RA3-6B2	BioLegend
	PE	RA3-6B2	BD
MHC Class II ($I\text{-}A^b$)	APC/PE/FITC	AF6-120.1	BioLegend
CD86	FITC	GL-1	BioLegend
CD11a	Biotin	2D7	BD
CD49d	Alexa-Fluor 647	R1-2	BioLegend
Vβ 11 TCR	FITC	KT11	BioLegend
Vβ 5.1/2 TCR	Biotin	MR9-4	BD
CCR2	Alexa Fluor 700	475301	R&D Systems
CX_3CR_1	APC		
Ly6C	APC-Cy7	HK1.4	BioLegend
Gr-1	PE	RB6-8C5	BD
CD11b	APC	M1/70	Biolegend
	Biotin	M1/70	BioLegend
CD31	APC	MEC13.3	BioLegend
Streptavidin-	PE, APC, FITC, PE-Cy5, APC-Cy7		Biolegend

2.1.3.7. RT-PCR primer and probe sequences

Table 2: RT-PCR Primer and probes (purchased from Sigma-Aldrich)

HPRT	Fw (5`-3`)	TGCTCGAGATGTCATGAAGG
	Rev (5`-3`)	TATGTCCCCCGTTGACTGAT
	Probe	[6FAM]ATCACATTGTGGCCCTCTGT[TAM]
Integrin alpha L	Fw (5`-3`)	AATGACGCTGGCAACAGATG
	Rev (5`-3`)	GAGGTAAGTGTTCTGATCGCATGT
	Probe	[6FAM]CTTTTGGCCTGTGACCCTGGACTGCT[TAM]
Integrin alpha 4	Fw (5`-3`)	CGAGTTTCAAGCAGTGGAGAGA
	Rev (5`-3`)	TGGTATGTGGCCTCTACATGAATG
	Probe	[6FAM]CACACCAGGCATTCATGCGGAAAGAC[TAM]
IFNy	Fw (5`-3`)	TCAAGTGGCATAGATGTGGAAGAA
	Rev (5`-3`)	TGGCTCTGCAGGATTTTCATG
	Probe	[6FAM]TCACCATCCTTTTGCCAGTTCCTCCAG [TAM]
GM-CSF	Fw (5`-3`)	GGGCGCCTTGAACATGAC
	Rev (5`-3`)	CGCATAGGTGGTAACTTGTGTTTC
	Probe	[6FAM]CCCCCCAACTCCGGAAACGGA [TAM]
IL-17A	Fw (5`-3`)	ACTTTCAGGGTCGAGAAGATGCT
	Rev (5`-3`)	TTCTGAATCTGCCTCTGAATCCAC
	Probe	[6FAM]TGGGTGTGGGCTGCACCTGC [TAM]
ALCAM	Fw (5`-3`)	ACGCGACTGTGGTGTGGAT
	Rev (5`-3`)	CCTGATAATGAAGACTGGAAAAGGA
	Probe	[6FAM]AAGGATAACATCCGGCTCCGGTCCA [TAM]
ICAM	Fw (5`-3`)	GCCAAGCCCACGCTACCT
	Rev (5`-3`)	TCTCTGGGATGGATGGATACCT
	Probe	[6FAM]TCACCGTTGTGATCCCTGGGCCT [TAM]
VCAM	Fw (5`-3`)	CAGAGTGTACAGCCTCTTTATGTCAAC
	Rev (5`-3`)	GGACTGCCCTCCTCTAGTATAGGA
	Probe	[6FAM]TTGCCCCCAAGGAAACCACCATC [TAM]
CCR7	Fw (5`-3`)	CAGCCTTCCTGTGTGATTTCTACA
	Rev (5`-3`)	ACCACCAGCACGTTTTTCCT
	Probe	[6FAM]CAGAGCACCATGGACCCAGGGAAAC [TAM]
CXCR3	Fw (5`-3`)	CCAAGCCATGTACCTTGAGGTTAG
	Rev (5`-3`)	AATCGTAGGGAGAGGTGCTGTTT
	Probe	[6FAM]ATGCCTCGGACTTTGCCTTTCTTCTGG [TAM]
CXCR4	Fw (5`-3`)	ACCTCTACAGCAGCGTTCTCATC
	Rev (5`-3`)	TGTTGGTGGCGTGGACAATA
	Probe	[6FAM]TGGCCTTCATCAGCCTGGACCG [TAM]
CCR5	Fw (5`-3`)	TGCTCAACCTGGCCATCTCT
	Rev (5`-3`)	CCCACTCATTTGCAGCATAGTG
	Probe	[6FAM]CTGCTCTTCCTGCTCACACTACCATTCTGG [TAM]
CCR6	Fw (5`-3`)	TCGTCCAGGCAACCAAATC
	Rev (5`-3`)	CCACACTGCCACACAGATGAC
	Probe	[6FAM]TTCCGGGTACGCTCCAGAACACTGA [TAM]
CCL5	Fw (5`-3`)	GGAGTATTTCTACACCAGCAGCAA
	Rev (5`-3`)	CACACACTTGGCGGTTCCTT
	Probe	[6FAM]TGCTCCAATCTTGCAGTCGTGTTTGTCA [TAM]
CXCL10	Fw (5`-3`)	CATCCCTGCGAGCCTATCC
	Rev (5`-3`)	CATCTCTGCTCATCATTCTTTTTCA
	Probe	[6FAM]CCCACGTGTTGAGATCATTGCCACG [TAM]
CXCL12	Fw (5`-3`)	CAAGCATCTGAAAATCCTCAACAC

	Rev (5`-3`)	GCACACTTGTCTGTTGTTGTTCTTC
	Probe	[6FAM]AAACTGTGCCCTTCAGATTGTTGCACG [TAM]

2.2. Methods

2.2.1. Mouse strains

All mice strains were bred in IVC cages (SPF conditions) in the central animal facility of the University Medicine School Göttingen. Experiments were performed with at least 10 weeks old mice. All mice were on the C57bl/6 background. Animal experiments were approved by the responsible authorities in Lower Saxonia.

Table 3: Used mouse strains

Name	**used for**	**Ref.**
C57bl/6	Host	
RFP	Crossed to various mouse strains	[170]
GFP	Crossed to various mouse strains	[171]
2D2	Isolation of MOG-specific T cells	[77]
2D2RFP	Isolation of RFP^+ MOG-specific T cells	
2D2GFP	Isolation of GFP^+ MOG-specific T cells	
Th	Isolation of MOG-specific B cells	[143]
ThRFP	Isolation of RFP^+ MOG-specific B cells	
THGFP	Isolation of GFP^+ MOG-specific B cells	
B1.8 (NP)	Isolation of NP-specific B cells	[172]
OT-II	Isolation of OVA-specific T cells, used as host	[173]
OT-II x NP	Host	
mb1Cre	Crossed to XBP-1 $^{fl/fl}$	[174]
XBP-1 $^{fl/fl}$		[175]
MHC-$II^{-/-}$	Crossed to Th mice for isolation of MHC-$II^{-/-}$ MOG-specific B cells	[176]
FOXP3-eGFP	Crossed to 2D2RFP for isolation of MOG-specific FOXP3 eGFP reporter T cells	[177]

2.2.2. Antigen-specific T and B cell isolation

Briefly, lymph nodes and spleens were taken out from the respective donor animals (Table 3) and single cell suspension was prepared by smashing the organs through a mesh (40µm pore size). After centrifugation (1200rpm, 8 min., 4°C) the cells were resuspended at a concentration of $1x10^8$ cells/ml in isolation buffer. $CD3^+$ T cells and $CD19^+$ B cells were isolated using the EasySep Mouse T Cell Enrichment Kit (Stemcell) or the EasySep Mouse B Cell Isolation Kit, respectively. Cell isolation was performed following the manufacturer's protocol for the "Big Easy" silver EasySep magnet. Cell purity was routinely determined by flow cytometry and was always higher than 90%. After purification, the cells were used for various *in vitro* or *in vivo* experiments.

2.2.3. Adoptive transfer experiments

For adoptive cell transfer the cells were injected intravenously (i.v.) in different cell numbers depending on the experiment in a total volume of 200µl PBS into different host mice. For C57BL/6 experiments $7\text{-}8x10^6$ T cells were injected either alone or together with $1x10^7$ MOG-specific B cells if not otherwise stated. For OT-II or OT-II x NP experiments, $3.5x10^6$ MOG-specific T cells were either injected alone or together with $1x10^7$ MOG-specific B cells. Two days post adoptive cell transfer, the host mice were immunized with the respective antigen emulsified in CFA like described in chapter 2.2.5.2..

2.2.4. CFSE-labeling

To assess T cell proliferation *in vitro* or *ex vivo*, isolated antigen-specific T cells were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE). Briefly, the respective lymphocytes were incubated at a concentration of $2x10^6$ cells/ml PBS containing 0.25µM CFSE for 10 min at 37°C. The reaction was stopped by addition of 10ml FCS and the cells were washed twice in PBS containing 5% FCS. According to the experiment, the CFSE labeled cells were either injected i.v. into host animals to determine the proliferation *ex vivo* at different time points or cultured with different conditions (chapter 2.2.7.1.) for three days. The proliferation, indicated by the CFSE dilution with every cell division, was assessed by flow cytometry.

2.2.5. EAE induction

2.2.5.1. Antigens

The 2D2 TCR epitope MOG_{p35-55} (MEVGWYRSPFSRVVHLYRNGK) was purchased from Charité, Institute for Medical Immunology. 4-Hydroxy-3-nitrophenylacetyl hapten conjugated to OVA (ovalbumin) protein (NP-OVAL) was purchased from Biosearch Technologies. A plasmid construct encoding the extracellular domain of rat MOG protein (MOG amino acids 1–125) was provided by C. Linington (University of Glasgow, UK) and purified from bacterial inclusion bodies [178]. Expression plasmidpQE-12 containing rat MOG 1-125 was grown in LB medium containing ampicillin (100 mg/ml) and kanamycin (25 mg/ml). MOG expression was induced with isopropyl thiogalactoside (IPTG) before the cells were pelletted. The pellet was resuspended in sonibuffer with lysozyme, sonicated and mechanically disrupted and homogenized (all used buffers for rrMOG purification are listed in chapter 2.1.2.). Lysed samples were washed, resuspended in solubilization buffer, and loaded onto the Ni-NTA column prepared with chelating sepharose and 1% $NiCl_2$. The column was washed with column washbuffer and the protein was eluted with column elution buffer. The MOG protein was refolded in several dialysis steps. For the first 24-48h it was dialyzed against dialysis buffer 1 followed by a dialysis against dialysis buffer 2 ON. Final dialysis was performed against 1x PBS for 48h. The protein concentration was measured on a Nanodrop at 280nm.

2.2.5.2. Immunization and scoring

Two days post adoptive cell transfer (chapter 2.2.3.) EAE was induced by immunization with respective antigen in at least 10 weeks old mice.
Mice were anesthetized with 100µl of 10% ketamine, 5% xylariem in 0,9% NaCl per 10g body weight. Mice were injected (50µl/side) subcutaneously (s.c.) at the tail base with an emulsion containing 50µg complete freund's adjuvant (CFA) and 50µg MOG_{p35-55} in PBS. CFA was prepared by addition of 10mg mycobacterium tuberculosis (strain H37 Ra) to 10 ml incomplete freund`s adjuvant (1mg/ml). Pertussis toxin (PTX) (200ng/day) was injected intraperitoneal (i.p.) on days 0 and 2. Concentrations of other antigens were different in each experiment and are stated in the figure legend.

Animals were scored daily for clinical signs of EAE, based on a scoring system reaching from 0 – 10 points with the individual points defined as follows:

0 = healthy
1 = reduced tail tonicity
2 = flaccid tail paralysis
3 = loss of righting reflex
4 = kinetic ataxia
5 = slight paralysis of hind legs
6 = plegia of one leg or moderate paralysis of both hind legs
7 = paraplegia with complete paralysis of both hind legs
8 = tetraparesis with additional (slight) paralysis of both forelegs
9 = moribund
10 = dead With a score of >7 the animals had to be sacrifice.

2.2.6. Tissue preparation for cell isolation

2.2.6.1. Lymphocyte isolation from lymph nodes and spleen

To analyze the injected antigen-specific T and B cells *ex vivo*, lymph nodes (inguinal and paraaortic as draining lymph nodes, axilliary and cervical lymph nodes as non-draining lymph nodes) and spleen were isolated from the respective host mice and single cell suspension was prepared like described above (chapter 2.2.2.). Due to expression of fluorescent markers (either RFP or GFP) it was possible to track the cells with flow cytometry (chapter 2.2.11.) or cell sorting (chapter 2.2.12.) after recovery.

2.2.6.2. Lymphocyte isolation from peripheral blood

If animals were euthanized, blood was taken by heart puncture and mixed in a 1:2 ratio with 2mM ethylenediaminetetraacetic acid (EDTA) in PBS to avoid thrombocyte aggregation. In case animals were kept alive, blood was taken by retro-orbital puncture. Lymphocytes were separated via density gradient centrifugation using lymphocyte separation medium (LSM) at 1500rpm, 20 min, 4°C. The interphase between plasma and separation solution containing the lymphocytes was transferred into a new tube and washed with PBS.

2.2.6.3. Lymphocyte isolation from spinal cord and brain

Euthanized mice were perfused through the left cardiac ventricle with cold 0,9% NaCl. The spinal cord and brain were extracted and kept in PBS. Single-cell suspensions were prepared by application of mechanical disruption and subsequent resuspension in spinal cord resuspension buffer. After centrifugation, the pellet was resuspended in 30% Percoll solution and mononuclear cells were separated from myelin via Percoll density gradient centrifugation (70/45/30% Percoll in spinal cord resuspension buffer)(2300rpm, 20 min, 4°C w/o break). The interphases (30:45 % and 45:70 %) containing the lymphocytes were transferred into a new tube and washed with PBS.

For isolation of perivascular lymphocytes, spinal cord tissue was prepared following the spinal cord microvessel endothelial cell isolation protocol and T cells were sorted according to their RFP^+ expression.

2.2.6.4. Isolation of spinal cord microvessel endothelial cells

The spinal cord was extracted and kept in EH medium. To prepare a cell suspension, the spinal cord was cut in small pieces (1-5mm^2) and homogenized in a 7ml Dounce tissue grinder (Kimble/Kontes), first with a larger clearance pestle (0.071-0,119mm) and then with a smaller clearance pestle (0,02-0,056mm). The homogenate was then spun down (1200rpm, 8 min, 4°C) and resuspended in 6ml of an 18% (w/v) dextran solution (MW 70000) in EH medium. To get rid of the myelin proteins, the resuspension was spun down at 10000xg for 20 min and the supernatant was removed. The pellet was resuspended in EH medium and washed (1200rpm, 8 min, 4°C). To digest the microvessels, the pellet was resuspend in 600µl 1% collagenase and gently shaked at 37°C for 20 min. Afterwards, the suspension was transferred into a FACS tube, washed with FACS buffer and stained for CD31 for $CD31^+$ cell sorting.

2.2.7. *In vitro* experiments

2.2.7.1. *In vitro* co-culture experiments

To study the interaction of antigen-specific T and B cells in vitro, respective T and B cells were isolated as described in chapter 2.2.2. in sterile conditions. $1x10^6$ T cells and $2x10^6$ B cells were co-cultured for 3 days with increasing concentrations of different antigens in a range from 0-10µg/ml in a 24-well plate in ReMed in an incubator at 37°C and 5% CO_2. To determine soluble factors in the supernatant, it was removed 3 days post culture and stored in -80°C until analysis. Depending on the experiment, either the T cells or the B cells were CFSE labeled (chapter 2.2.4.) before co-culture and proliferation was analyzed 3 days post culture.

2.2.7.2. *In vitro* restimulation experiments

To investigate the cytokine production of transferred T cells *ex vivo*, single cell suspension was prepared from draining lymph nodes and spleen. Erythrocytes were lysed from splenocytes by adding 1ml erythrocyte lysis buffer for 5min on ice. Afterwards, $5x10^5$ total lymph node cells and splenocytes were cultured with increasing concentrations of MOG_{p35-55} (0, 1, 5, 10 µg/ml) in 100µl ReMed in a 96-well plate for 72h (incubator, 37°C, 5% CO_2). The cell culture supernatant was collected and stored at -80°C until cytokine determination via ELISA.

2.2.8. Cell count

Total cell numbers from single cell suspensions were determined using a Neubauer cell chamber. Trypan blue containing 1% acidic acid for erylysis was used to distinguish between viable and non-viable cells.

cell number: N x dilution factor x volume x 10^4 N= number of living cells

Cell number of adoptively transferred RFP^+ or GFP^+ cells was determined with BD Calibrite APC beads by flow cytometry. A defined number of beads was solved in PBS and 50µl of the suspension was added to 200µl of an organ single cell suspension with defined volume. Weight of respective organs (lymph nodes, spleen and spinal cord) was determined before smashing to enable calculation of cell number per gram.

Total cell number was calculated according to the equation:

$$\left(\frac{beads\ in\ 50\mu l\ x\ acquired\ cells}{acquired\ beads}\right)\ x\ dilution\ factor$$

To determine the cell number per gram organ weight, the total number had to be divided by organ weight (g).

2.2.9. Enzyme linked immunosorbent assay (ELISA)

2.2.9.1. Cytokine ELISA

The amounts of IFNγ, IL-17A and GM-CSF were determined from supernatants of restimulated total draining lymph node cells and splenocytes (chapter 2.2.7.2.) using BD OptEIA mouse ELISA Kits for IFNγ or capture antibody, detection antibody and standard from R&D Systems for IL-17A and GM-CSF following the manufacturer`s protocol. ELISA plates were coated with 50µl of respective capture antibody diluted 1:250 in ELISA coating buffer ON at 4°C. For quantification, supernatant samples were diluted (IFNγ 1:50; IL-17A 1:5; GM-CSF 1:20) and analyzed in triplicates. For HRP enzymatic reaction 100µl 3,3', 5,5' tetramethylbenzidine (TMB) was added for 30 min and the reaction was stopped by addition of 50µl 1M H_2SO_4. The absorbance was measured at 450nm with correction wavelength of 620nm in an ELISA reader.

2.2.9.2. Serum antibody ELISA

For determination of α-MOG IgM or IgG antibodies, ELISA plates were either coated with 4µg/ml rrMOG or 10µg/ml MOG_{p35-55} in PBS ON at 4°C. For determination of α-NP IgM or IgG antibodies, plates were coated with 5µg/ml NP-OVAL or 5µg/ml OVA as control. To determine antibody titers, sera samples were diluted with dilution buffer 1:100 and 1:1000 and analyzed in triplicates. HRP-conjugated detection antibodies against mouse IgM and IgG were used in a 1:250 dilution. Further analysis steps were performed as described above.

2.2.10. Cytometric Bead Array (CBA)

With the Mouse Th1/Th2/Th17 Cytokine Kit the cytokines IL-2, IL-4, IL-6, IFN-γ, TNF-α, IL-17A, IL-10 were assessed in the supernatants of in vitro co-culture assays. The

supernatants of different T cell–B cell in vitro co-culture experiments were collected after 72h and stored in -80°C until cytokine quantification. Before analysis, supernatant samples were diluted 1:10 with dilution buffer (provided from the Kit).
10 µl of each supernatant dilution was mixed with 10 µl Capture Beads (beads with different fluorescence intensities linked to antibodies against the 7 different cytokines) and 10 µl PE- detection solution (PE-conjugated antibodies). Furthermore, a dilution series from a standard (from kit, consisting of cytokines) was provided and also mixed with capture beads and PE- detection solution. After an incubation time of 2 h at RT in the dark, the samples were washed once in washing buffer (from kit), resuspend in 50 µl washing buffer and analyzed with flow cytometry. Cytokine concentrations were determined with the help of the standard curve and the FCAP Array v3.0.1 Software.

2.2.11. Flow cytometry

1-5x10^6 cells per sample was surface stained in 100 µl FACS buffer with combinations of monoclonal antibodies labelled with fluorochromes for 15 min at 4°C. After staining, the samples were washed and resuspended in FACS buffer. Stained cells were either analyzed on a BD FACSCalibur or on a BD FACSAria II cell sorter. Final analysis was performed using FlowJo V10 (Tree Star) software. The injected antigen-specific T and B cells could be tracked due to their expression of either RFP or GFP. All used antibodies are listed in Table1.

2.2.12. Cell sorting

Cell sorting was performed with the BD FACSAria II cell sorter. For RNA isolation, cDNA synthesis and subsequent RT-PCR, *ex vivo* isolated MOG-specific $CD4^+$ T cells from different organs (chapter 2.2.6.) were identified and sorted by their simultaneous expression of either GFP or RFP and CD4. The sorted cells were collected in EH medium containing 10% FCS and subsequently resuspended in RLT buffer (chapter 2.2.13.) for RNA extraction. Endothelial cells from spinal cord microvessels were sorted according to their CD31 expression.

2.2.13. RNA isolation, cDNA synthesis and RT-PCR

RNA isolation was performed using Qiagen RNeasy Micro Kit for <100000 cells or Qiagen RNeasy Mini Kit >100000 cells following the manufacturer`s protocol. As the total RNA amount from less than 100.000 cells was very low, the RNA concentration

could not be determined and whole eluat was used for subsequent cDNA synthesis. cDNA synthesis was performed using RevertAid First Strand cDNA Synthesis Kit with random hexamer primer as described in the manufacturer`s protocol. RT-PCR was performed with the qPCR Master Mix on a StepOnePlus Real-Time PCR System. RT-PCR was performed with target specific FAM and TAMRA labeled TaqMan probes. Analysis was performed using StepOnePlus Software v2.0. Expression was normalized to that of the housekeeping gene hypoxanthine phosphoribosyltransferase 1 (HPRT). The best working forward and reverse primer concentration was titrated and used primer and probe sequences are listed in Table 2.

2.2.14. Statistics

Statistical analysis was performed with GraphPad Prism 5. Data are depicted as mean±SEM (standard error of the mean). Respective statistical analysis methods are always depicted in the figure legend. P-value of statistical difference was indicated like: **ns**: $p \geq 0.05$; *: $p \leq 0.05$; **: $p \leq 0.01$; ***:$p \leq 0.001$

3. Results

3.1. Establishment of the EAE model

Several publications suggest an interaction of T and B cells to be involved in the pathogenesis of EAE. Most of the studies make use of the C57bl/6 active immunization with MOG antigens (MOG_{p35-55} (MOG peptide), rhMOG (recombinant human MOG), rrMOG (recombinant rodent MOG)) EAE model where the amplification of respective myelin-specific T and B cells is triggered. However, by their investigation of the endogenous T and B cell pool they do not exclusively focus on myelin-specific lymphocytes. We now developed a system enabling us to track and analyze MOG-specific T and B cells during the whole course of EAE *ex vivo*.

As the antigen-specificity of pathogenic lymphocytes is critically associated with the development of EAE, it is advantageous to generate a system which facilitates the tracking of antigen-specific T and B cells during the course of EAE. For this purpose, we isolated MOG-specific T cells from 2D2 mice [77] and MOG-specific B cells from Th mice [143]. Beside the transgenic TCR or BCR, these cells simultaneously express either red fluorescent protein (RFP) or green fluorescent protein (GFP), enabling the cell tracking during the whole course of disease and to *ex vivo* analyze them with the help of different techniques (flow cytometry, FACS sorting, 2-photon intravital imaging). To study the cellular behavior during the course of EAE, we transferred the cells intravenously (i.v.) into different host animals followed by active immunization with different antigens. Initial experiments were performed with C57bl/6 (WT) recipient mice. To study the influence of MOG-specific B cells on MOG-specific T cells in detail we utilized OT-II mice as recipients. These mice harbor >90% of endogenous OVA-specific T cells allowing to minimize the endogenous T cell responses to MOG antigens. To investigate the effect of different genetically modified B cells on EAE, we utilized OT-II x NP as these mice are devoid of endogenous pathogenic T cells and B cells.

To evaluate the activation status of freshly isolated MOG-specific T cells, we performed antibody-labeled surface staining for flow cytometry analysis before i.v. transfer. To ensure that the transferred MOG-specific T cells can be primed antigen-specific *in vivo*, we analyzed their proliferation behavior *ex vivo* upon MOG_{p35-55} or OVA protein immunization.

3.1.1. Freshly isolated MOG-specific T cells have a naïve phenotype and exhibit an antigen-specific response *in vivo*

To initially characterize MOG-specific T cells before transfer, they were surface stained with antibodies against CD3, CD4, CD8 (Fig. 4 A) and the surface markers CD62L, CD69 and CD44 (Fig. 4 B) followed by flow cytometry analysis. CD62L is highly expressed by naïve T cells, whereas CD69 is considered as an early activation marker. CD44 is mainly expressed by antigen-experienced T cells. For lymphocyte isolation, lymph nodes and spleens from the respective donor mice were prepared and cell isolation was carried out using a negative selection kit to avoid TCR or BCR cross-linking and cell activation.

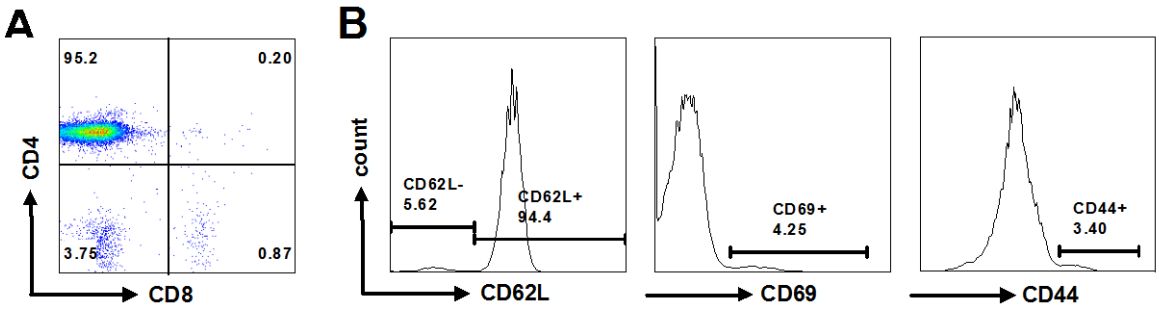

Figure 4: Freshly isolated MOG-specific T cells from 2D2 mice have a naïve phenotype. MOG-specific T cells were isolated from 2D2 mice and antibody-labelled against **(A)** CD4 and CD8 (gated on CD3) and **(B)** CD62L, CD69 and CD44 (gated on CD4) for flow cytometry analysis. Histogram gates depict the percentage of surface marker positive cells.

Approximately 95% of the isolated $CD3^+$ T cells were $CD4^+$ (Fig. 4 A). The majority (94 %) of the $CD4^+$ T cells were $CD62L^+$ and only a minor proportion of the isolated T cells expressed the activation marker CD69 (4.25%) or the memory marker CD44 (3.39%) (Fig. 4 B) assuming that the vast majority of the transferred MOG-specific T cells had a naïve phenotype.

To proof whether the transferred MOG-specific T cells respond specifically to the MOG peptide antigen *in vivo*, freshly isolated RFP^+ MOG-specific T cells were labeled with CFSE and injected i.v. into C57bl/6 WT mice. Two days later, the host mice were immunized either with 50µg MOG_{p35-55} or with 50µg OVA protein as control. CFSE is a membrane-permeable fluorescent dye, which is non-fluorescent in its native form. Due to enzymatic modification within the cell, it is rendered highly fluorescent and also unable to diffuse out of it. Upon one cycle of cell division the

stain is halved in each of the daughter cells, which can be detected by flow cytometry. Five days post immunization (d p.i.), single cell suspension from spleen and draining lymph node (inguinal) was analyzed for CFSE dilution by flow cytometry.

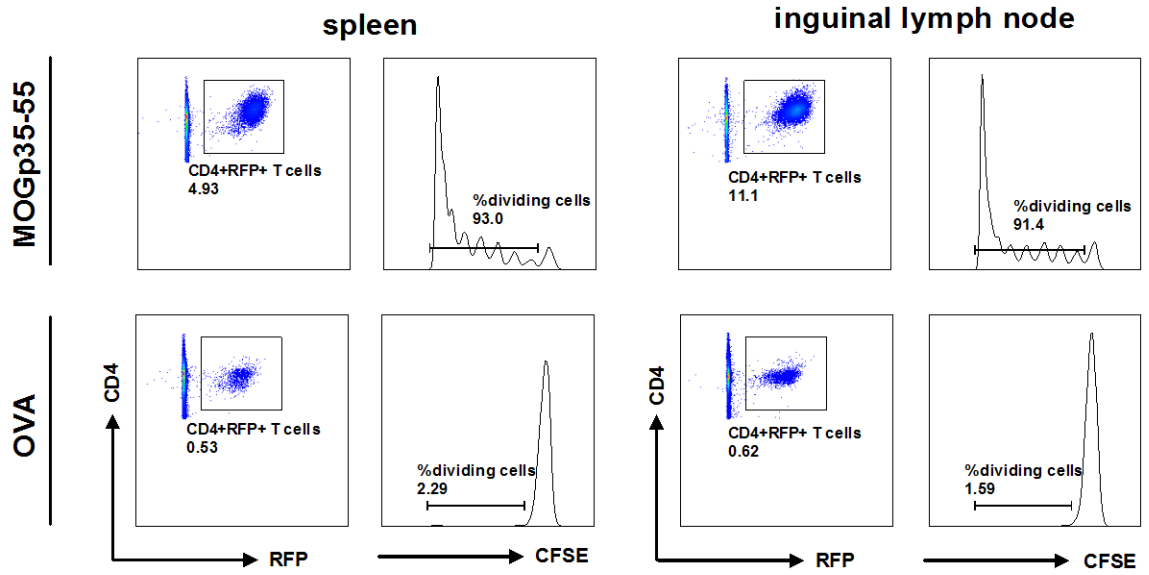

Figure 5: Transferred MOG-specific T cells show antigen-specific proliferation. RFP^+ MOG-specific T cells were isolated from 2D2 mice, CFSE labeled and i.v. injected in C57bl/6 WT mice two days before immunization with either MOG_{p35-55} (upper row) or OVA protein (lower row). Five days p.i., single cell suspension from the spleen (left) and the draining lymph node (right) was prepared and labeled with an antibody against CD4 for flow cytometry analysis. Transferred MOG-specific T cells were identified by their simultaneous expression of CD4 and RFP (dotplots) and the proportion of CFSE dilution was examined with a histogram plot.

The transferred MOG-specific T cells exclusively proliferated after immunization with MOG_{p35-55} (Fig. 5 upper row) but not after immunization with a foreign antigen (Fig. 5 lower row) as measured by CFSE dilution. The specific amplification of MOG-specific T cells after immunization with MOG_{p35-55} can not only be demonstrated by the dilution of CFSE (histogram plots) but also by the frequency of RFP^+CD4^+ T cells among the total $CD4^+$ T cell pool (dot plots). These basic observations ensure that we transferred naïve MOG-specific T cells which have to be activated *in vivo* like the endogenous T cell pool. In addition we ensure that the transferred MOG-specific T cells exclusively respond in an antigen-specific manner.

3.1.2. Characterization of the antigen-specific T and B cell interaction *in vitro*

As we were interested in the antigen-specific interplay of T and B cells, we performed a series of *in vitro* experiments. We wondered whether the antigen presentation by B cells is sufficient to trigger T cell proliferation *in vitro*. Therefore, we co-cultured B cells with CFSE labeled T cells in the presence of antigen and determined T cell proliferation after 3 days. To investigate whether also B cells become activated upon T cell interaction, we determined MHC-II and CD86 expression on B cells. To study the contribution of antigen-specificity on the interplay of T and B cells, we co-cultured different antigen-specific T and B cells with increasing concentrations (0, 0.01, 0.1, 1, 10 µg/ml) of different antigens (MOG_{p35-55}, rrMOG, NP-OVAL). We co-cultured either MOG-specific T cells or OVA-specific T cells (isolated from OVA-specific TCR transgenic mice (OT-II mice) [173]) with MOG-specific B cells or with NP-specific B cells (isolated from B1.8 mice (in this study always termed “NP mice”) [172]).

MOG-specific T cells proliferated in the presence of MOG-specific B cells and MOG_{p35-55} but also in the presence of NP-specific B cells (Fig. 6 A left). In contrast, in the presence of rrMOG, only MOG-specific B cells could trigger MOG-specific T cell proliferation (Fig. 6 A middle). OVA-specific T cells proliferated neither in the presence of MOG_{p35-55} nor in presence of rrMOG, but they responded to even very low NP-OVAL concentrations when presented by NP-specific B cells (Fig. 6 A right). The results regarding the T cell proliferation led to the conclusion that a T cell response can just be initiated in the presence of the appropriate antigen. Both, MOG-specific B cells and NP-specific B cells upregulated CD86 and MHC-II at high MOG_{p35-55} concentration, but exclusively in the presence of MOG-specific T cells (Fig. 6 B+C left). In the presence of rrMOG, MOG-specific B cells upregulated CD86 and MHC-II at very low antigen concentration, but just when they acquired T cell help of the same antigen specificity (Fig. 6 B+C middle). In contrast, NP-specific B cells upregulated CD86 and MHC-II in the presence of NP-OVAL and OVA-specific T cells, but also in the presence of antigen-unspecific T cells (Fig. 6 B+C right). In summary, the activation of B cells was not as strictly as observed for the T cells, as NP-specific B cells also upregulated MHC-II in the presence of an irrelevant antigen and of antigen-unspecific T cells. However, the interaction of T and B cells resulted in an activation of both interaction partners.

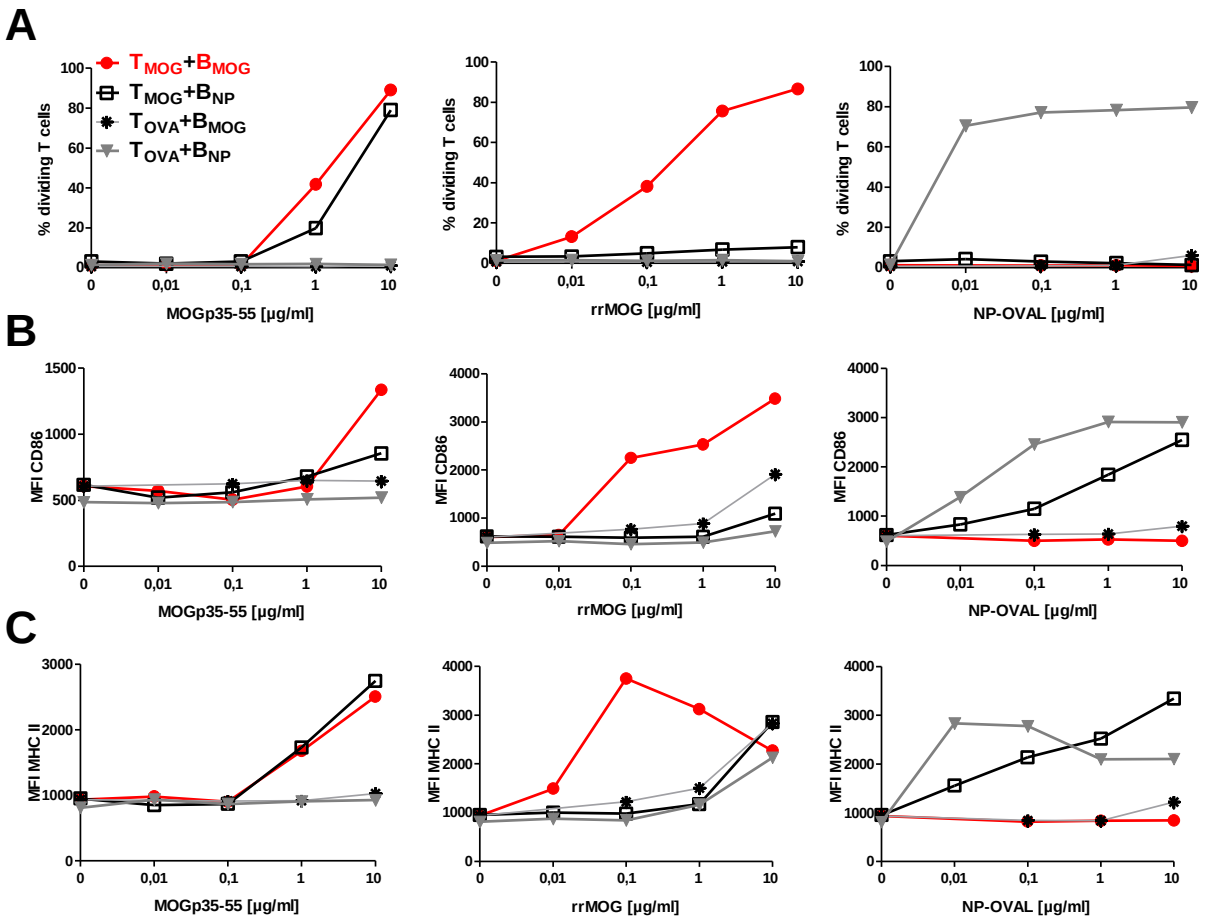

Figure 6: *In vitro* analysis of antigen-specific T and B cell interaction. 1x10^6 MOG-specific T cells were co-cultured with either 2x10^6 MOG-specific B cells (red line) or NP-specific B cells (open quad), as control OVA-specific T cells were co-cultured with MOG-specific B cells (black line) or NP-specific B cells (grey line) with increasing MOG_{p35-55} (left), rrMOG (middle) or NP-OVAL (right) concentrations (0; 0.01; 0.1; 1; 10 µg/ml) for 72h. **(A)** T cell proliferation was determined by CFSE dilution. **(B+C)** Co-cultured B cells were stained for B220 and CD86 **(B)** or for B220 and MHC-II **(C)** and analyzed for their mean fluorescence intensity (MFI) of respective marker expression by flow cytometry. Shown is one representative experiment out of three independent experiments.

3.1.3. MOG-specific B cells accelerate the disease onset in actively induced EAE

To investigate whether the cell transfer had a direct effect on the course of EAE, we transferred 8x10^6 MOG-specific T cells, 5-10x10^6 MOG-specific B cells or both together into C57bl/6 WT mice followed by immunization with MOG_{p35-55} and monitored the clinical outcome for 18 days.

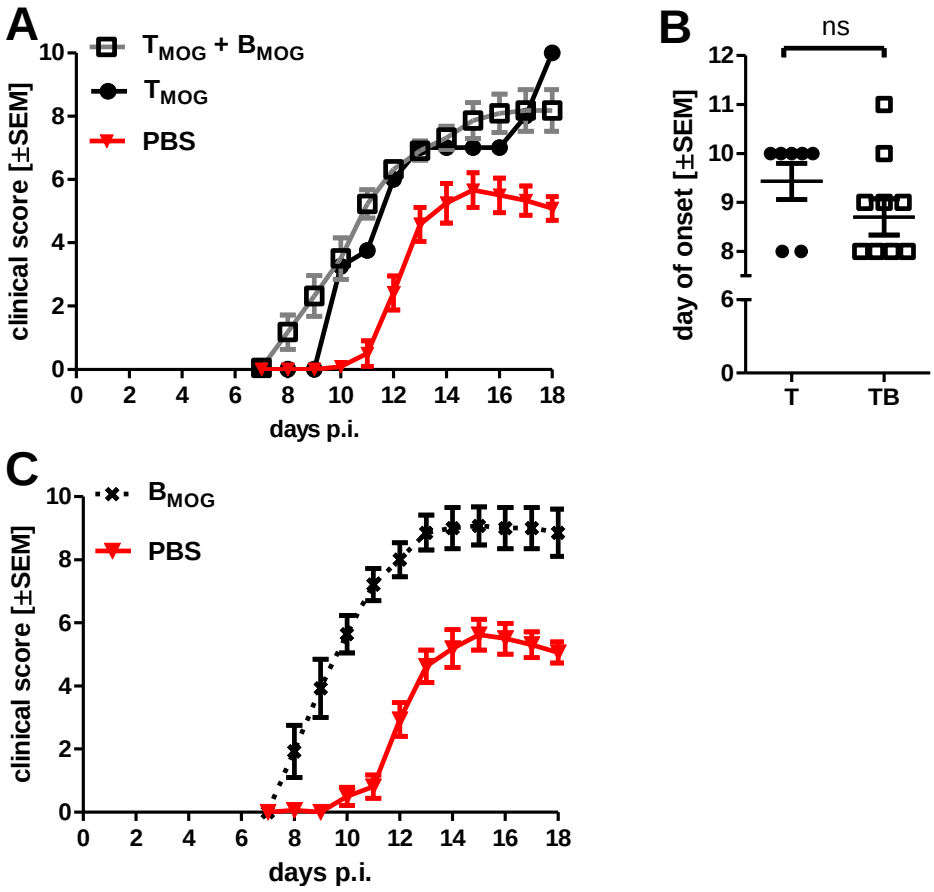

Figure 7: Clinical outcome of MOG-specific T and/or MOG-specific B cell transfer into WT mice and immunization with MOG_{p35-55}. MOG-specific T and B cells were isolated from respective donor mice and i.v. transferred into WT mice followed by immunization with MOG_{p35-55} two days later. **(A)** EAE disease course of T and B cell (grey line), T cell (black line) or PBS-supplemented (red line) mice. **(B)** Day of onset of each animal per T or TB group. Shown are two pooled experiments with each 8 (T group), 10 (TB group) and 6 (PBS) animals. **(C)** EAE disease course of B cell (black broken line) and PBS (red line) supplemented mice. Shown is one experiment with 7 mice per group. Clinical graphs show mean ± SEM. Non-significant differences between means are indicated: ns p> 0,05, Mann-Whitney nonparametric *t* test.

Control PBS-injected WT mice started to develop first clinical signs of EAE at about day 10-11 post immunization and peak disease severity on day 15 followed by a partial recovery (Fig. 7 A+C, red line). The transfer of either MOG-specific T cells (Fig. 7 A, black line), MOG-specific B cells (Fig. 7 C, black broken line) or both together (Fig. 7 A, grey line) accelerated the disease onset and also exacerbated disease severity as almost all animals had to be sacrified during the experiment. Though the transfer of MOG-specific T cells and MOG-specific B cells alone each had an effect on the day of disease onset, the simultaneous transfer of both cell types could just slightly, but not significantly, promote the clinical outcome as depicted in direct comparison of the day of onset (Fig. 7 B). We assumed that the contribution of endogenous T and B cells to disease severity make it difficult to further increase the clinical outcome. To overcome this problem, we copied the

model to OT-II mice as hosts, as they harbor >90% of OVA-specific T cells enabling us to minimize endogenous T cell effects as we know from in vitro experiments, that OVA-specific T cells do not respond to MOG antigens (Fig. 6 A left+ middle). To minimize endogenous T and B cell effects, we crossed OT-II mice to NP mice (termed OT-II x NP), resulting in host mice mainly harboring OVA-specific T cells and NP-specific B cells.

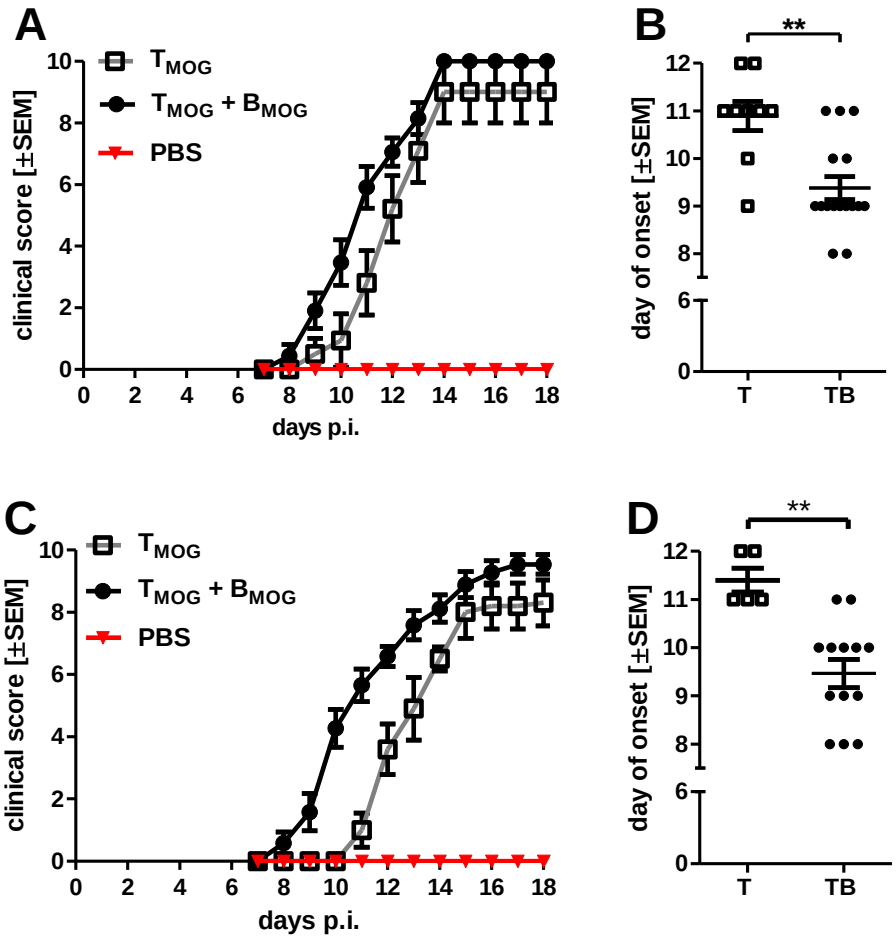

Figure 8: MOG-specific B cells accelerate the onset of EAE in OT-II and OT-II x NP mice. MOG-specific T and B cells were isolated from respective donor mice and i.v. transferred into OT-II **(A,B)** or OT-II x NP **(C,D)** mice followed by immunization with MOG_{p35-55} two days later. **(A)** EAE disease course of T (grey line), TB (black line) or PBS supplemented (red line) OT-II mice. **(B)** Day of onset of each animal per T or TB group. Shown are four pooled experiments with 14 (T group), 16 (TB group) and 6 (PBS) animals. **(C)** EAE disease course of T (grey line), TB (black line) or PBS supplemented (red line) OT-II x NP mice. **(D)** Day of onset of each animal per T or TB group. Shown are three pooled experiments with each 14 (T group), 15 (TB group) and 3 (PBS) animals. Clinical graphs show mean ± SEM, healthy mice were not included in the analysis. Significant differences between means are indicated: **p< 0,01, Mann-Whitney nonparametric *t* test.

Both, OT-II and OT-II x NP mice were not susceptible for EAE induction without previous cell transfer (Fig. 8 A+C red line). However, the resistance could be overcome by the transfer of MOG-specific T cells (Fig. 8 A+C grey line) or the

simultaneous transfer of MOG-specific T and B cells (Fig. 8 A+C black line). In addition, the disease onset could be significantly accelerated by the simultaneous transfer of MOG-specific T and B cells in OT-II (Fig. 8 B, Table 4) and in OT-II x NP mice (Fig. 8 D, Table 4), leading to the conclusion that MOG-specific B cells had a disease promoting effect in this experimental setting. Furthermore, the additional transfer of MOG-specific B cells increased the incidence for EAE (in OT-II: T: 61.5%, TB: 100%; in OT-II x NP: T: 35.7%, TB: 86.7%) as summarized in Table 4. Nevertheless, the disease severity was not increased by the additional transfer of MOG-specific B cells, because as soon as T cell-injected mice developed first clinical signs, the clinical progress was similar to that observed in mice receiving T and B cells. Furthermore, cell transfer followed by active immunization always resulted in sacrifice of the animals due to a clinical score >7, thus making it impossible to study the recovery phase. Preliminary data also reveal the same effect of MOG-specific B cells in rrMOG-induced EAE (data not shown).

Table 4: Influence of cell transfer into OT-II and OT-II x NP mice on EAE susceptibility

Host	Cell transfer	Mean day of onset (±SEM)	Incidence
OT-II	T cells	10.9 ± 0.3	61,5%
	T+B cells	9.4 ± 0.24	100%
OT-II x NP	T cells	11.4 ± 0.24	35,7%
	T+B cells	9.5 ± 0.3	86,7%

Although several publications suggest a dispensable role for B cells in MOG peptide-induced EAE, our established OT-II EAE model as well as the OT-II x NP EAE model reveal an effect of MOG-specific B cells after MOG_{p35-55} immunization. As we observed a disease promoting effect reflected by an earlier disease onset and a higher incidence in both models, we rather suggest a pro-inflammatory role for the transferred B cells than a role for B cells as regulatory cells. We now wanted to clarify the underlying mechanisms utilized by B cells to promote the development of EAE.

Most of the experiments described in the PhD-thesis were performed in OT-II mice and critical findings were repeated in OT-II x NP mice as the B cell-mediated effect was more pronounced in this model. For our further analysis, we always compared

the phenotype of T cells injected alone (in graphs always depicted as “T”) with the T cells which were injected together with MOG-specific B cells (in graphs always depicted as “TB”).

3.2. Influence of MOG-specific B cells during the activation phase of EAE

In contrast to other professional antigen-presenting cells like dendritic cells or macrophages, B cells express an antigen-specific B cell receptor on their surface enabling them to capture even low amounts of antigen, process it and present it bound on MHC-II molecules to $CD4^+$ T cells. Therefore, several publications suggest a role for B cells as antigen-presenting cells during EAE [78, 79, 139]. By presenting antigen to T cells, B cells can induce T cell proliferation, activation or their differentiation into a helper T cell direction, which is crucial for the development of EAE. In active EAE, myelin-specific T cells get activated within the first 4-5 days post disease induction in secondary lymphoid organs. According to the side of immunization, different lymph nodes arise as draining lymph nodes where T cell-priming predominantly occurs. The subcutaneous immunization into the tail base determines the inguinal (ing LN) and the paraaortic lymph nodes (para LN) as draining lymph nodes, whereas the axillary (ax LN) and the cervical lymph nodes (cer LN) are non-draining lymph nodes. To clarify whether MOG-specific B cells exert their disease-promoting function within the first four days after immunization, we characterized the injected MOG-specific T cells in terms of proliferation, activation and differentiation within the first four days post immunization (schematically depicted in Fig. 9).

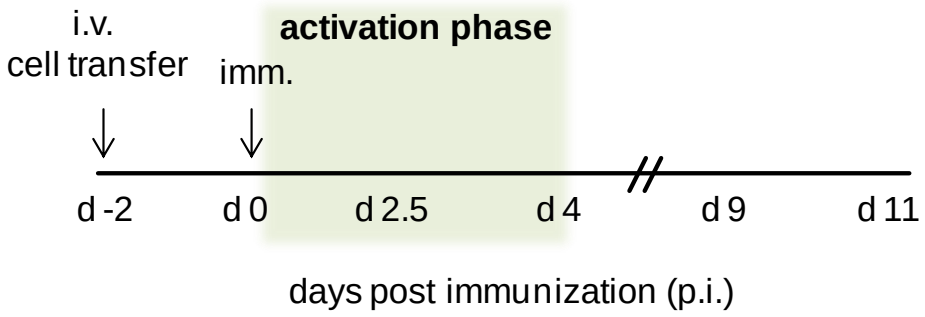

Figure 9: Schematic overview of experimental design with focus on the activation phase. MOG-specific T cells +/- MOG-specific B cells were i.v. transferred into OT-II recipients (d-2) and immunized with MOG_{p35-55} 2 days later (d0). Cells were analyzed either 2.5d or 4d p.i.

3.2.1. MOG-specific B cells do not enhance antigen-specific T cell proliferation *in vivo*

Although some publications suggest a dispensable role for B cells in enhancing T cell proliferation during EAE [139, 154], we wanted to investigate whether MOG-specific B cells enhance the proliferation of MOG-specific T cells in our experimental model. Freshly isolated RFP-expressing MOG-specific T cells were labeled with CFSE and injected either alone or together with MOG-specific B cells in OT-II recipients followed by immunization with MOG_{p35-55} two days later. The CFSE dilution of the injected MOG-specific T cells was analyzed ex vivo 2d, 2.5d and 4d post immunization by flow cytometry. Both, T cells injected alone or together with MOG-specific B cells started to proliferate at about 48h to 60h post immunization in the draining lymph nodes (Fig 10 A+B) and displayed similar proliferative responses during the subsequent time points in the draining lymph nodes, non-draining lymph nodes and in the spleen (Fig. 10 B). As it is possible that strongly proliferated cells were intangible with this analysis because they have already left the lymph node, we additionally determined the number of transferred T cells in the different lymph nodes, in the spleen and in the blood, as well as the frequency of RFP^+ $CD4^+$ T cells among the total $CD4^+$ T cell pool four days post immunization (Fig. 10 C+D). At this time point we could not detect any differences in the cell number, neither in the secondary lymphoid organs, nor in the blood (Fig. 10 C), which was consistent with the frequency of RFP^+ T cells among total $CD4^+$ T cells (Fig. 10 D).

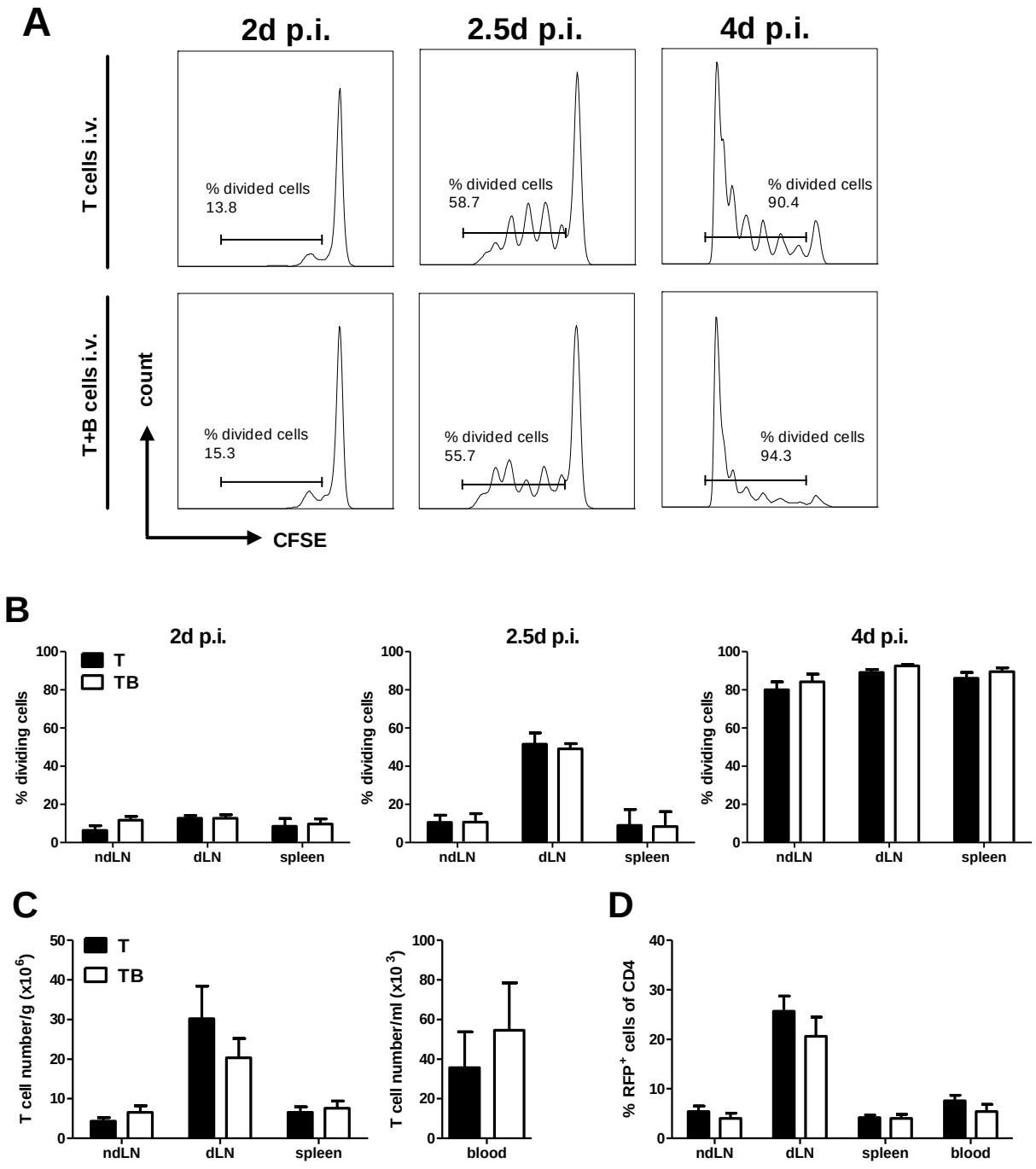

Figure 10: MOG-specific B cells do not enhance T cell proliferation *in vivo*. (**A+B)** CFSE-labeled MOG-specific T cells were ex vivo analyzed for CFSE dilution via flow cytometry **(A)** representative flow cytometry histogram plots of T cell CFSE dilution analyzed from the draining lymph node 2, 2.5 and 4 days p.i. either injected alone (upper row) or together with MOG-specific B cells (lower row). **(B)** Quantification of CFSE dilution of T cells analyzed from different lymph nodes and spleen of three pooled independent experiments, determination of % divided cells was performed like depicted in A. **(C)** absolute numbers of transferred MOG-specific T cells were determined like described in chapter 2.2.8 for lymph nodes, spleen and blood 4d p.i. **(D)** Frequency of RFP^+ $CD4^+$ among total $CD4^+$ was determined with flow cytometry 4d p.i.. Data represent >4 animals per group. Data are depicted as mean ± SEM.

3.2.2. MOG-specific B cells do not enhance antigen-specific T cell activation *in vivo*

Activated T cells can be discriminated form naïve T cells by their expression of different surface molecules. Upon antigen encounter within the lymph node, T cells downregulate their CD62L expression enabling them to leave the lymph node and migrate into the blood. CD69 is one of the earliest surface markers which is upregulated by T cells following activation. CD44 is an adhesion molecule and one characteristic marker of antigen-experienced T cells. In order to test whether the *in vivo* interaction of MOG-specific T and B cells increase the frequency of activated T cells, we stained them with fluorescently labeled antibodies against CD69, CD62L and CD44 and analyzed the percentage of positive cells with flow cytometry four days p.i..

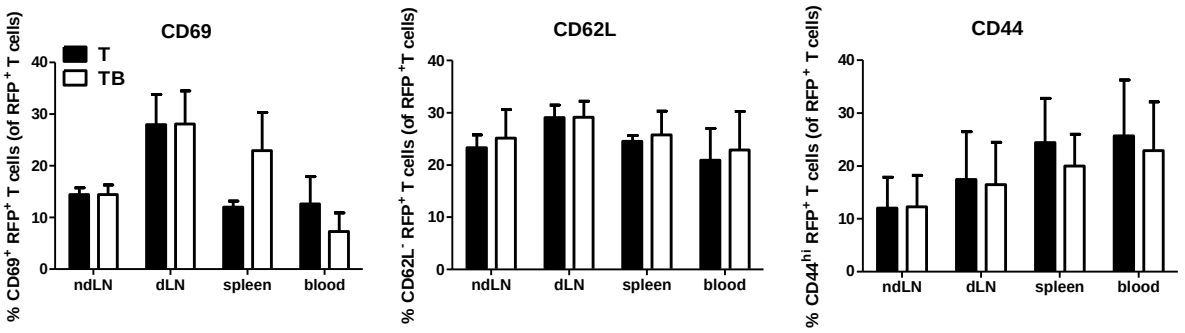

Figure 11: MOG-specific B cells do not influence T cell activation *in vivo*. Single cell suspensions of different lymph nodes, spleen and blood of mice were stained against CD69, CD62L and CD44 4d p.i.. Injected MOG-specific T cells were identified as $CD4^+$ RFP^+. Figure depicts the percentage of positive (in case of CD62L: negative) cells among the $CD4^+$ RFP^+ cell pool. Data represent two independent experiments with 4 animals per group. Data are depicted as mean ± SEM.

The presence of MOG-specific B cells did not increase the frequency of $CD62L^-$ RFP^+ T cells (Fig. 11 middle) or of $CD44^{hi}$ RFP^+ T cells (Fig. 11 right) within the different lymph nodes, the spleen or the blood. In contrast, there was a slight increase of $CD69^+$ T cells when injected together with MOG-specific B cells within the spleen (Fig. 11 left), but not in the lymph nodes or in the blood and it is questionable whether this difference accounts for the accelerated disease onset. Nevertheless, in comparison to activation marker expression of T cells before transfer (Fig. 4), the cells became activated upon EAE induction within the first four days p.i..

3.2.3. MOG-specific B cells do not promote early antigen-specific T cell differentiation *in vivo*

Upon antigen encounter within a pro-inflammatory milieu, naïve $CD4^+$ T cells differentiate into helper T cells mainly characterized by their produced effector cytokines. TH1 and TH17 T cells are the critical helper T cell subsets in EAE. B cells have been shown to amplify T helper cell subsets in EAE and thus, contribute to disease pathogenesis [56, 78, 79, 139]. We therefore wanted to investigate whether the additional transfer of MOG-specific B cells influence the cytokine production of the transferred T cells in our experimental model. For this purpose, we sorted the transferred MOG-specific T cells according to their RFP and CD4 expression from the draining lymph nodes 2.5 days p.i. or from draining lymph nodes and spleen 4 days p.i. and determined the mRNA expression level of IFNγ, IL-17A and GM-CSF via RT-PCR. Sorted RFP^+CD4^+ MOG-specific T cells from the draining lymph nodes of T and B cell-injected mice did not show an increased IFNγ and IL-17A expression 2.5 days p.i. (Fig. 12 A). On that time point, GM-CSF mRNA was not detectable in both groups. In addition, mRNA expression of IFNγ, IL-17A and GM-CSF was not statistically different 4 days p.i. (Fig. 12 B). Nevertheless, in comparison to mRNA expression of T cells before transfer (naïve T cells, grey bars), *in vivo* activated MOG-specific T cells showed an upregulation of IFNγ, IL-17A and GM-CSF within the first four days after immunization (Fig. 12 B).

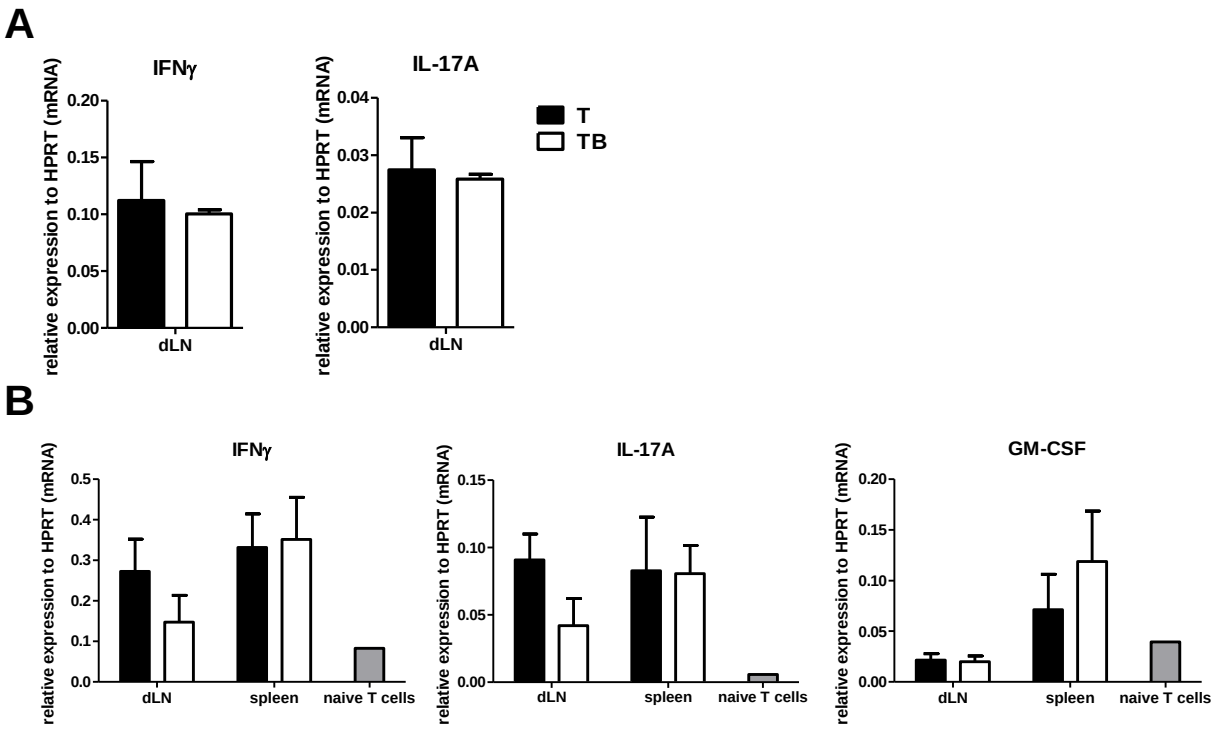

Figure 12: MOG-specific B cells do not increase the expression of TH1 and TH17 specific cytokines on mRNA level. Cytokine expression (mRNA) of sorted RFP^+CD4^+ T cells 2.5d p.i. **(A)** or 4d p.i. **(B)** or from RFP^+CD4^+ T cells before transfer (grey bar). Graphs show the expression of respective mRNA relative to the endogenous housekeeping gene hypoxanthine phosphoribosyltransferase 1 (HPRT). Data in (A) and (B) represent 3 and 4 animals per group, respectively. Data are depicted as mean ± SEM.

To further evaluate whether MOG-specific B cells affected T cell differentiation on the protein level, we restimulated total lymph node cells and splenocytes isolated from T cell or T and B cell-injected OT-II mice 4d p.i. *in vitro* with increasing MOG_{p35-55} antigen concentrations. We assessed the amounts of secreted IFNγ, IL-17A and GM-CSF after 72h in the supernatant via ELISA. As the frequency of RFP^+ MOG-specific T cells was similar between the two groups at that time point (Fig. 10 D), we assumed that an observed effect was due to an increased cytokine production or an increased number of differentiated T cells, but not due to a higher number of MOG-specific T cells in total. Total lymph node cells and splenocytes from T and B cell-injected mice did not show an enhanced reactivity in response to MOG_{p35-55} in comparison to restimulated cells from T cell-injected mice. They neither showed an enhanced sensitivity to lower antigen concentrations, nor a higher production of IFNγ (Fig. 13 A), IL-17A (Fig. 13 B) or GM-CSF (Fig. 13 C) at the same antigen concentration.

Therefore, we exclude a critical role for B cells in enhancing T cell proliferation, activation and differentiation within the activation phase of EAE.

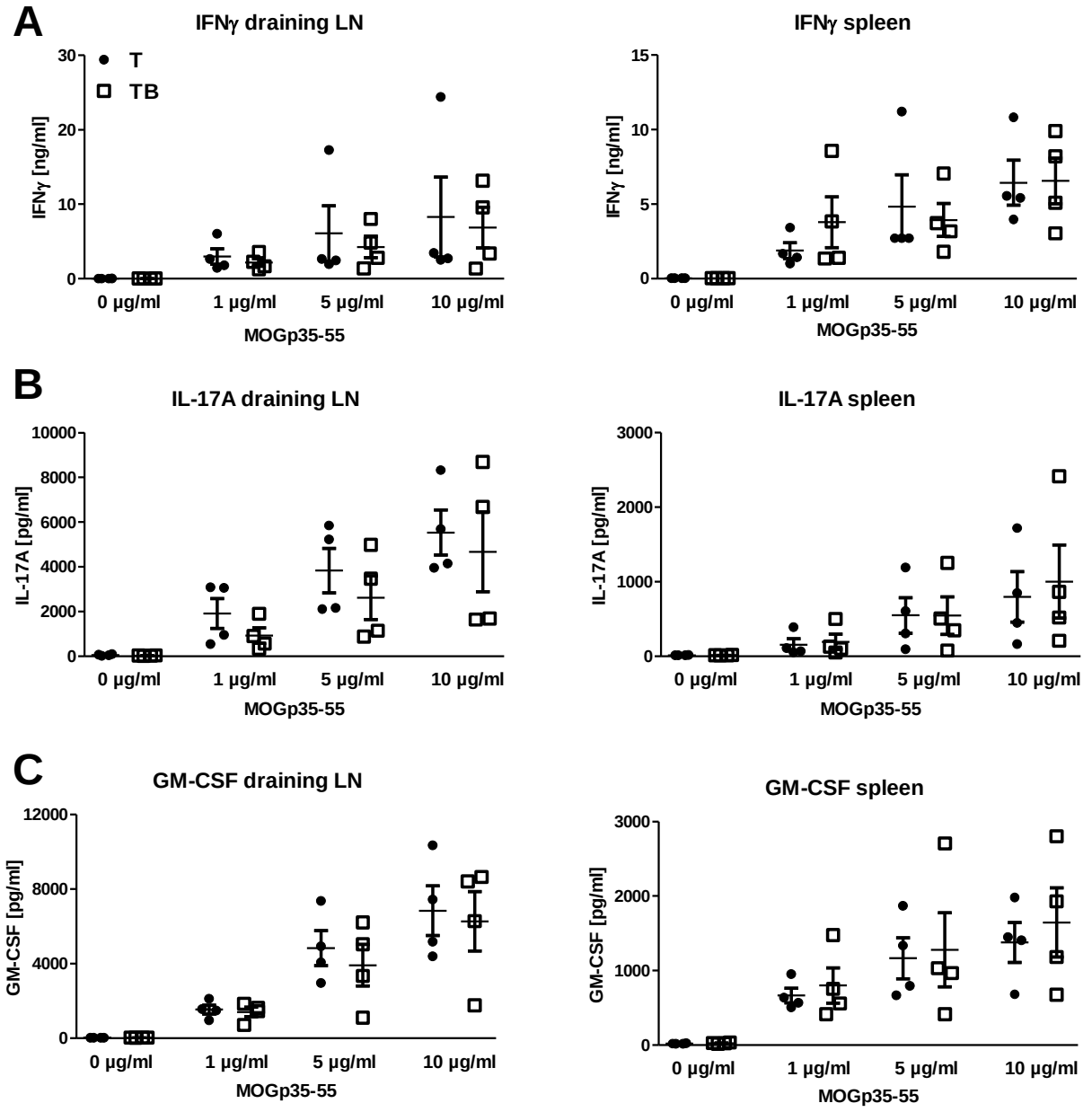

Figure 13: MOG-specific B cells do not enhance reactivity of T cells to MOG_{p35-55}. Total draining lymph node cells and splenocytes were isolated 4d p.i. from T cell (black circles) or T and B cell (white quad) injected mice and restimulated in vitro with increasing MOG_{p35-55} concentrations for 72h. Supernatant was collected and the amount of IFNγ **(A)**, IL-17A **(B)** and GM-CSF **(C)** was determined by ELISA. Each symbol represents an individual mouse out of two independent experiments, with the bar indicating the mean ± SEM.

3.3. Characterization of T cell behavior at the onset of EAE

As we could not detect any obvious differences in the T cell activation, in their proliferation and in their T helper cell differentiation during the activation phase of actively induced EAE, we assumed that the MOG-specific B cells exert their pro-inflammatory functions at later time points. Hence, we characterized the transferred T cells at day 9 p.i., the disease onset of the T and B cell-injected OT-II mice (as schematically depicted in Fig. 14). We were interested whether B cells might affect T cell activation or the peripheral T cell numbers shortly before the appearance of first clinical signs. Furthermore, as it is suggested that MOG-specific B cells enhance T cell differentiation, we also investigated whether the presence of MOG-specific B cells influence MOG-specific T cell differentiation at later time points. Moreover, we were intending to uncover the reason why T and B cell-injected mice develop EAE earlier than T cell-supplemented mice. Therefore, we focused on early events in the CNS (before appearance of clinical signs) to figure out whether B cells play a critical role in T cell reactivation within the CNS or whether they promote T cell infiltration by influencing their migration behavior.

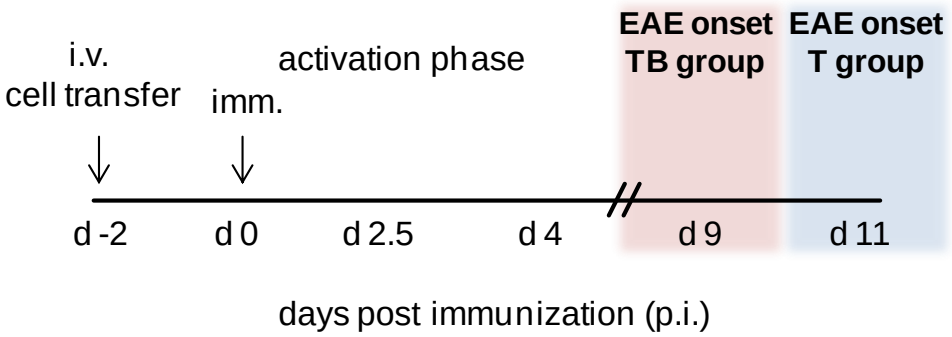

Figure 14: Schematic overview of experimental design with focus on the onset of EAE. MOG-specific T cells +/- MOG-specific B cells were i.v. transferred into OT-II recipients (d-2) and immunized with MOG_{p35-55} 2 days later (d0). Cells were analyzed 9d p.i. (onset of TB group) or d11 (onset of T group).

3.3.1. MOG-specific B cells do not influence antigen-specific T cell number and activation before onset

As we could not detect any difference in the T cell phenotype within the activation phase (2-4d p.i.), we wanted to investigate whether this observation changed during the progress of the pre-clinical phase, precisely on the day of EAE onset. We were interested whether the T cell phenotype of healthy animals with extravasated T cells (shortly before appearance of clinical symptoms) differs from these of animals without extravasated T cells. In order to determine the presence of extravasated T cells, my colleague Tanja Litke performed two-photon intravital imaging of the spinal cord. With this technique it is possible to visualize the fluorescently labeled T cells, both extravasated or intraluminal within the leptomeningeal vessels of the spinal cord. Starting from day 8 p.i., we analyzed 12 animals in total (6xT, 6xTB) for the following 2 days. During this time, 4 animals of the TB group had extravasated T cells without clinical manifestation of disease and one animal with a clinical score. In contrast, no animal of the T group had a clinical score and just one had extravasated T cells (Fig. 15 A). For further flow cytometry analysis, the RFP^+ cell number within the CNS and peripheral lymphoid organs of T cell-injected animals without extravasated T cells and T and B cell-injected animals with extravasated T cells was compared. We could detect a difference in cell number within the CNS (Fig. 15 B), but this was neither reflected in the blood, nor in different non-draining (ax: axillary LN, cer: cervical LN), draining lymph nodes (ing: inguinal LN, para: paraaortic LN) nor in the spleen (Fig. 15 B). In line with this, also the frequency of RFP^+ T cells among the $CD4^+$ T cell pool was not different between the two groups (Fig. 15 C). We could further not detect a striking difference in the frequency of $CD69^+$ RFP^+ T cells, $CD62L^-$ RFP^+ T cells or $CD44^{hi}$ RFP^+ T cells between the two groups, neither in the blood, nor in the different lymph nodes or in the spleen (Fig. 15 D). From these observations, we conclude that MOG-specific B cells did not amplify the peripheral MOG-specific T cells or increase the frequency of activated T cells.

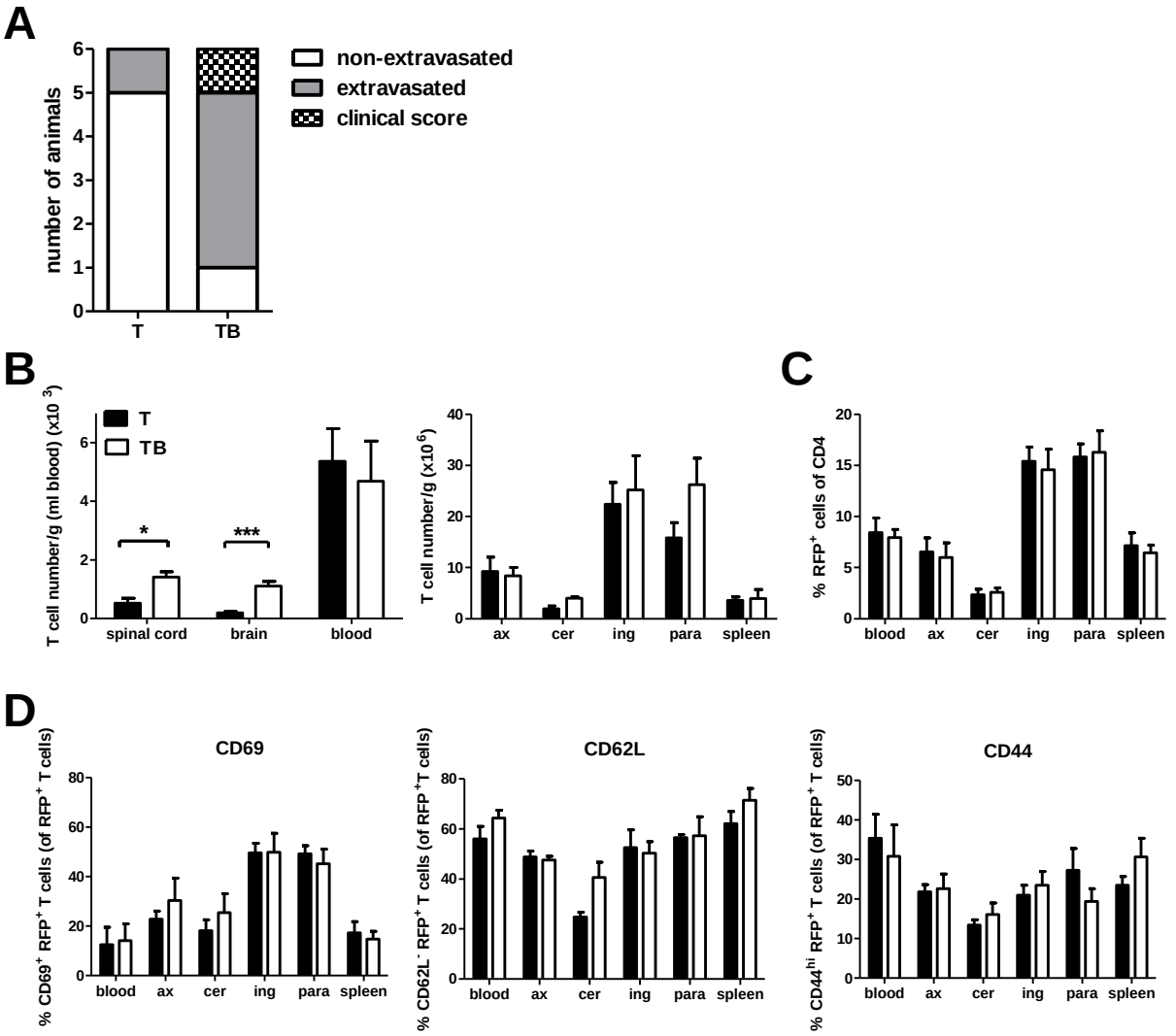

Figure 15: MOG-specific B cells do not influence T cell number or activation at the onset of the disease. Injected OT-II mice were evaluated for the presence of infiltrated RFP^+ T cells at the onset of EAE by two-photon microscopy and further analyzed with flow cytometry 8-10d p.i.. **(A)** Number of animals with non-extravasated (white), extravasated (grey) T cells or clinical score (dotted). **(B-D)** For analysis, T cell-injected animals without extravasated cells (T, black bars) were compared to T and B cell-injected animals with extravasated cells (TB, white bars) **(B)** Number of transferred MOG-specific T cells was determined like described in chapter 2.2.8. for spinal cord, brain, blood, lymph nodes and spleen **(C)** Frequency of RFP^+ $CD4^+$ among total $CD4^+$ was determined with flow cytometry. **(D)** Flow cytometry analysis of CD69, CD62L and CD44 expression of $CD4^+$ RFP^+ T cells. Data represent >4 animals per group and are depicted as mean ± SEM, Significant differences are depicted: *p<0.05, ***p< 0.001, unpaired students *t* test

3.3.2. MOG-specific B cells do not promote T cell differentiation before disease onset

We know from our investigations concerning the cytokine expression of T cells within the activation phase that B cells are dispensable for the initial differentiation of naïve T cells into either TH1 or TH17 cells. Nevertheless, it is possible that they are crucial during the later time points as also suggested by other studies [56, 139]. In order to assess whether MOG-specific B cells increase pro-inflammatory cytokine production of the transferred MOG-specific T cells at later time points, we sorted them shortly before the onset of EAE according to their simultaneous RFP and CD4 expression from peripheral lymphoid organs and from the blood and performed RT-PCR. To asses cytokine secretion on protein level, we restimulated total draining lymph node cells and splenocytes of either T cell-injected or T and B cell-injected OT-II mice 9 days p.i. *in vitro* with increasing MOG_{p35-55} concentrations for 72h and quantified IFNγ, IL-17A and GM-CSF from the supernatant by ELISA. We could not detect any difference in the expression of IFNγ, IL-17A and GM-CSF on mRNA level of T cells, which were injected alone (Fig. 16 A black bars) or together with MOG-specific B cells (Fig. 16 A white bars). As a lot of publications compare the cytokine expression between sick and healthy animals, we also included a third group, T cells sorted from T and B cell-injected mice with clinical score (Fig. 16 red bars), in our analysis. Interestingly, even MOG-specific T cells sorted from sick animals did not show an increased cytokine mRNA expression level. This was, at least in part, in contrast to the data we obtained by quantification of the different cytokines after restimulation of lymph node cells or splenocytes *in vitro*. Here, we could detect an increased amount of IFNγ of restimulated lymph node cells from sick T and B cell-injected mice (Fig. 16 B left, red circles) and an even more pronounced IL-17A amount of restimulated splenocytes (Fig. 16 C right). These contradictory results lead to the conclusion, that the expression of cytokines is not upregulated on the single cell level during EAE, but rather that the number of cytokine-expressing cells increased in total. Nevertheless, this could not account for the accelerated disease onset, because there was no difference when we compared cells isolated from healthy animals, neither in the amount of IFNγ (Fig. 16 B), IL-17A (Fig.16 C) nor of GM-CSF (Fig. 16 D). Lymph node cells and splenocytes restimulated from T and B cell-injected mice neither showed an increased sensitivity for the antigen, which would be reflected by an increased cytokine amount at a lower antigen concentration, nor a higher cytokine

production than restimulated cells from T cell-injected mice. From that we conclude, that MOG-specific B cells did not accelerate the onset of EAE by influencing T cell differentiation before onset of clinical EAE.

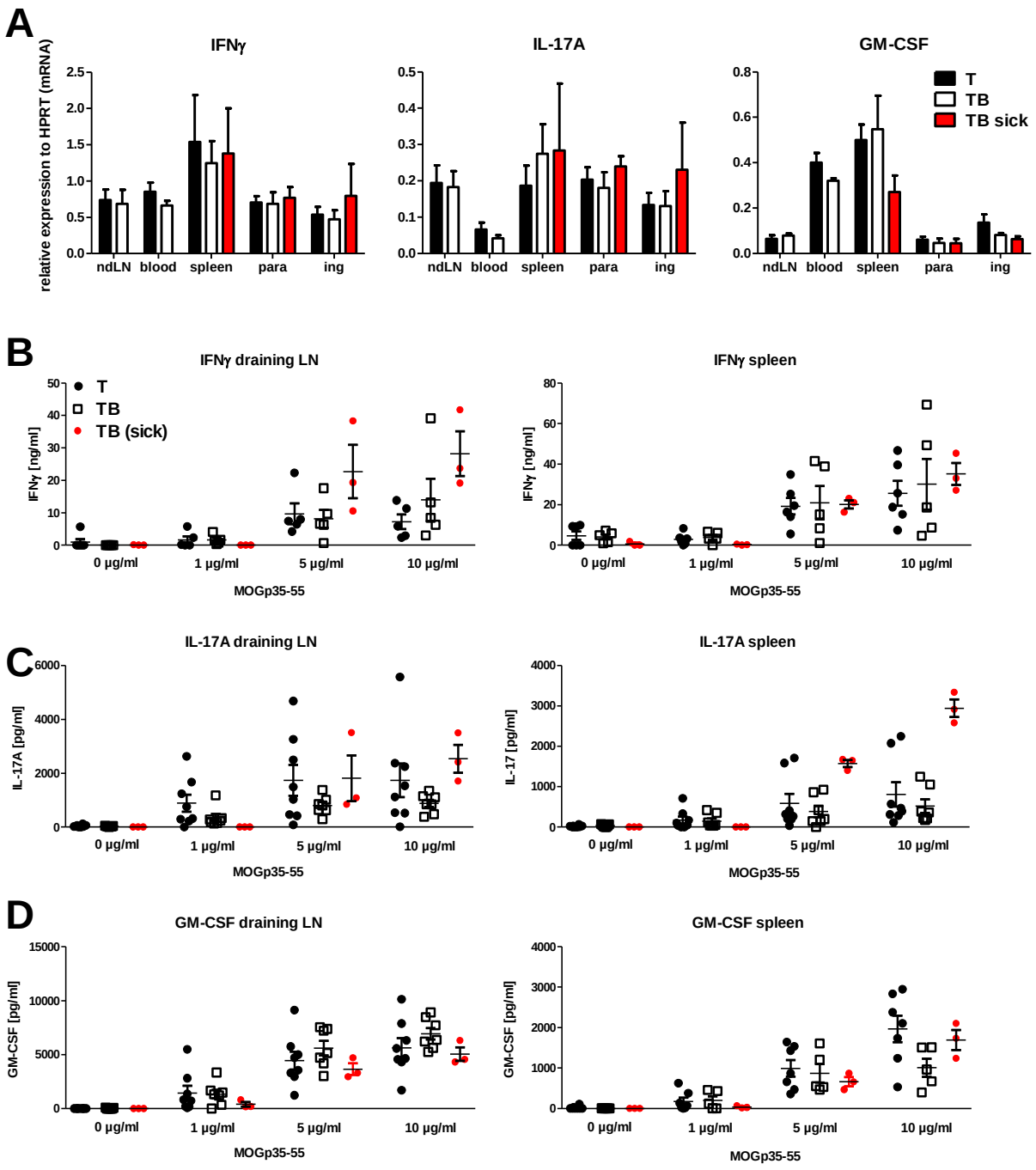

Figure 16: MOG-specific B cells do not trigger T cell differentiation before onset of EAE. **(A)** Cytokine expression (mRNA) of sorted RFP$^+$ cells isolated from blood and peripheral lymphoid organs of healthy T cell (black bars) or T and B cell (white bar) or sick (Score 4) T and B cell (red bars) injected mice 9 days p.i. Data represents n=6 for T and TB out of three independent experiments and n=3 for TB sick **(B-D)** total draining lymph node cells (left) or splenocytes (right) were taken from respective animals (healthy T inj.: black circle, healthy TB inj: white quad, sick TB inj.: red circle) 9d p.i. and restimulated *in vitro* with increasing MOG_{p35-55} concentrations for 72h. Supernatants were collected for **(B)** IFNγ, **(C)** IL-17A or **(D)** GM-CSF analysis by ELISA. Each symbol represents an individual mouse out of three independent experiments, with the bar indicating the mean ± SEM.

3.3.3. MOG-specific B cells promote the earlier infiltration of T cells into the CNS

We know from our clinical observations, that B cells accelerate the disease onset resulting in first clinical signs about 9.5 days post immunization (Table 4). Therefore, we wondered why the animals which received MOG-specific T and B cells develop EAE earlier than mice which just received MOG-specific T cells. There are two main possible mechanisms known which can account for an earlier disease onset. On the one hand, it is possible that B cells promote T cell infiltration into the spinal cord and thus, lead to an earlier disease initiation. On the other hand, it was reported that rather the activation status than the number of infiltrating T cells determine the clinical outcome, as antigen-unspecific T cells can infiltrate the CNS tissue in equal numbers than myelin-specific T cells, but due to the failed reactivation, they are not able to induce clinical symptoms [115]. Therefore, it is possible that B cells influence T cell activation during the reactivation phase within the CNS**.** As B cells are the most prevalent MHC-II$^+$ cell type within the naïve CNS, they cannot only act as APCs in the periphery, but it is suggested that they play a role in the initial steps of T cell reactivation and thus lead to the recruitment of further cells into the CNS [154].

To investigate whether an earlier T cell infiltration accounts for the earlier onset, we determined the number of spinal cord infiltrated RFP$^+$ cells 8.5 to 9 days post immunization of animals either injected with T cells and animals injected with T and B cells. To really figure out the situation before disease onset, we did not include animals with clinical symptoms in our analysis. To isolate the initial infiltrated T cells, which were located within the perivascular spaces, we did not implement the percoll gradient method, because this is more suitable for lymphocytes located within the CNS parenchyma. Instead, we adapted the spinal cord microvessel endothelial cell isolation method for that purpose.

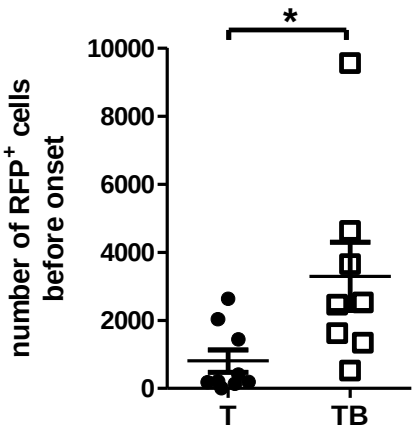

Figure 17: MOG-specific B cells promote the infiltration of MOG-specific T cells into the CNS. Number of RFP^+ cells isolated from spinal cord 9 days p.i.. Each symbol represents an individual mouse, with the bar indicating the mean ± SEM. (n= 9 for T and 8 for TB), Significant differences between means are indicated: *p< 0.05, unpaired students *t* test

Most of the animals receiving T and B cells, analyzed shortly before the onset of clinical EAE, had a higher number of infiltrated T cells in contrast to animals who just received MOG-specific T cells (Fig. 17). To further exclude a difference in the reactivation status of infiltrated cells, we sorted the RFP^+ T cells of both, T cell-injected and T and B cell-injected-mice and determined the expression of IFNγ, IL-17A and GM-CSF on mRNA level by RT-PCR. Here, it is important to mention, that we sorted the initial infiltrates of T and B cell-injected mice 9 days post immunization and of T cell-injected mice 11-12 days post immunization to ensure the comparison of comparable cell numbers. The initial infiltrates of mice receiving MOG-specific T cells alone and of those, who received both cell types, displayed comparable mRNA expression for IFNγ, IL-17A and GM-CSF (Fig. 18 A). Moreover, when we transferred GFP^+ MOG-specific B cells, we never detected them within the spinal cord of healthy animals 9 days p.i. (Fig. 18 B left) although they were present within the periphery (spleen). And even in sick animals, the presence of infiltrated GFP^+ B cells was very rare (Fig. 18 B right). From these observations we conclude that MOG-specific B cells promote the infiltration of T cells and are not crucial for the reactivation step within the spinal cord in our experimental setting. Furthermore, we can exclude a critical role for B cells in amplifying peripheral total T cell numbers or increasing the frequency of activated or differentiated T cells. Therefore, we were interested whether B cells influence T cell migration in our experimental setting.

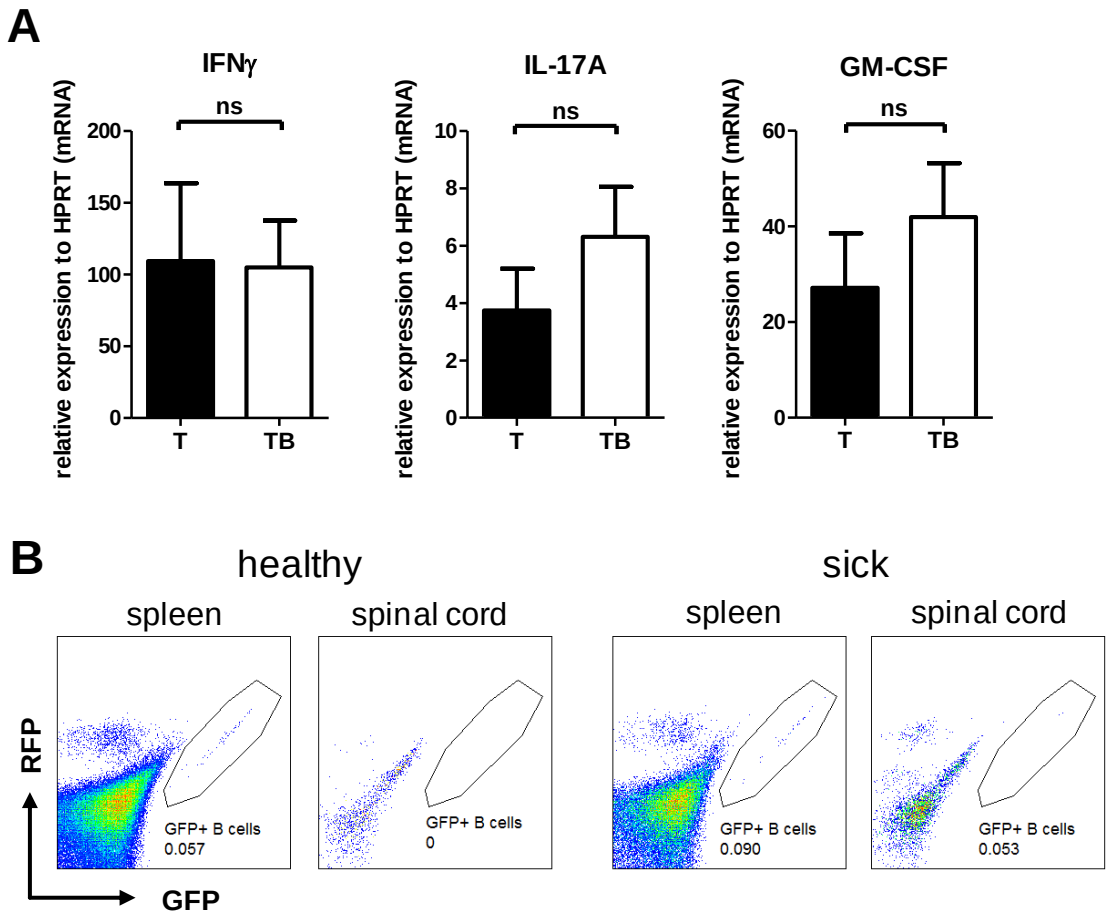

Figure 18: No difference in activation status of RFP$^+$ initial infiltrated T cells. **(A)** Cytokine expression (mRNA) of sorted RFP$^+$ cells isolated from spinal cord 11-12 days p.i. for T group and 9 days p.i. for TB group. **(B)** Frequency of GFP$^+$ B cells in spleen and spinal cord of an healthy animal (left) and a sick animal (Score 4,5) (right) 9d p.i.. Data in A represent three independent experiments with each 2 animals per group. Data are depicted as mean ± SEM. Non-significant differences are depicted: ns p>0.05, unpaired students *t* test

3.3.4. Effect of MOG-specific B cells on T cell migration into the CNS

Our previous results led us to suggest that the accelerated onset in the presence of MOG-specific B cells is rather due to an increased T cell infiltration (Fig. 17) than to an increased reactivation (Fig. 18). Therefore, we focused on mechanisms required for T cell migration.

3.3.4.1. MOG-specific T cells show an enhanced expression of CCR6 and even pronounced expression of CXCR4 in the presence of MOG-specific B cells

The interaction of chemokines and their respective receptors, expressed by T cells, is a critical step for T cell migration from the blood into various tissues. In the context of MS and EAE, several chemokine receptors have been found to be crucial for the infiltration into the CNS. Upon inflammation, TH1 cells upregulate the chemokine receptors CCR5 and CXCR3 and they were shown to be required for T cell migration into the CNS [118-122], whereas TH17 cells infiltrate the CNS in a CCR6-dependent manner [135]. Also T cells expressing homeostatic chemokine receptors like CXCR4 and CCR7 have been shown to be located within MS lesions, also suggesting a role for these receptors in CNS infiltration [120, 179]. Although it is not described yet for B cells, it is possible that the antigen-specific interaction of T and B cells lead to a kind of T cell imprinting. This is for example described for the interaction of dendritic cells from the payers patch with T cells resulting in an upregulation of integrins and chemokine receptors required for their homing into the gut [180]. To investigate whether the MOG-specific interaction of T and B cells results in a different chemokine receptor regulation than of T cells injected without B cell supplementation, we determined the chemokine receptor expression, precisely CCR7, CXCR3, CXCR4, CCR5 and CCR6 on mRNA level of initial CNS-infiltrated T cells. Like described in chapter 3.3.3., we sorted the initial RFP^+ T cells of T and B cell-injected animals 9d p.i. and these of T cell-injected animals 11-12d p.i. according to their RFP-expression.

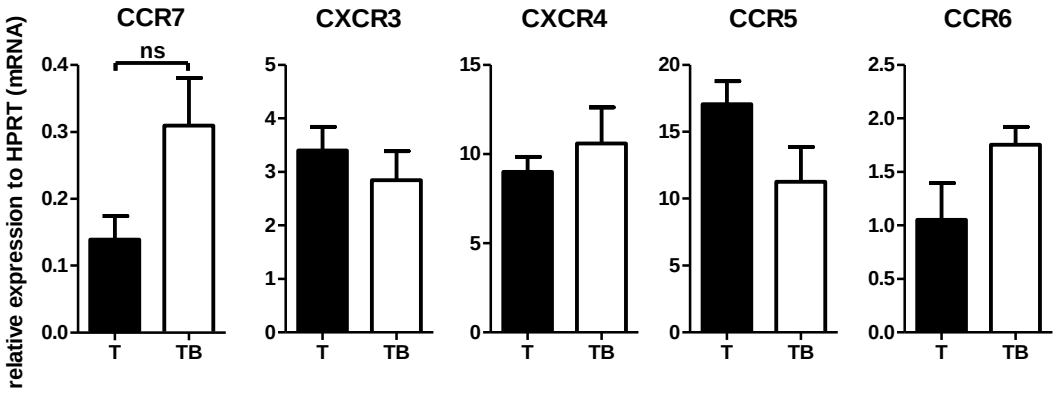

Figure 19: Initial infiltrated T cells utilize the same chemokine receptor for CNS infiltration. Chemokine receptor expression (mRNA) of sorted RFP$^+$ cells isolated from spinal cord 11-12 days p.i. for T group and 9 days p.i. for TB group. Data represent three independent experiments with each 2 animals per group and are depicted as mean ± SEM. Non-significant differences between means are indicated: ns p> 0.05, unpaired students *t* test

We could not detect a significant difference of the chemokine receptor expression by the initial infiltrates of T cell-injected mice (Fig. 19 black bars) and T and B cell-injected mice (Fig. 19 white bars). However, there was a tendency that T cells from T and B cell-injected mice showed a higher CCR6 (TH17 cells) and CCR7 expression (although statistically not significant), in contrast to T cell-injected alone, which showed a higher expression of CCR5 (TH1 cells). This is in line with the cytokine expression of the initial infiltrated T cells (Fig. 18) as the T cells from T and B cell-injected animals showed a higher IL-17A and GM-CSF expression (but not statistically different). We were interested whether this observation could be reflected by T cells from the periphery. For this purpose, we sorted the RFP$^+$ MOG-specific T cells either from T cell-injected or from T and B cell-injected mice from peripheral lymphoid organs and from blood 9d p.i. and performed chemokine receptor expression analysis by RT-PCR. In contrast to the initially infiltrated T cells from T and B cell-injected mice, T cells sorted from the periphery of the same group did not reveal an increased CCR7 expression, but a slight increase in CCR6 expression of T cells isolated from the spleen (Fig. 20). As CCR6$^+$ TH17 cells are known to be crucial for the initiation of EAE, it is likely that MOG-specific B cells promote EAE development by triggering either CCR6 upregulation or amplification of CCR6$^+$ T cells. But CCR6$^+$ T cells infiltrate the CNS through blood-cerebrospinal fluid barrier and therefore it is questionable that we did not detect an increased CCR6-expression of T cells isolated from the blood [135]. This observation has to be elaborated in detail to come to a definitive answer. Moreover, we could not detect a difference in

CXCR3 and CCR5 expression of sorted RFP$^+$ MOG-specific T cells between the two groups (Fig. 20). However, RFP$^+$ T cells sorted from spleen, blood and draining lymph nodes of T and B cell-injected mice significantly upregulated CXCR4 (Fig. 20). As the role of CXCR4 in lymphocyte migration into the CNS is controversial, this observation is difficult to interpret, especially because the initial infiltrated T cells did not show an increased CXCR4 expression (Fig. 19).

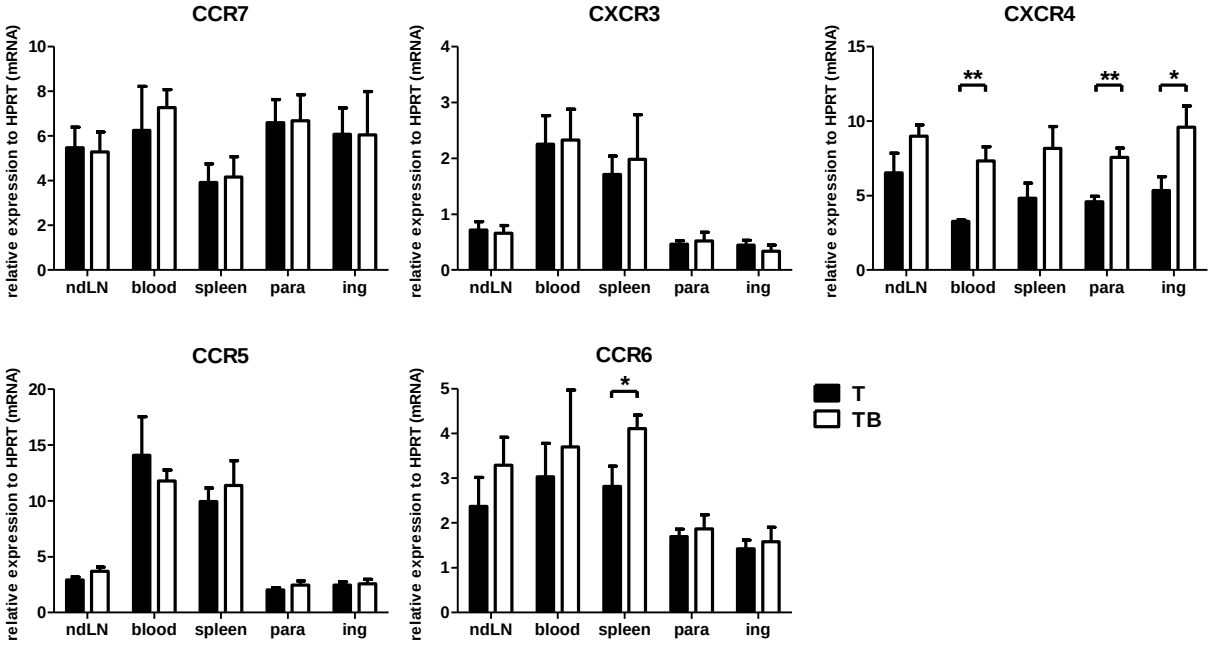

Figure 20: MOG-specific B cells influence CXCR4 expression of T cells but have no effect on the other chemokine receptors. Chemokine receptor expression (mRNA) of sorted RFP$^+$CD4$^+$ T cells from blood, non-draining lymph nodes, spleen and draining lymph nodes 9d p.i.. Data represent three independent experiments with each 2 animals per group. Significant differences are indicated: *p< 0.05 and **p< 0.01, unpaired students *t* test, shown are means ± SEM.

3.3.4.2. MOG-specific B cells do not influence adhesion molecule expression of T cells

Adhesion molecules like VLA-4 and LFA-1 expressed by T cells have been shown to be crucial for the initial interaction of T cells with endothelial cells of CNS microvessels as well as for their transmigration [99, 106]. Therefore, we investigated whether MOG-specific B cells trigger the upregulation of either LFA-1 or VLA-4 on T cells or whether they expand LFA-1^+ or VLA-4^+ MOG-specific T cells in our experimental setting. For expression analysis we sorted RFP^+CD4^+ T cells 9d p.i. from either T cell-injected mice (Fig. 21 A black bars) or T and B cells-injected mice (Fig. 21 A white bars) and determined integrin alpha 4 (alpha subunit of VLA-4) or integrin alpha L (alpha subunit of LFA-1) mRNA expression by RT-PCR. When we compared the mRNA expression of T cells sorted from lymph nodes, spleen and blood, we could neither detect a significant difference in integrin alpha 4 nor in integrin alpha L expression between the two groups (Fig. 21 A). As it is possible that the expression is not regulated on mRNA level, but that B cells rather amplify VLA-4- or LFA-1-positive T cells, we determined the number of positive cells within the blood and spleen between day 7 and day 9 p.i. (Fig. 21 B+C). The number of VLA-$4^+RFP^+CD4^+$ T cells increased over time in the blood as well as in the spleen (Fig. 21 B), but there was no difference in the T cell number of mice injected with T cells and of these injected with T and B cells. In accordance to that, we could not detect any difference in LFA-$1^+RFP^+CD4^+$ T cell number (Fig. 21 C). We therefore conclude that MOG-specific B cells do not influence T cell migration into the CNS by affecting their expression of chemokine receptors or integrins.

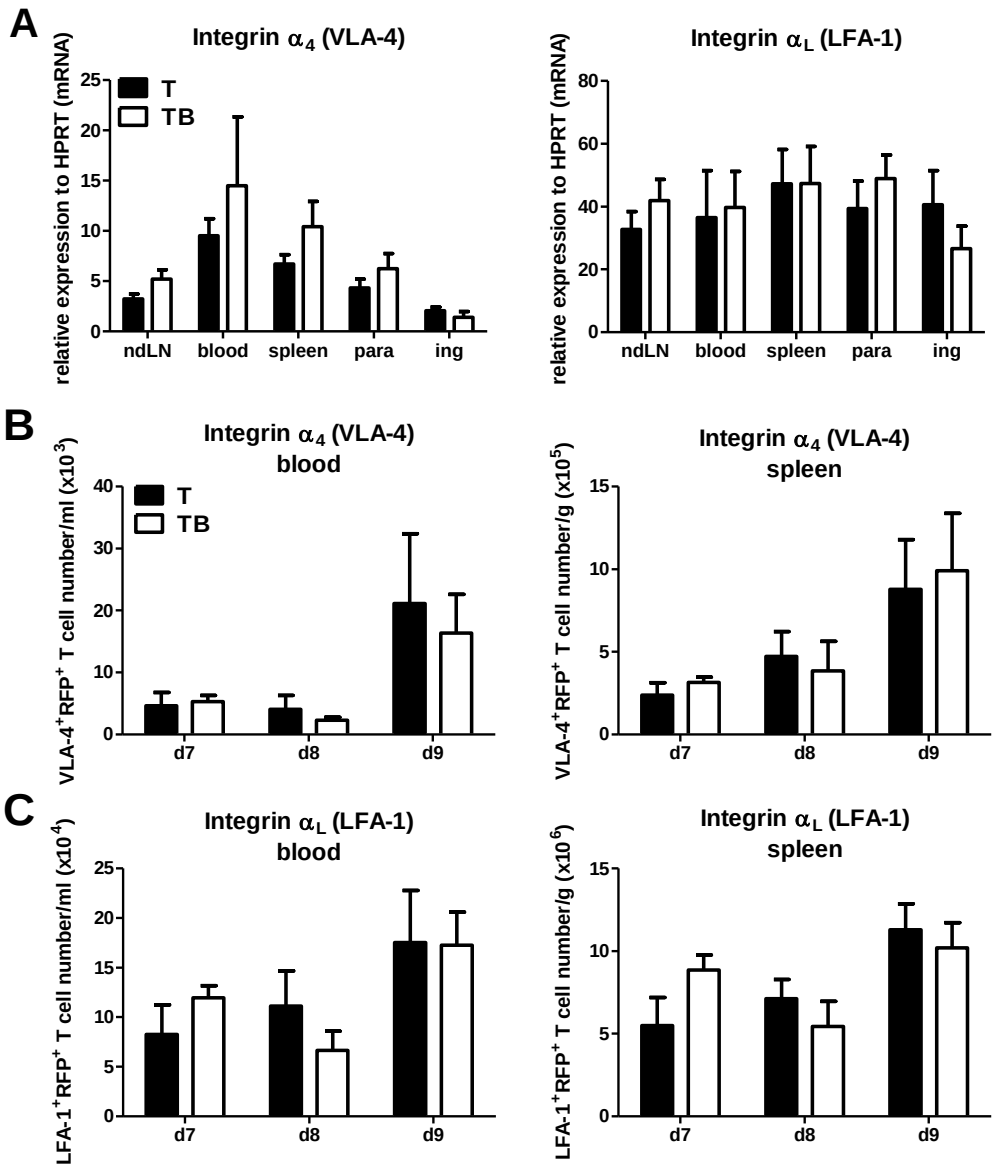

Figure 21: MOG-specific B cells do not influence integrin expression of T cells. **(A)** Integrin expression (mRNA) of sorted RFP^+CD4^+ T cells from blood, non-draining lymph nodes, spleen and draining lymph nodes 9d p.i. **(B)** Quantification of VLA-4^+ RFP^+CD4^+ T cells from the blood (left) and from the spleen (right) with flow cytometry on d7-d9 p.i. **(C)** Quantification of LFA-1^+ RFP^+CD4^+ T cells from the blood (left) and from the spleen (right) with flow cytometry on d7-d9 p.i. Data in A represent three independent experiments with each 2 animals per group. Data in B+C represent two independent experiments with at least 2 animals per group. Shown are mean ± SEM

3.4. MOG-specific B cells do not influence mobilization of myeloid cells

As we could not detect any differences in the T cell phenotype which would allow their earlier infiltration into the CNS, we determined whether MOG-specific B cells might influence other cell types responsible for the accelerated disease onset. Several publications suggest a crucial role for monocytes in disease initiation. EAE studies with $CCR2^{-/-}$ mice, the chemokine receptor predominantly expressed by inflammatory monocytes, reveal that the impaired CNS infiltration of monocytes is accompanied by a reduced number of infiltrating T cells [165, 167]. In addition, the increase of $Ly6C^{+}$ monocyte number in the blood was associated with an earlier EAE disease onset [181]. In concordance, it was shown that B cells can influence monocyte mobilization in acute myocardial infarction [182]. Therefore, we were interested whether monocyte mobilization is affected in our experimental setting. Monocytes mainly appear in the blood as two populations: inflammatory monocytes are $CD11b^{+}Ly6C^{+}CCR2^{+}$ whereas resident monocytes are $CD11b^{+}Ly6^{int}CX3CR1^{+}$ [4]. As these populations originate from a common precursor, the simultaneous expression of CCR2 and CX3CR1 is not unusual. To investigate whether the frequency of monocyte subpopulations is altered between T cell-injected and T and B cell-injected mice before EAE onset, we determined the number of the different monocyte populations in the blood, the spleen and the spinal cord on day 7 and 8 p.i. by flow cytometry. Different monocyte subpopulations were gated like depicted in Fig. 22 A. Seven days p.i. we detected a slight increase of total $CD11b^{+}Ly6C^{hi}$ cells, which was reflected in both, an increase of $CD11b^{+}Ly6C^{hi}CCR2^{+}$ and of $CD11b^{+}Ly6C^{hi}CCR2^{+}CX3CR1^{+}$ in the blood, but not in the spleen and spinal cord of T and B cell-injected mice (Fig. 22 B). One day later the picture completely changed with an increased number of $CD11b^{+}Ly6C^{hi}$ monocyte subpopulations in the blood of T cell-injected mice (Fig. 22 C left), but a tendency of an increased number of that subpopulations in the spinal cord of T and B cell-injected mice (Fig. 22 C right). However, as we could not detect a unique increase of the $CD11b^{+}Ly6C^{hi}$ monocyte subpopulations in the blood as described in the literature, we conclude that B cells do not influence monocyte mobilization in our experimental setting.

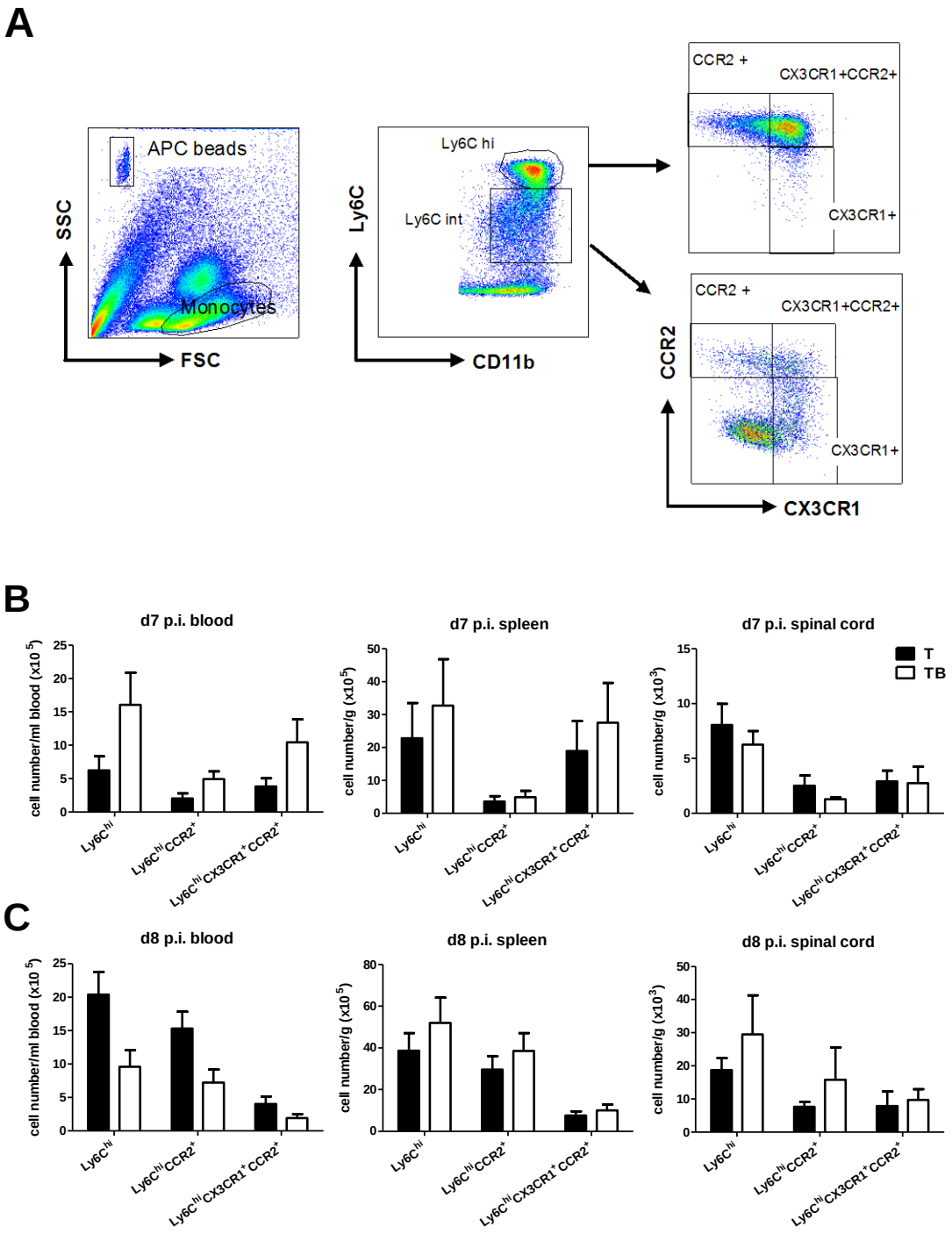

Figure 22: MOG-specific B cells do not influence monocyte mobilization. **(A)** Flow cytometry gating strategy for different monocyte subpopulations. **(B-C)** different monocyte subpopulations (all gated on CD11b) were quantified in blood, spleen and spinal cord of T cell-injected (black bars) and T and B cell injected mice (white bars) **(B)** 7d p.i. and **(C)** 8d p.i.. Data represents 2 independent experiments with each 2 animals per group. Shown are mean ± SEM

3.5. MOG-specific B cells do not mediate upregulation of adhesion molecules and chemokines by spinal cord microvessel endothelial cells

During EAE, endothelial cells of spinal cord microvessels have been shown to upregulate the expression of several chemokines and adhesion molecules required for lymphocyte attraction, adhesion and transmigration [112, 125, 126, 129]. Therefore, it is possible that B cells trigger chemokine or adhesion molecule upregulation, for example through soluble factors like cytokines, and thus promote lymphocyte infiltration into the CNS. In line with this, it was shown that B cells are the major source of IL-6 in secondary lymphoid tissues and that absence of IL-6 during EAE results in an impaired upregulation of ICAM-1 and VCAM-1 of CNS endothelial cells [183]. To investigate whether the presence of MOG-specific B cells promote the upregulation of adhesion molecules and chemokines before onset of EAE, we isolated spinal cord microvessels and sorted the endothelial cells according to their CD31 (PECAM-1) expression of T cell-injected and T and B cell-injected mice 8 days p.i.. We quantified their ICAM-1, VCAM-1, ALCAM, CXCL10, CXCL12, CCL5 mRNA expression by RT-PCR. To ensure that endothelial cells were isolated from mice at the same preclinical stage, we additionally determined the number of infiltrated RFP^+ cells and only endothelial cells with comparable low numbers of infiltrated cells (~30-60 cells) were used for gene expression analysis. Otherwise, we could not exclude that the presence of already infiltrated cells and their ongoing reactivation contributed to an increased expression of adhesion molecules and chemokines.

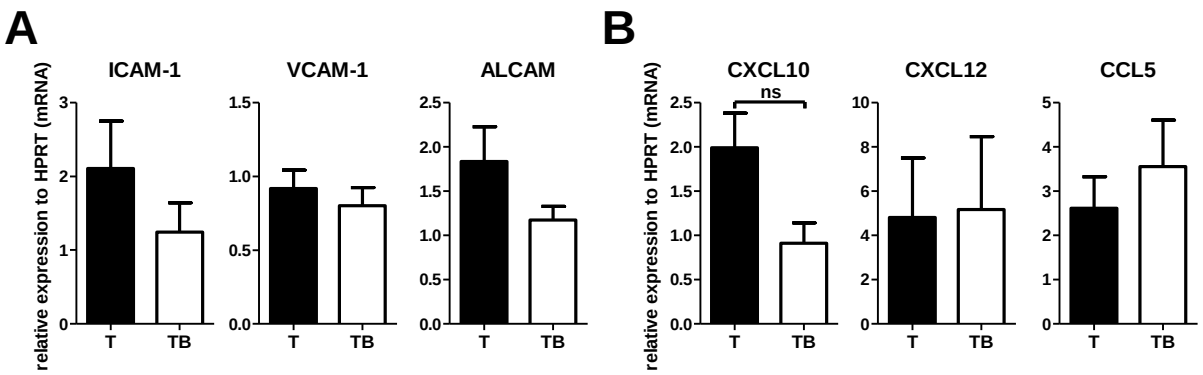

Figure 23: MOG-specific B cells do not trigger the upregulation of adhesion molecules and chemokines by spinal cord microvessel endothelial cells. Spinal cord microvessels were isolated from T cell-injected and T and B cell-injected OT-II mice with similar CNS infiltrated RFP^+ cell numbers and endothelial cells were sorted according to their CD31 expression 8d p.i.. **(A)** Adhesion molecules (ICAM-1, VCAM-1, ALCAM) and **(B)** chemokine (CXCL10, CXCL12, CCL5) (mRNA) expression analysis was performed by RT-PCR. Data represent 2 independent experiments with 4 animals per group. Data are depicted as mean ± SEM. Non-significant differences between means are indicated: ns p> 0.05, unpaired students *t* test

Although we observed an increased number of CNS infiltrated T cells upon additional transfer of MOG-specific B cells, these cells did not trigger the upregulation of adhesion molecules (Fig. 23 A) or chemokines (Fig. 23 B) by spinal cord microvessel endothelial cells. On the contrary, endothelial cells isolated from T cell-injected mice even showed an enhanced expression of ALCAM and of CXCL10 on mRNA level in contrast to T and B cell-injected mice. Therefore, it is unlikely that B cells promote T cell infiltration into the CNS by influencing microvessel endothelial cells.

3.6. The antigen-specific interaction of T and B cells determine the accelerated disease onset

As we could not detect any obvious or detectable difference in the T cell phenotype or an effect of B cells on other cell types like monocytes or spinal cord microvessel endothelial cells, we wanted to search for other mechanisms by which B cells contribute to the accelerated disease onset. We were interested whether the presence of activated, but not MOG-specific B cells is sufficient to accelerate the disease onset or whether the antigen-specific interaction of T and B cells determines the effect on EAE onset. For that purpose, we supplemented OT-II mice with different combinations of T and B cells and immunized them with NP-OVAL and MOG_{p35-55} (Table 5) and monitored their clinical outcome.

Table 5: Overview of clinical experiment

Cell transfer	Antigen
MOG-specific T cells	NP-OVAL + MOG_{p35-55}
MOG-specific T and B cells	NP-OVAL + MOG_{p35-55}
MOG-specific T cells and NP-specific B cells	NP-OVAL + MOG_{p35-55}

To investigate whether the presence of activated, but not MOG-specific B cells is sufficient to accelerate the onset of EAE, we transferred NP-specific B cells together with MOG-specific T cells into OT-II hosts and immunized them with a combination of NP-OVAL and MOG_{p35-55}. We know from *in vitro* studies that NP-specific B cells become activated by the NP-OVAL in the presence of OVA-specific T cells (endogenous cells) (Fig 6 B+C right). We compared the clinical outcome to either MOG-specific T cell-injected mice or to MOG-specific T and B cell-injected mice, both immunized with NP-OVAL and MOG_{p35-55}, but we did not expect an effect of NP-OVAL on the disease development in these groups. To proof that the NP-specific B cells became activated *in vivo*, we determined their IgG production against OVA, rrMOG and NP-OVAL and could show that they specifically produced anti-NP-OVAL IgG (Fig. 24 C).

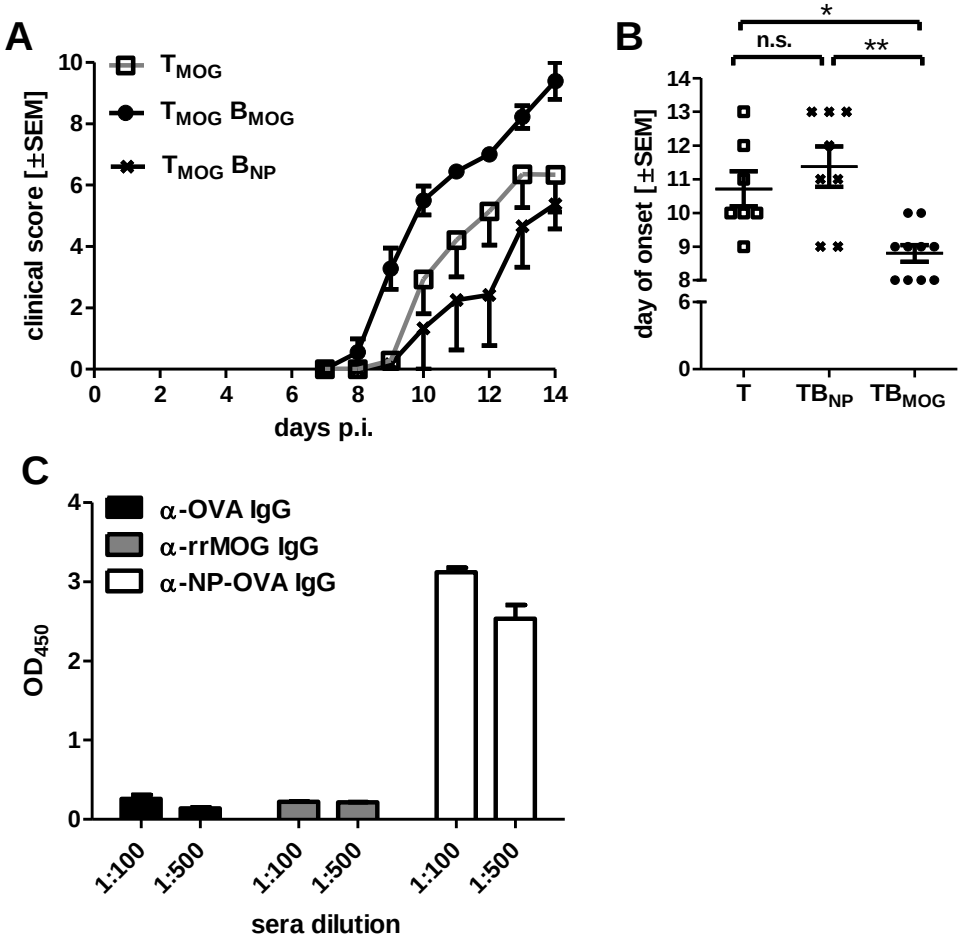

Figure 24: The antigen-specific interaction of T and B cells determine the clinical outcome of EAE. OT-II mice were injected either with MOG-specific T cells, MOG-specific T and B cells or MOG-specific T cells and NP-specific B cells followed by immunization with NP-OVAL and MOG_{p35-55}. **(A)** EAE disease course of OT-II injected mice **(B)** Day of onset of each animal per T or TB_{MOG} or TB_{NP} group. Shown are two pooled experiments with 9 animals per group. **(C)** Serum anti-OVA, anti-rrMOG and anti-NP-OVAL IgG titer of MOG-specific T and NP-specific B cell-injected OT-II mice obtained 10-12d p.i. from peripheral blood, pooled data from 5 animals. Data show mean ± SEM. Significant differences between means are indicated: ns $p>0.05$, *$p< 0.05$ **$p< 0.01$, 1way ANOVA

Whereas MOG-specific T and B cell-injected mice developed first clinical signs at about day 8.8 ± 0.24, the presence of *in vivo* activated NP-specific B cells had no effect on the onset compared to MOG-specific T cell-injected mice (TB_{NP}: day 11.38 ± 0.59, T: day 10.71 ± 0.52) (Fig. 24 A+B). We therefore assume, that the antigen-specific interaction of MOG-specific T and B cells rather determine the clinical outcome than the presence of activated MOG-unspecific B cells.

3.7. MHC-II deficiency of MOG-specific B cells do not exclusively affect their antigen-presenting capacity

We know from the previous clinical experiment that activated, so-called “bystander” B cells had no effect on the onset of EAE. Therefore, we were interested about the mechanism which is based on the antigen-specific interaction of T and B cells. To interfere with this mechanism, we crossed Th mice to MHC-II$^{-/-}$ mice to generate MHC-II$^{-/-}$ MOG-specific B cells with the final intention to use these B cells for adoptive transfer experiments. To ensure that MHC-II$^{-/-}$ MOG-specific B cells were really impaired in their antigen-presenting capacity, we first tested them in an *in vitro* proliferation assay. We cultured CFSE-labeled MOG-specific T cells either with MOG-specific B cells or with MHC-II$^{-/-}$ MOG-specific B cells in the presence of increasing concentrations of either MOG_{p35-55} or rrMOG and assessed MOG-specific T cell proliferation after 72h. In addition, we analyzed *in vitro* B cell proliferation to investigate whether the defective antigen-presentation also impaired B cell function. We observed that MHC-II$^{-/-}$ B cells were impaired in triggering T cell proliferation (Fig. 25 A). Moreover, MHC-II deficiency resulted in defective B cell response as their antigen-specific proliferation was also impaired (Fig. 25 B). As MHC-II deficiency not only resulted in their impaired antigen-presenting capacity, but also affected their cellular behavior, we wondered whether this also had consequences *in vivo*. A recent publication observed that MHC-II deficiency of B cells was accompanied by an impaired secretion of antibodies [139]. Therefore, we injected MOG-specific B cells or MHC-II$^{-/-}$ MOG-specific B cells into NP mice and immunized them with a high dose of rrMOG (40µg) and determined anti-rrMOG IgG levels 8d and 18d p.i.. We used NP mice recipients for that analysis as they are devoid of endogenous antibody-secreting MOG-specific B cells (Fig. 25 C lower row PBS ctrl). We detected substantial amounts of anti-rrMOG IgGs in serum of MOG-specific B cell-injected NP mice even after 8d p.i.. In contrast, MHC-II$^{-/-}$ MOG-specific B cells did not show an antibody response 8d p.i. or at later time points (18d p.i.), suggesting that the MHC-II deficiency not only affected their cellular-, but also their humoral immune response. Thus, we proposed that these B cells were not suitable to investigate the exclusive impact of antigen presentation on the course of EAE.

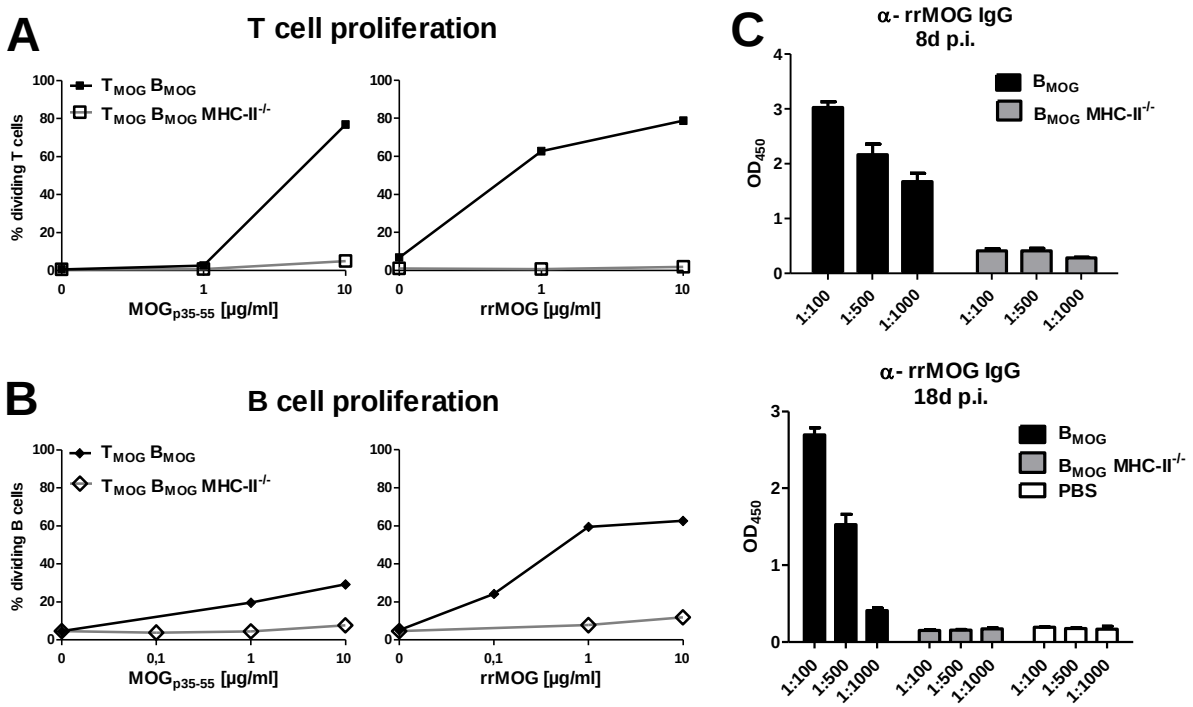

Figure 25: Ablation of MHC-II of B cells results in impaired B cell function.
(A+B) $1x10^6$ MOG-specific T cells were co-cultured with either $2x10^6$ MOG-specific B cells (black line) or MHC-$II^{-/-}$ MOG-specific B cells (grey line) with increasing MOG_{p35-55} (left) or rrMOG (right) concentrations (0; 0,1; 1; 10 µg/ml) for 72h. **(A)** T cell and **(B)** B cell proliferation was determined by CFSE dilution. **(C)** MOG-specific B cells, MHC-$II^{-/-}$ MOG-specific B cells or PBS were i.v. injected into NP mice and immunized two days later with 40µg rrMOG. Sera were obtained 8d p.i. (upper row) and 18d p.i. (lower row) and anti- anti-rrMOG IgG was quantified by ELISA, graphs are depicted as mean ± SEM.

3.8. The role of antibodies in MOG_{p35-55} induced EAE

3.8.1. MOG-specific B cells produce anti-rrMOG IgG upon MOG_{p35-55} immunization

As we have analyzed the phenotype of the MOG-specific T cells in the previous experiments extensively during the activation phase and before the disease onset and could not detect any striking difference, we suppose that the disease-accelerated effect is mediated by the MOG-specific B cells. That assumption is supported by the observation that activated MOG-unspecific B cells did not accelerate the disease onset (Fig. 24 A+B). Of course, MOG-specific B cells and NP-specific B cells differ in their antibody specificity. Although several publications suggest that anti-MOG antibodies are not produced after MOG_{p35-55} immunization [139, 151], we investigated whether this also holds true in our experimental setting. Because we know that NP-specific B cell mice were devoid of endogenous antibody-secreting MOG-specific B cells, we used these mice as hosts to analyze whether the transferred MOG-specific B cells were in general able to exhibit an humoral immune response after MOG peptide immunization. We injected MOG-specific B cells or PBS as control into NP-host mice and immunized them two days later with MOG_{p35-55}, obtained blood 9d p.i. and assessed the amount of anti-MOG_{p35-55} IgM and IgG and of anti-rrMOG IgM and IgG by ELISA.

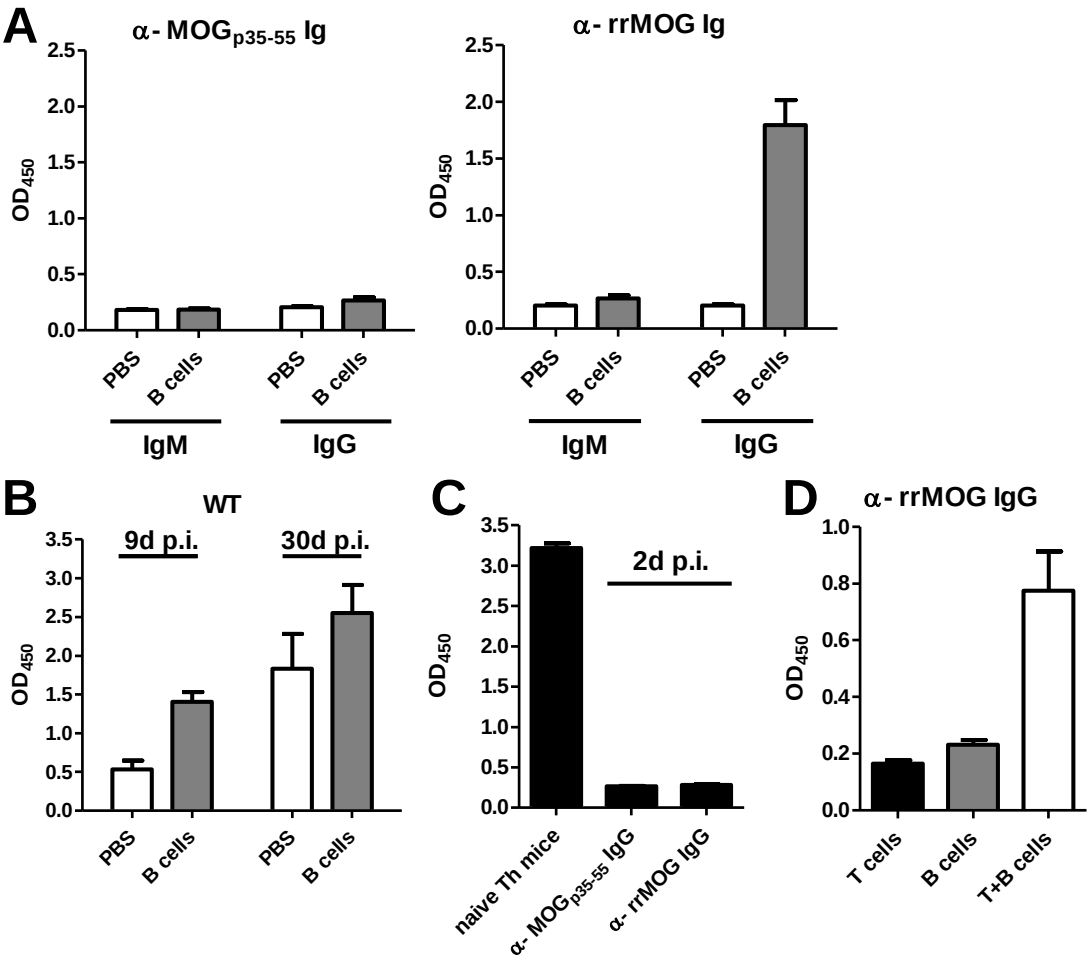

Figure 26: MOG-specific B cells produce anti-rrMOG IgG upon MOG_{p35-55}. immunization. **(A)** MOG-specific B cells or PBS were i.v. injected into NP mice and immunized two days later with MOG_{p35-55}. Sera were obtained 9d p.i. and anti-MOG_{p35-55} IgM and IgG (left) and anti-rrMOG IgM and IgG (right) was quantified by ELISA. **(B)** MOG-specific B cells or PBS were i.v. injected into WT mice and immunized two days later with MOG_{p35-55}. Sera were obtained 9d and 30d p.i. and anti-rrMOG IgG was quantified by ELISA **(C)** Serum was obtained from naïve Th mice or MOG-specific B cell-injected WT mice 2d p.i. and anti-MOG_{p35-55} IgG and anti-rrMOG IgG (naïve Th mice) was quantified by ELISA. **(D)** MOG-specific T cells, MOG-specific B cells or MOG-specific T and B cells were i.v. injected in OT-II, immunized with MOG_{p35-55} and serum was obtained 9-12d p.i.. Anti-rrMOG IgG was quantified by ELISA. Data in A and B represent 4 mice per group, in C 2 mice per group and in D 6 mice per group, shown are mean ± SEM.

Transferred MOG-specific B cells did not secrete anti-MOG_{p35-55} IgM or IgG (Fig. 26 A, left) 9 days post immunization, which is not surprising because B cells are known to rather recognize structural epitopes than short amino acid sequences. However, although we immunized with MOG peptide, we detected anti-rrMOG IgG (Fig. 26 A right). As we could not detect any anti-rrMOG specific IgGs in PBS injected mice, we assumed that the transferred B cells were the source of antibodies. When we repeated the experiment in WT mice, we observed that the transferred MOG-specific B cells exhibited a stronger antibody response than the endogenous B cells at an

earlier time point (Fig. 26 B), but even endogenous B cells produced anti-rrMOG IgGs after MOG peptide immunization (30d p.i.). It is known that naïve Th mice, the donor of MOG-specific B cells, harbor a high titer of anti-rrMOG IgG (Fig. 26 C). Therefore, it is possible that the detected anti-rrMOG IgG are an artificial effect of transferring already differentiated antibody-secreting plasma blasts. However, upon transfer of MOG-specific B cells, they did not immediately (2d p.i.) produce and secrete anti-rrMOG specific IgGs (Fig. 26 C). Thus, we assumed that the transferred MOG-specific B cells became activated *in vivo* by immunization. To verify whether these observations also hold true in our experimental setup, we transferred either MOG-specific T cells, MOG-specific B cells or both together into OT-II mice, immunized them with MOG_{p35-55} two days later and determined anti-rrMOG IgG from sera obtained 9-12d p.i.. The transfer of MOG-specific T cells did not trigger anti-MOG IgG production of the endogenous B cell pool (at least not until this early time point) and also MOG-specific B cells did not secrete any anti-rrMOG IgG, presumably due to the absent antigen-specific T cell help (Fig. 26 D). However, the simultaneous transfer of MOG-specific T and B cells resulted in an increased anti-rrMOG IgG production (Fig. 26 D). Conclusively, although several publications suggest that B cells do not exhibit an humoral immune response after MOG peptide immunization, we were indeed able to detect MOG-specific antibodies in our experimental setup. Moreover, the transfer of MOG-specific T cells alone did not facilitate an humoral immune response against MOG of the endogenous B cell pool leading to the assumption that the antibodies in theory can account for an accelerated disease onset in OT-II mice.

3.8.2. Mice with XBP-1 deficient B cells lack anti-rrMOG specific Igs and show a delayed disease onset upon rrMOG immunization

To investigate the role of antibodies in our experimental setting, we made use of a mouse system where B cells are incapable to develop into antibody-secreting plasma cells. The transcription factor X-box binding protein 1 (XBP-1) is required for the differentiation of B cells to plasma cells and XBP-1 deficient lymphoid chimeras are devoid of immunoglobulin of any isotype and of plasma cells [184]. To generate mice where XBP-1 deficiency is restricted to the B cell compartment, we crossed mb1Cre mice with $XBP\text{-}1^{fl/fl}$ mice resulting in Cre expression under the control of the B cell-

specific mb1 promotor and subsequent specific deletion of XBP-1 [174]. Before crossing these mice to Th mice to obtain MOG-specific XBP-1-deficient B cells, we performed an initial clinical experiment to test their functionality. XBP-1$^{fl/fl}$mb1Cre^{-} mice served as control mice in this experiment. As it is suggested that the secretion of pathogenic antibodies is rather mediated by protein antigens than by peptides, we immunized them with 20µg rrMOG and obtained blood 7d, 11d and 27d p.i. and quantified anti-rrMOG IgM and IgG levels.

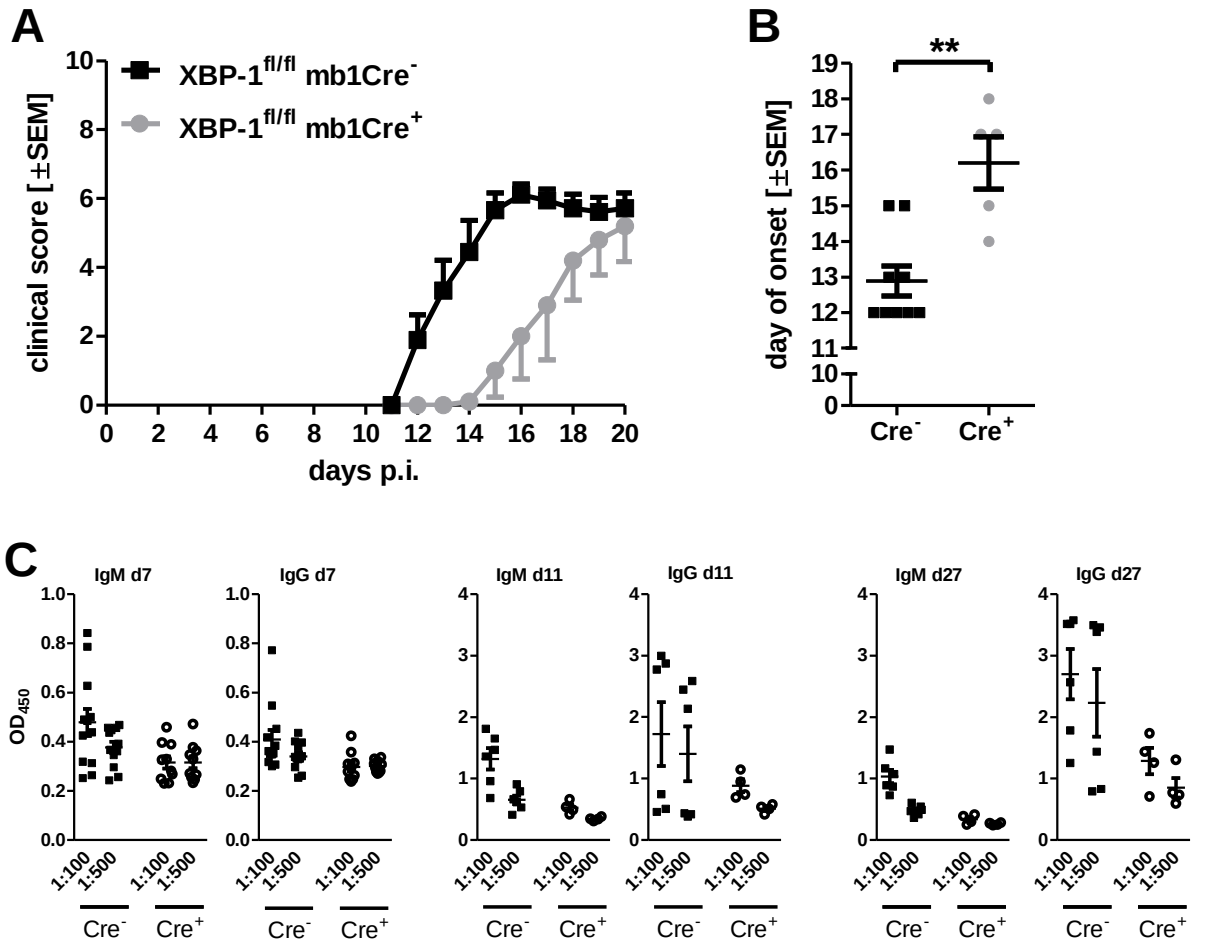

Figure 27: Absence of anti-rrMOG Igs results in a delayed EAE onset. **(A+B)** Clinical outcome of XBP-1$^{fl/fl}$mb1Cre^{-} or XBP-1$^{fl/fl}$mb1Cre^{+} mice immunized with 20µg rrMOG. Healthy mice were not included in the analysis. Clinical data represent 9 animals for Cre^{-} group and 5 animals for Cre^{+} group from 2 independent experiments **(C)** Sera obtained from XBP-1$^{fl/fl}$mb1Cre^{-} or XBP-1$^{fl/fl}$mb1Cre^{+} mice 7, 11 and 27d p.i. and anti-rrMOG IgM and IgG quantification by ELISA.. Each symbol represents an individual mouse in C out of two independent experiments. Data are shown in mean ± SEM. Significant differences between means are indicated: **p< 0,01, Mann-Whitney nonparametric *t* test.

During our initial analysis of the XBP-1$^{fl/fl}$mb1Cre^{+} mice we recognized that these mice had a reduced B cell compartment in contrast to XBP-1$^{fl/fl}$mb1Cre^{-} mice. Therefore, the outcome of the clinical data has to be interpreted carefully and might not exclusively be base on the absence of antibodies. But as we planned to use these mice as donor for B cells and not as hosts for cell transfer experiments, the reduced B cell number did not affect our experiments. EAE induction with rrMOG revealed a difference in the clinical outcome of XBP-1-sufficient and XBP-1-deficient B cell mice (Fig. 27 A+B). XBP-1$^{fl/fl}$mb1Cre^{-} mice started to develop first clinical signs about four days earlier than XBP-1$^{fl/fl}$mb1Cre^{+} mice (Cre^{-}: 12.89± 0.42; Cre^{+}: 16.2± 0.73). In addition, the different genotype affected the susceptibility for EAE as 47.3% of Cre^{-} mice developed EAE whereas only 29.4% of Cre^{+} mice showed clinical symptoms. In line with this was the reduction of anti-rrMOG IgM and IgGs mainly emphasized on day 11 and 27 p.i. (Fig. 27 C). But as mentioned before, we cannot rule out that the observed clinical effect was rather mediated by the reduced B cell compartment than by the lower antibody titer. In addition, it is also questionable whether the reduced antibody titer is due to deficient plasma cell differentiation or the general reduction in B cell numbers.

3.8.3. XBP-1-deficient MOG- specific B cells do not accelerate the onset of EAE

To investigate whether MOG-specific B cells contribute to the accelerated disease onset by their production and secretion of high-affinity anti-rrMOG antibodies of the IgG isotype, we crossed the XBP-1$^{fl/fl}$mb1Cre^{+} mice to Th mice (for simplicity designated as "XBP-1$^{-/-}$ MOG-specific B cells") with the final intention to use these B cells for adoptive transfer experiments. To ensure that the XBP-1 deficiency exclusively affected the humoral immune response of B cells without affecting their cellular immune response, we first tested their antigen-presenting capacity *in vitro*. We co-cultured them with CFSE-labeled MOG-specific T cells with increasing concentrations of either MOG$_{p35-55}$ or rrMOG and analyzed T cell proliferation 72h post culture. We additionally assessed MHC-II and CD86 expression of B cells by flow cytometry as readout for B cell activation.

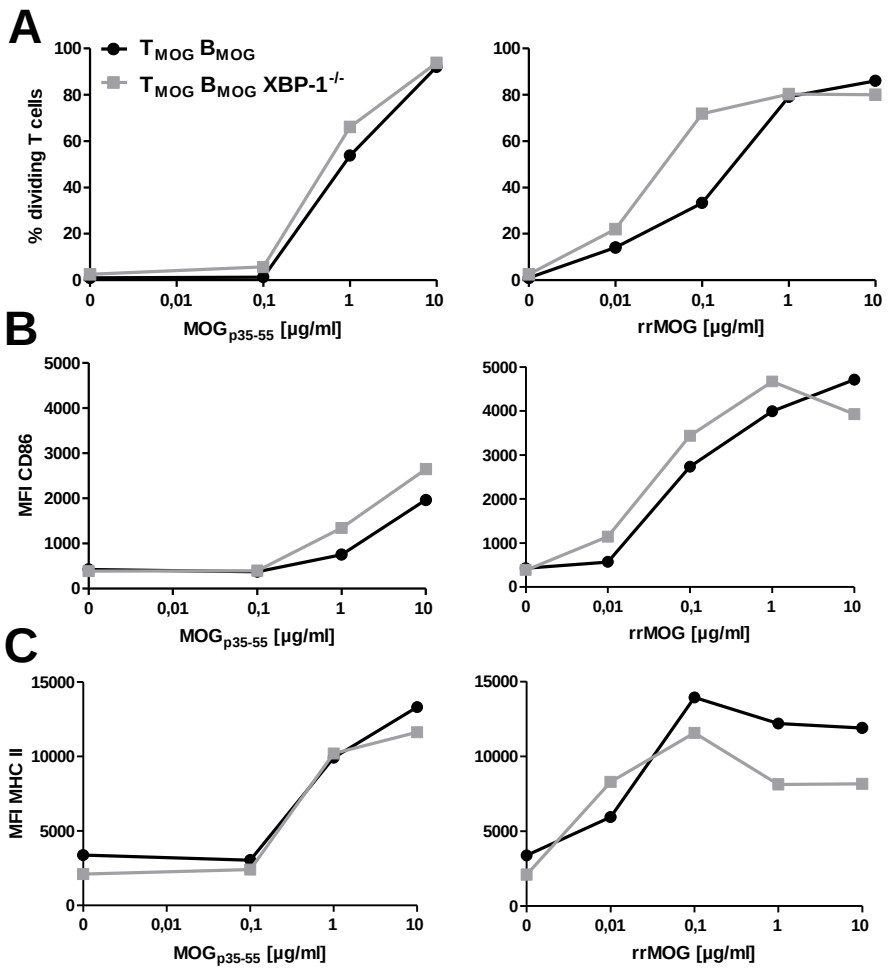

Figure 28: XBP-1 deficiency does not affect the antigen-presenting capacity of B cells. 1x10^6 MOG-specific T cells were co-cultured with either 2x10^6 MOG-specific B cells (black line) or XBP-1$^{-/-}$ MOG-specific B cells (grey line) with increasing MOG_{p35-55} (left) or rrMOG (right) concentrations (0; 0,01; 0,1; 1; 10 µg/ml) for 72h. **(A)** T cell proliferation was determined by CFSE dilution. **(B+C)** Co-cultured B cells were stained for B220 and CD86 **(B)** or for B220 and MHC-II **(C)** and analyzed for their mean fluorescence intensity (MFI) of respective marker expression by flow cytometry. Shown is one experiment.

XBP-1 deficiency did not influence the antigen-presenting capacity of MOG specific B cells. Both, XBP-1-deficient and XBP-1-sufficient B cells triggered antigen-specific T cell proliferation at the same antigen concentrations (Fig. 28 A). In addition, both B cell genotypes expressed MHC-II and CD86 with the same kinetic (Fig. 28 B+C). Minor differences in MHC-II expression as observed in Fig. 28 C (right) rather arise from natural variability. Thus, we conclude that the antigen-presenting capacity was not affected by the XBP-1 deficiency and that XBP-1-deficient B cells arise as appropriate B cells to study the role of antibodies in EAE. As the clinical outcome of T and B cell supplementation was more pronounced in the double-transgenic OT-II x NP host mice (Fig. 8 C+D, table 4), we used these mice as hosts for the clinical

experiment. We transferred MOG-specific T cells either together with MOG-specific B cells or with XBP-1-deficient MOG-specific B cells into OT-II x NP host mice, immunized them two days later with MOG_{p35-55} and monitored their clinical outcome for 20 days.

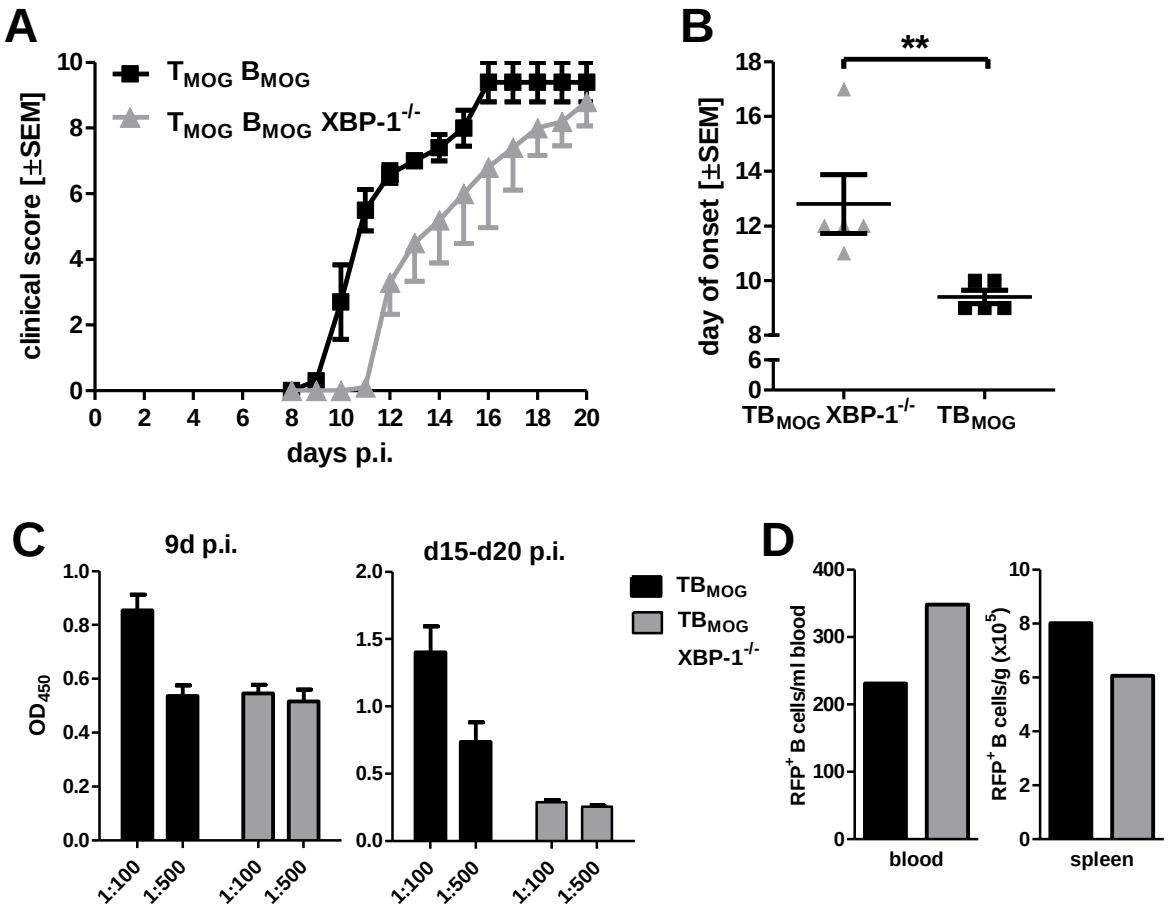

Figure 29: MOG-specific XBP-1-deficient B cells do not accelerate the onset of EAE and do not produce anti-rrMOG specific IgGs. MOG-specific T cells were injected either with RFP^+MOG-specific B cells or RFP^+MOG-specific XBP-1-deficient B cells into OT-II x NP mice and immunized with MOG_{p35-55}. **(A+B)** Clinical outcome of cell transfer **(C)** Sera obtained from MOG-specific T and B cell-injected mice or MOG-specific T and XBP-1-deficient B cell-injected mice 9d p.i. and at end of experiment (depending on the clinical score between d15 and d20 p.i.) and anti-rrMOG IgG quantification by ELISA **(D)** Quantification of total RFP^+ B cell numbers 9d p.i. in blood and spleen. Data in A and B represent 5 mice per group, in C 2 mice per group and in D one mouse per group. Data are shown in mean ± SEM. Significant differences between means are indicated: **p< 0,01, Mann-Whitney nonparametric *t* test.

The transfer of XBP-$1^{-/-}$ MOG-specific B cells did not accelerate the disease onset like observed with XBP-1-sufficient MOG-specific B cells (Fig. 29 A+B). Precisely, mice which received MOG-specific T cells and XBP-1-deficient B cells developed first clinical signs at about day 12.8± 1.0, whereas the transfer of MOG-specific T cells together with XBP-1- sufficient B cells resulted in a clinical onset at about day 9.4±

0.24. In contrast to that, the different B cell genotype did not impact the susceptibility to EAE, as both groups developed EAE with an incidence of 83%. To ensure that XBP-1-deficient B cells were really impaired in antibody production, we obtained blood from two animals per group 9 days p.i. and from all animals after they had to be sacrified due to a too high clinical score and determined serum anti-rrMOG IgG level. Mice which received XBP-1-sufficient MOG-specific B cells showed an increased anti-rrMOG IgG serum level already 9 days p.i. (Fig. 29 C, left), which was even enhanced at later time points, whereas serum anti-rrMOG IgG levels of XBP-1-deficient B cell-injected mice did not exceed background level (Fig. 29 C, right). As mentioned before, Th XBP-$1^{fl/fl}$mb1Cre^{+} mice (donor mice of MOG-specific XBP-1-deficient B cells) have an impaired B cell compartment with low numbers of peripheral B cells. To exclude that the observed clinical effect and the accompanied low serum anti-rrMOG IgG level was due to an e.g. increased apoptosis rate of the transferred B cells, we determined the number of transferred RFP^{+} B cells of one mouse per group 9 days p.i.. At that time point, the number of transferred B cells was similar in the spleen and in the blood (Fig. 29 D) between the two groups, leading to the conclusion that the deficiency of XBP-1 rather affected the development of B cells, but did not impact the survival of mature B cells with the consequence that isolated B cells stay viable upon transfer. In summary with the *in vitro* data, these data provide evidence that the transferred MOG-specific B cells promote the development of EAE through the secretion of MOG-specific IgGs. However, how these antibodies exert their pathogenic potential remains elusive.

4. Discussion

4.1. Establishment of the EAE model

Although MS is known to be a T cell-mediated autoimmune disease, beneficial effects of B cell-depleting therapies of MS patients with Rituximab, an anti-CD20 monoclonal antibody, reveal evidence that B cells are involved in the pathogenesis of MS. Several publications disclose divergent roles for B cells during the course of MS and EAE, the mouse model of MS. On the one hand, B cells can exert their pro-inflammatory function by their secretion of pro-inflammatory cytokines [55, 56, 154] or by triggering T cell activation through antigen-presentation [139]. Moreover, auto-antibodies are also considered to be involved in MS and EAE [53, 54, 140]. On the other hand, a regulatory subpopulation of B cells and plasma cells were shown to secrete anti-inflammatory cytokines like IL-10 and IL-35 and thus dampen down the autoimmune response [157, 159, 160]. In addition to the different roles suggested for B cells, the critical time point when B cells participate in the immune response also seems to be uncertain. Whereas some publications suggest a role for B cells in T cell priming in secondary lymphoid tissues [139], others suggest a critical role for B cells in the reactivation phase of EAE in the CNS [154]. Similarly, the critical time point of regulatory B cell contribution also seems to be controversial. Whereas some reports reveal a critical requirement for regulatory B cells during the EAE recovery phase [157], others suggest an important role for these cells in the EAE induction phase [161]. Hence, there is an increasing interest in studying the role and the critical time point of B cell contribution to the pathogenesis of MS. This would help to develop drugs exclusively targeting the B cell-dependent mechanism without affecting the total cell lineage.

We have now developed a system enabling us to track and analyze T and B cells of the same antigen-specificity *in vivo* during the course of EAE. Fluorescently labeled (RFP or GFP) MOG-specific T and B cells were isolated from respective donor mice and injected i.v. into different recipients followed by immunization with MOG peptide (MOG_{p35-55}). This allowed the re-isolation and characterization of the cells during the whole course of actively induced EAE from the periphery, as well as from the CNS. We did not manipulate the course of EAE by transferring e.g. activated T cells (Fig. 4) and the antigen-specific T cells responded specifically to their appropriate antigen *in vivo* (Fig. 5). This ensured that the injected cells became primed *in vivo* in a similar

manner as the endogenous lymphocytes and that co-injected T and B cells could interact with each other. We first established the model in C57bl/6 WT mice, where we observed a disease promoting effect of transferred MOG-specific T cells, but also of transferred MOG-specific B cells (Fig. 7 A, C), leading to the assumption that both lymphocyte populations participate in the autoimmune response. Thus, we thought that the simultaneous transfer of MOG-specific T and B cells might even enhance the observed effects. Although we observed a trend that the simultaneous transfer of MOG-specific T and B cells accelerate and exacerbate the course of disease in comparison to MOG-specific T cell transfer alone, the difference was not statistically significant (Fig. 7 A, B). We assumed that the contribution of the endogenous MOG-specific T cell pool to the disease progression was too strong to further significantly enhance the immune response. Therefore, we employed OT-II host mice, as it is known that OVA-specific T cells do not respond to MOG antigens and our *in vitro* data confirmed this observation (Fig 6 A left and middle). In line with this, OT-II mice were not susceptible for EAE induction with MOG_{p35-55} (Fig. 8 A), leading to the conclusion that the participation of OVA-specific T cells in the autoimmune response against MOG and thus in the initiation of EAE is negligible. To minimize the endogenous T and B cell effects simultaneously, we crossed the OT-II mice to NP mice resulting in OT-II x NP mice. These mice, mainly harboring OVA-specific T cells and NP-specific B cells, enabled us to widely exclude any effects from endogenous T and B cells after immunization with MOG peptide. As observed with OT-II mice, OT-II x NP mice were not susceptible for EAE induction either. The protection of EAE could be overcome by the transfer of MOG-specific T cells (Fig. 8 A) in OT-II host mice, as well as in OT-II x NP host mice (Fig. 8 C). In both models, the additional transfer of MOG-specific B cells to MOG-specific T cells significantly accelerated the disease onset and increased the susceptibility for EAE (Fig. 8 A-D, Table 4), although the disease-accelerated effect of MOG-specific B cell supplementation was more pronounced in the OT-II x NP model (Table 4, day of onset). Thus, both models exhibited several advantages: First, they facilitated a focus on the pathogenic MOG-specific T and B cell population during the course of EAE, not only in the periphery, but also in their target organ, the CNS. Second, they permitted us to study the impact of MOG-specific B cells on the course of EAE, because the accelerated onset revealed an effect of the transferred MOG-specific B cells in OT-II mice and, even more pronounced, in OT-II x NP mice. Furthermore, we avoided the usage of host

mice lacking e.g. B cells (µMT) or even T and B cells (RAG2$^{-/-}$) as these mice are known to have an impaired secondary lymphoid organ structure and injected T cells can undergo homeostatic proliferation [185, 186]. The traditional view is that peptide antigens are predominantly presented by professional APCs like DCs, whereas B cells require the presence of protein antigens for presentation [187, 188]. In line with this, several publications reveal that the presence or absence of B cells in MOG-peptide induced EAE is completely irrelevant for the course of EAE [138, 139]. Our own observations showed the reverse. We could clearly show that the supplementation of C57bl/6 WT mice with MOG-specific B cells (Fig. 7 C) or the simultaneous transfer of MOG-specific T and B cells into OT-II and OT-II x NP mice and subsequent MOG peptide immunization accelerated the onset of EAE and increased susceptibility in comparison to non-supplemented mice or T cell-supplemented mice, respectively (Fig. 8 A-D). We also established the above described models (C57bl/6 WT and OT-II) with rrMOG protein immunization (data not shown) and also observed an effect of supplemented MOG-specific B cells on the disease course. However, we were more interested in the role of B cells in MOG peptide induced EAE, because this is contradictory to most of the publications [138, 139]. It is suggested in the literature that peptide antigens can be presented by B cells irrespective of their antigen receptor specificity by binding on surface MHC-II molecules [78, 79, 139]. Our *in vitro* results also reveal that NP-specific B cells can trigger MOG-specific T cell proliferation in the presence of MOG_{p35-55} almost as efficiently as MOG-specific B cells (Fig. 6 A left), whereas they fail to induce T cell proliferation in the presence of rrMOG (Fig. 6 A middle). This led to the conclusion that the antigen-specificity of the B cells is irrelevant for triggering T cell proliferation in the presence of MOG peptide antigens *in vitro*. However, the simultaneous transfer of MOG-specific T cells and NP-specific B cells into OT-II hosts and subsequent immunization with MOG_{p35-55} did not lead to the accelerated disease onset observed with MOG-specific B cells (preliminary data, Suppl. Fig. S1). This led to the assumption that the *in vivo* interaction of MOG-specific T and B cells in the presence of MOG_{p35-55} did not simply affect T cell proliferation, but rather might result in a more complex impact on the entire autoimmune response. In line with this, we observed a diminished EAE susceptibility of T cell-injected OT-II x NP mice in contrast to T cell-injected OT-II mice (Table 4; 61.5% vs. 35.7%). This observation also emphasizes the critical role of B cell receptor antigen-specificity because if the antigen-specificity

of the endogenous B cell pool is irrelevant for peptide-induced EAE, it might not affect the clinical outcome of EAE. It should be mentioned here that we did not experimentally exclude a difference in the residual antigen-presenting cell pool of the two different host mice, which could possibly also account for a different EAE susceptibility.

In summary the newly established EAE model not only enabled us to study the behavior of MOG-specific T and B cells during the course of EAE, but also highlights the importance of antigen-receptor specificity even in MOG peptide-induced EAE.

4.2. The role of MOG-specific B cells in the activation phase of EAE

There are several possible mechanisms of how B cells can influence the course of EAE. It is considered that B cells act as antigen-presenting cells and thereby prime encephalitogenic T cells [139]. In addition, B cells influence T cell differentiation by their provision of pro-inflammatory cytokines such as IL-6 [56]. Moreover, B cells can secrete high affinity anti-MOG-specific antibodies [140]. In contrast to the described pro-inflammatory roles for B cells in EAE, several publications also suggest a regulatory role for some B cell subpopulations during disease progression [159, 160]. However, in all of our experimental models (C57bl/6 (Fig. 7), OT-II, OT-II x NP hosts (Fig. 8)), we observed a disease promoting effect of the transferred B cells and in none of them did we find an implication for a regulatory role of B cells. Furthermore, we tried to identify regulatory B cells ($CD19^{+}CD1d^{hi}CD5^{+}$) by FACS staining in naïve C57bl/6 and Th mice and could not at all detect a distinct population. Therefore, we rather suggest a pro-inflammatory role for the transferred B cells in our system. We were interested whether the transferred MOG-specific B cells participate as antigen-presenting cells in the priming phase (d2- d4) of EAE. We rather expected a supportive than a critical role of the B cells, as the role of B cells in priming naïve T cells *in vivo* is controversially discussed [189, 190] and conditional MHC-II expression of B cells in EAE also reveals a limited role for B cells as antigen-presenting cells [38]. In line with this we observed that the transfer of MOG-specific T cells alone is sufficient to drive the development of EAE. When we focused on T cell proliferation between d2 and d4, we could not detect a difference of T cells injected alone or together with MOG-specific B cells (Fig. 10 A+B), which was in line with the T cell

number (Fig. 10 C) and frequency among the $CD4^+$ T cell pool (Fig. 10 D). Moreover, the presence of MOG-specific B cells did not increase the frequency of activated T cells (Fig. 11). In general, antigen-presentation by B cells can induce T cell differentiation into a T helper cell direction [191] and several publications suggest that B cells promote predominantly TH17, but also TH1 differentiation in EAE [56, 139, 151]. However, we did not detect a difference in IFNγ, IL-17A or GM-CSF expression on mRNA level early after disease induction (2.5d p.i.; Fig. 12 A) and later (4d p.i.; Fig. 12 B). Furthermore, the presence of B cells neither expanded already differentiated T cells nor increased their sensitivity to lower antigen concentrations (Fig. 13). Analyses with MOG-specific FOXP3-GFP reporter T cells also revealed no impaired conversion of naïve T cells into induced regulatory T cells in the presence of B cells (Suppl. Fig. S2).

From these observations we conclude that MOG-specific B cells did not modulate T cell proliferation, activation or differentiation during the priming phase of EAE.

4.3. The role of MOG-specific B cells before onset of EAE

As we could not detect a contribution of MOG-specific B cells to promoting encephalitogenic T cell amplification, activation or differentiation in the priming phase of EAE, we focused on the time point immediately before EAE onset. We know from experiments with inducible conditional CD28 knockout mice that T cell priming is not entirely completed after the activation phase of EAE [212]. Thus, it is possible that B cells exert their pro-inflammatory function later during preclinical progression. As B cells accelerate EAE onset from day 11 to day 9 in our EAE model (Fig. 8 A+B, Table 4), we hypothesized that we would find a difference in the T cell phenotype shortly before the manifestation of clinical symptoms of MOG-specific T and B cell-injected mice. We used the technique of two-photon intravital imaging to distinguish mice with intraluminal and extraluminal T cells in the CNS. Almost all of the analyzed T and B cell-injected mice were found to have already extravasated T cells, which resembled the clinical outcome (Fig. 15 A). However, when we compared T and B cell-injected mice with extravasated T cells and T cell-injected mice without extravasated T cells with further flow cytometric analyses we did not detect any difference in the peripheral T cell number (Fig. 15 B) or frequency of activated T cells (Fig. 15 C)

between the two groups. This was in line with a study published where no difference could be detected in peripheral T cell activation in the absence or presence of MOG-specific B cells of healthy mice [78]. As mentioned previously, it is traditionally thought that B cells drive the development of TH17 cells [56, 139]. We could not detect any difference in the expression level of IFNγ, IL-17A and GM-CSF on mRNA level of peripheral MOG-specific T cells 9d p.i. (Fig. 16 A). In addition, there was no difference in cytokine secretion of restimulated lymph node cells and splenocytes re-isolated either from T cell or T and B cell-injected mice before clinical manifestation of EAE (9d p.i.) (Fig. 16 B-D). However, we found increased levels of IFNγ and IL-17A (Fig. 16 B, C) from sick T and B cell-injected mice (also re-isolated 9d p.i.). Notably, most of the publications which suggest a role for B cells in driving T cell differentiation, analyzed cytokine expression in late phases of disease progression (between d14 and 32 p.i.) when mice already display a difference in the clinical progress [56, 139, 151]. But whether this was a specific B cell effect or due to the overall increased pro-inflammatory milieu remains questionable.

When we focused on the underlying reason why T and B cell-injected mice displayed an accelerated EAE onset, we could clearly detect an increased number of CNS-infiltrated encephalitogenic T cells (Fig 17). As we could never detect any of the injected MOG-specific B cells in the CNS of healthy mice (by flow cytometry or by two-photon imaging performed by my colleague Tanja Litke) and just very few of them in mice with fulminant EAE (Score 4.5) (Fig. 18 B), we exclude a role at least for the injected B cells in the reactivation step. This was in contrast to a recent publication, which suggests that the initial interaction of T and B cells in the CNS account for an enhanced pro-inflammatory milieu leading to further cell attraction [154]. Moreover, the presence of meningeal ectopic B cell follicles was also reported in some MS patients [51]. But in our study also initial CNS-infiltrated T cells (~3000 cells) isolated before the occurrence of clinical signs, displayed similar IFNγ, IL-17A and GM-CSF mRNA levels (Fig. 18 A). This was a quite challenging analysis, because it was not feasible to accurately identify the first 50-100 infiltrated cells. Therefore we cannot completely exclude that the very first infiltrated T cells became reactivated more efficient in T and B cell-injected mice, which would then lead to further cell attraction with the consequence of a higher number of detected infiltrated cells before the onset of EAE. Our analyses did clearly show that the earlier onset was due to a higher number of infiltrated T cells and not due to a general enhanced

reactivation in the presence of B cells. However, the higher number of infiltrated cells was neither accompanied by an increased peripheral T cell number, nor by an enhanced T cell activation or differentiation in the periphery.

4.4. Effect of MOG-specific B cells on T cell migration

The migration behavior of T cells is determined by their expression of distinct chemokine receptors and adhesion molecules. It is widely accepted that expression of CCR9 and LPAM (integrin $\alpha_4\beta_7$) by T cells is responsible for their gut homing [192-194], whereas skin-homing is mediated by E and P-selectin ligands and CCR4 and CCR10 [195, 196]. The upregulation of the particular homing receptors is mediated by the interaction of T cells with respective tissue-specific dendritic cell subsets [180, 197]. Chemokine receptor-dependent T cell attraction is also critically involved in the pathogenesis of EAE. Whereas TH1 cells preferentially utilize CXCR3 and CCR5, TH17 cells are characterized by their expression of CCR6. Interference or complete absence of the chemokine receptors or their ligands results in attenuated disease course or complete resistance to EAE [120, 121, 135]. Beside the upregulation of pro-inflammatory chemokine receptors, also homeostatic chemokine receptors such as CCR7 and CXCR4 have been shown to be involved in CNS migration [103, 120, 130]. Therefore, the critical chemokine receptor required for CNS infiltration does not seem to be as strictly defined as observed in gut and skin homing. To get an idea which chemokine receptor was preferentially utilized by the pathogenic T cells in our model, we determined chemokine receptor expression of initial infiltrated T cells on mRNA level. We did not detect a statistically significant difference in chemokine receptor expression of T cells either injected alone or simultaneously with MOG-specific B cells. However, there was a tendency of a higher CCR7 and CCR6 expression (Fig. 19) of T cells injected together with B cells. Interestingly, these cells also showed an enhanced, but not statistically different, expression of the TH17 specific cytokines IL-17A and GM-CSF (Fig. 18 A). Therefore, one can argue that the peripheral interaction of MOG-specific T cells with MOG-specific B cells somehow drive the development of the TH17 phenotype. It is possible that the experimental approaches we used (RT-PCR of sorted T cells from periphery and in vitro antigen-specific restimulation) to determine T helper cell differentiation in the periphery before clinical onset were not sensitive enough to detect already differentiated TH17 cells or

that these cells were not terminally differentiated into TH17 cells before they became reactivated in the CNS by perivascular APCs. In agreement with this finding, we also detected an increase in CCR6 expression of splenic T cells (Fig. 20). $CCR6^+$ CD4 T cells (TH17 cells) have been shown to be crucial for the initiation of EAE. They infiltrate the CNS at the site of the choroid plexus into the brain, where their reactivation results in an overall increased pro-inflammatory milieu whereby they trigger further CCR6-independent lymphocyte infiltration [135]. As we also detected increased T cell numbers in the brain before the onset of clinical EAE (Fig. 15 B), this scenario can also account for the accelerated onset in our experimental model. We are planning to investigate this observation in detail, for example by determining the number of T cells in the CSF. Furthermore, we observed a significant upregulation of CXCR4 of T cells re-isolated from peripheral lymphoid organs and blood (Fig. 20). The role of CXCR4 and its ligand CXCL12 in the context of EAE is controversial. CXCL12 is expressed by spinal cord endothelial cells, among other sites, where it is mainly secreted at the basolateral surface into the perivascular space. Here, it has been suggested, CXCL12 traps $CXCR4^+$ lymphocytes and prevents their migration deeper into the CNS parenchyma [130]. Although treatment studies with AMD3100, an antagonist of CXCR4 signaling, reveal worsening of clinical symptoms due to an increased number of infiltrated mononuclear cells deeper into parenchymal tissue after treatment, CXCR4 blockade had no effect on the onset of the disease. Therefore, it is also likely that a different CXCR4 expression kinetic of the pathogenic T cells can account for the earlier disease onset observed in our experimental model. In theory, the $CXCR4^+$ T cells become attracted into the perivascular space, where they become reactivated by resident APCs, which in turn leads to the upregulation of various chemokines and cytokines enabling further cell infiltration. In line with this, we observed massive upregulation of CXCL10 and CCL5 on mRNA level of spinal cord microvessel endothelial cells isolated 9-11d p.i. when initial CNS infiltrates were detected in T and B cell-injected, but also in some T cell-injected mice (Suppl. Fig S3). The absent CXCR4 expression of the initial infiltrated cells (Fig. 19) can be explained by a downregulation during reactivation. However, it is experimentally challenging to find further evidence supporting our CXCR4 theory, as functional interference with CXCR4 would mainly target the altered T cell trapping rather than the initial infiltration. It is feasible to re-isolate $CXCR4^+$ RFP^+ T cells shortly before EAE onset from the periphery, transfer them into new host mice and investigate

whether they infiltrate the CNS earlier than control T cells, but it is questionable whether they maintain their CXCR4 expression until they arrive at the BBB.

As integrin expression by T cells is also critically required for CNS infiltration, we also focused on integrin α_L (LFA-1) and integrin α_4 (VLA-4) expressions before onset of EAE (Fig. 21 A). We detected neither an enhanced expression on mRNA level nor an increased number of VLA-4^+ T cells (Fig. 21 B) or LFA-1^+ T cells (Fig. 21 C). However, this was not surprising, as LFA-1 and VLA-4 are not exclusively involved in T cell migration, but are also involved in T cell–APC interaction, leading to several further factors which might influence their expression. Moreover, it is also likely that B cells do not influence the integrin expression of T cells, but rather their activation status. LFA-1 is normally expressed in a quiescent state on the surface of leukocytes and interacts weakly with its ligands ICAM-1 [198, 199]. Upon activation, e.g. by chemokine receptor signaling, it changes its conformational state into an active form, leading to a firm interaction with its endothelial cell expressed ligand [200]. As LFA-1 is also involved in the forming of an immunological synapse between T cells and APCs, it is in general possible that B cells can influence its activation state. At the moment we are establishing an *in vitro* BBB approach to test whether antigen-specific T and B cell interaction results in a higher number of firmly adhesive T cells.

4.5. Effect of MOG-specific B cells on myeloid cells

It is widely accepted that other leukocyte subsets are also critically involved in the pathogenesis of EAE. Myeloid cells do not only mediate tissue damage through secretion of toxic factors, but they also serve as antigen-presenting cells for the reactivation of infiltrated myelin-specific T cells [201-204]. Therefore, an absence of myeloid cell infiltration due to impaired chemokine signaling leads to decreased T cell infiltration and overall reduction in produced pro-inflammatory mediators [165-168]. The CD11b$^+$Ly6C^{hi} monocyte population is critically involved in EAE pathogenesis. This population is released from the bone marrow into the blood immediately before EAE relapse in a GM-CSF-dependent manner. Upon CNS infiltration the cells upregulate MHC-II and gain pro-inflammatory functions. Furthermore, the increase in circulating Ly6C^{hi} monocyte number was accompanied by an accelerated disease onset and an increase in clinical severity [169, 181]. As we did not detect a difference

in GM-CSF expression of the injected MOG-specific T cells after activation phase and before EAE onset (Fig. 12B, 13C, 16 A/D), we exclude that mechanism as accounting for a possible increase in monocyte number. However, it was shown that B cells attract monocytes to the heart after myocardial infarction in a CCL7-dependent manner. Therefore, we also wondered whether B cells can also influence monocyte mobilization in an autoimmune disease. We detected an increase in Ly6C^{hi} monocyte number in the blood of T and B cell-injected mice 7d p.i., but not in spleen and spinal cord (Fig. 22 B). However, this increase was not consistent during the following days. One can argue that the number of Ly6C^{hi} monocytes diminished in the blood of T and B cell-injected mice due to their enhanced infiltration into the CNS (Fig. 22 C+D), but one would assume a subsequent increased egress of monocytes from the bone marrow into the blood to compensate the reduced monocyte number in the blood. In summary, in our experiments the monocyte numbers were not as strikingly different as described in the literature. Therefore, we found no evidence that this mechanism is responsible for the accelerated disease onset.

4.6. Effect of MOG-specific B cells on spinal cord microvessel endothelial cells

CNS T cell infiltration can not only be influenced by their chemokine receptor expression, but also by chemokine and adhesion molecule expression of endothelial cells from spinal cord microvessels. The expression of chemokines and adhesion molecules is mainly regulated by different cytokines, for example IFNγ or IL-6 [183, 205-207]. As several chemokines become upregulated before the appearance of first clinical signs, it is possible that their different expression kinetic can account for an accelerated disease onset [183, 205-207]. However, we did not detect a difference in the expression of adhesion molecules (ICAM-1, VCAM-1, ALCAM) by spinal cord microvessel endothelial cells isolated from T cell or T and B cell-injected mice before first T cell infiltration (8d p.i.) (Fig. 23 A). In addition, sorted endothelial cells also displayed similar chemokine expression levels (Fig. 23 B), leading to the conclusion that the presence of MOG-specific B cells did not trigger T cell infiltration by influencing the expression of adhesion molecules and chemokines by spinal cord microvessel endothelial cells. However, we could confirm the observation that some

chemokines became upregulated before the appearance of clinical signs. When we determined adhesion molecule and chemokine expression of healthy animals with infiltrated cells, we observed an enhanced chemokine expression level, specifically of CXCL10 and CCL5, but this was a secondary effect due to the ongoing reactivation of infiltrated T cells (Suppl. Fig S3).

4.7. The antigen-specific interaction of T and B cells determine the accelerated disease onset

B cells can also be involved in the establishment of a pro-inflammatory milieu, not only by their antibody secretion or antigen-presenting capacity which would require antigen-specificity, but also by their secretion of cytokines, which are crucial to drive T cell differentiation [55, 56, 139]. Hence, also activated autoantigen-unspecific B cells can be involved in the initiation of an immune response [208, 209]. However, in the context of EAE, it was reported that the transfer of activated MOG-unspecific B cells cannot restore the EAE susceptibility of µMT mice as observed with transfer of activated MOG-primed B cells [140]. Therefore, we were also interested whether or not the presence of activated but antigen-unspecific B cells is sufficient to accelerate the onset of EAE in our experimental setting. The simultaneous immunization with NP-OVAL and MOG_{p35-55} ensures the activation of the transferred NP-specific B cells in presence of the endogenous OVA-specific T cells while also ensuring a sufficient priming of the transferred MOG-specific T cells. We confirmed the successful B cell activation by detection of a high serum anti-NP IgG titer (Fig 24 C). However, activated MOG-unspecific B cells failed to accelerate the EAE onset compared to MOG-specific B cells (Fig. 24 A+B). This finding again emphasized our observation that MOG-specific B cells play a supportive role even in MOG peptide-induced EAE. One can argue that the antigen-specific interaction of OVA-specific T cells and NP-specific B cells results in a different cytokine production profile than the antigen-specific interaction of MOG-specific T and B cells. Thus, we determined the cytokine levels of *in vitro* co-cultured T and B cells and even detected elevated levels of pro-inflammatory cytokines such as IFNγ, TNFα and IL-6 in OVA-specific T cell and NP-specific B cell co-culture (Suppl. Fig S4).

4.8. The role of antibodies in MOG_{p35-55} induced EAE

Previous experiments reveal that activated, MOG-unspecific B cells were not able to accelerate the onset of EAE as observed with MOG-specific B cells. Thus, we wondered about the disease accelerating mechanism which might be based on the antigen-specific interaction of T and B cells. Obviously, the MOG-specific interaction results in a different humoral immune response than the NP-OVAL-specific interaction. Although several publications suggest a dispensable role for B cells and auto-antibodies in MOG peptide-induced EAE [139, 140, 150, 151], we detected anti-rrMOG, but not anti-MOG_{p35-55} antibodies of the IgG isotype after MOG peptide immunization secreted by the transferred MOG-specific B cells before appearance of clinical signs (Fig 26. A). The anti-rrMOG IgG secretion required the presence of MOG-specific T and B cells suggesting that these cells interacted *in vivo* (Fig. 26 D). Our observation was supported by a publication which showed that B cell proliferation and isotype class switching can be induced by MOG-specific TH17 cells *in vitro* in the presence of MOG_{p35-55} [210]. The fact that we detected anti-MOG protein-specific IgGs even though we immunized with MOG peptide was presumably due to the specificity of the knock-in antibody of the transferred MOG-specific B cells. These antibodies were shown to accelerate and exacerbate EAE [143]. The role of antibodies in C57bl/6 MOG-induced EAE is controversial. Whereas a recent publication suggests a dispensability for antibodies upon rhMOG-immunization as well as for the development of spontaneous EAE [139], another study reveals a critical requirement for rhMOG-primed serum in restoring the susceptibility of EAE in µMT mice [140]. In addition, the presence of high endogenous MOG-specific serum autoantibody titer was shown to enhance clinical effects [143]. To investigate whether MOG-specific IgGs accounted for the accelerated disease onset in our experimental system, we employed XBP-$1^{fl/fl}$mb-$1Cre^{+}$ MOG-specific B cells for adoptive transfer experiments. XBP-1 is a transcription factor required for the terminal differentiation of B cells to plasma cells. In the literature it is described that XBP-$1^{-/-}$ B cell mice displayed low baseline serum levels of all Ig isotypes and an impaired humoral immune response upon immunization, but XBP-1-deficiency did not affect B cell activation and proliferation *in vitro* as well *in vivo* [184]. When we immunized XBP-$1^{fl/fl}$mb-$1Cre^{+}$ mice with rrMOG we observed a delayed EAE onset with a reduced disease severity and susceptibility compared to XBP-$1^{fl/fl}$mb-$1Cre^{-}$ control mice (Fig. 27 A+B), which was accompanied by reduced serum anti-rrMOG IgM and IgG levels

at several time points during disease progression (Fig. 27 C). Although peripheral B cell numbers were not affected when the Cre recombinase was expressed under the B cell-specific CD19 promotor [211], we detected reduced numbers of peripheral B cells in XBP-1$^{fl/fl}$mb-1Cre^{+} mice (as well as in Th XBP-1$^{fl/fl}$mb-1Cre^{+} mice). Therefore, it is not clear whether the clinical differences arose from the reduced B cell number or from the reduced antibody levels. When we adoptively transferred MOG-specific XBP-1$^{fl/fl}$mb-1Cre^{+} B cells, we observed that they did not accelerate the disease onset after MOG_{p35-55} immunization like observed with XBP-1-sufficient MOG-specific B cells (Fig. 29 A+B). The delayed disease onset was accompanied by reduced anti-rrMOG IgG serum levels before establishment of clinical EAE as well as on later time points (Fig. 29 C) without affecting transferred peripheral B cell numbers (Fig. 29 D). Our *in vitro* data reveal that XBP-1-deficiency did not influence the antigen-presenting capacity or activation of B cells (Fig. 28 A-C), assuming that the diminished humoral immune response accounted for the delayed clinical onset. However, one should keep in mind that the XBP-1 deficiency impairs the development of plasma cells and does not affect the antibody secretion directly. Since a recent publication reveals a novel additional role for CD138^{+} plasma cells during EAE and infectious diseases, it is also possible that plasma cells contribute to the accelerated onset in a different way than by the secretion of antibodies [159]. It remains elusive how these antibodies exert their pathogenic role in EAE. This question has not yet been addressed in detail in the literature. It is suspected that MOG-specific antibodies play a role in effector cell maturation and/or trafficking because the lack of antibodies is not accompanied by an impaired T cell priming (similar to our observation) [140]. Furthermore, earlier demyelination and larger cellular infiltrates were detected in Th mice (high titer of endogenous MOG-specific antibodies), suggesting a role for antibodies in demyelination [143].

5. Summary and Conclusion

Beneficial B cell-depleting therapy in MS patients reveals a critical role for B cells to be involved in the pathogenesis of MS. However, the mechanisms of how B cells contribute to the autoimmune responses are still elusive. We generated a model enabling us to visualize and analyze antigen-specific T and B cells during the course of actively induced EAE, both in the periphery and in the CNS, their target organ. For this purpose we isolated fluorescently labeled MOG-specific T cells from 2D2 mice and MOG-specific B cells from Th mice and injected them i.v. into OT-II mice followed by immunization with MOG_{p35-55}. OT-II mice were resistant to EAE induction. This resistance could be overcome by the transfer of MOG-specific T cells. An additional transfer of MOG-specific B cells resulted in an accelerated disease onset and an increased susceptibility for EAE. This experimental approach offers the advantage to focus on the pathogenic T and B cells due to their fluorescent label. Moreover, it permits the study of the direct impact of genetically modified lymphocytes on the course of EAE. We employed the active immunization model to ensure an antigen-specific *in vivo* activation. Intensive investigations on the impact of MOG-specific B cells on MOG-specific T cells during the course of EAE revealed that they did not play a critical role in enhancing T cell proliferation, activation or differentiation either in the activation phase or on the verge of the EAE onset. The accelerated disease onset in T and B cell-supplemented mice was accompanied by a higher number of CNS infiltrated T cells and not by an enhanced T cell reactivation, leading to the conclusion that MOG-specific B cells play a dispensable role in the reactivation step in the CNS. Moreover, MOG-specific B cells did not influence myeloid cell mobilization and adhesion molecule or chemokine expression of spinal cord microvessel endothelial cells. However, in the presence of MOG-specific B cells, peripheral MOG-specific T cells slightly upregulate the chemokine receptors CCR6 and CXCR4, leading to speculations about whether B cells might influence T cell trafficking. To investigate the mechanism utilized by B cells to contribute to the accelerated EAE onset, we performed different clinical experiments. Because activated NP-specific B cells had no impact on the EAE onset it is clear that the accelerated onset is determined by the antigen-specificity of B cells rather than by their activation status. Moreover, we detected MOG protein-specific antibodies of the IgG isotype in T and B cell-supplemented mice, but not in T cell-supplemented mice

before the appearance of first clinical signs. XBP-1 is a transcription factor critically involved in the development of plasma cells. Thus, we employed MOG-specific XBP-1-deficient B cells for adoptive transfer experiments to study the role of antibodies on the onset of EAE. XBP-1-deficient B cells did not accelerate the onset of EAE as XBP-1-sufficient B cells did, suggesting a disease-promoting role for anti-MOG protein specific antibodies in EAE. In conclusion, our established model provides a tool not only to track and analyze antigen-specific lymphocytes during EAE, but also to study the consequences of different lymphocyte supplementation on the course of disease. In our work, MOG-specific B cells and their utilized mechanisms did not enhance T cell proliferation, activation or differentiation in the periphery, but their interaction with MOG-specific T cells seem to trigger their CNS infiltration. Although several publications suggest a dispensable role for B cells in MOG peptide-induced EAE, our experimental data provide evidence that this may not be true: autoantigen-specific B cells can become stimulated by peptide antigens, resulting in their participation in the autoimmune response.

6. References

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212. Nadine Eckert Master Thesis 2014 "The role of the co-stimulatory molecule CD28 during different phases of the Experimental Autoimmune Encephalomyelitis"

7. Supplementary data

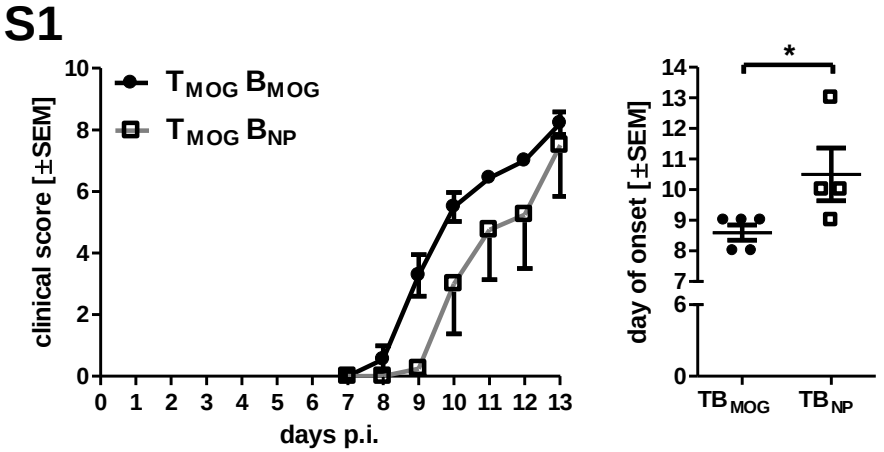

Figure S1: NP-specific B cells do not accelerate the onset of EAE. MOG-specific T and B cells and NP-specific B cells were isolated from respective donor mice and i.v. transferred (3,5x 10^6 MOG-specific T cells+ 1x 10^7 MOG-specific B cells or 3,5x 10^6 MOG-specific T cells+ 1x 10^7 NP-specific B cells) into OT-II mice followed by immunization with 50µg MOG + 50µg OVA protein two days later and monitored their clinical outcome for 13 days. Data represents mean ± SEM of 5 mice per B_{MOG} group and 4 mice per B_{NP} group of one experiment. Healthy animals were not included in the clinical data curve. Significant differences between means are indicated: *p< 0,05, Mann-Whitney nonparametric *t* test.

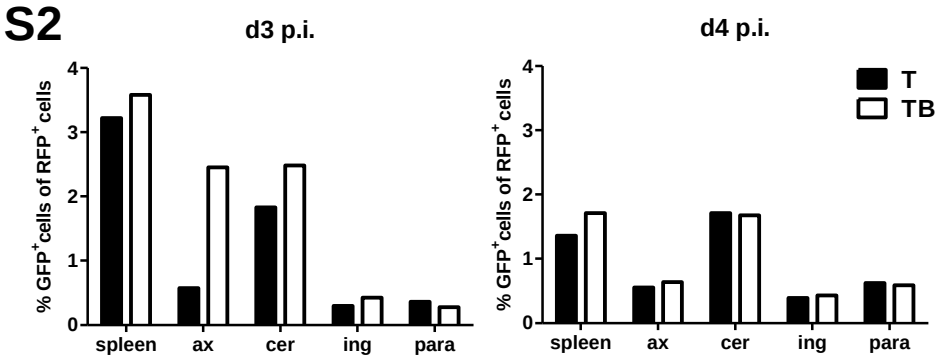

Figure S2: MOG-specific B cells do not inhibit the conversion of naïve MOG-specific T cells into induced regulatory T cells within the activation phase. RFP^+ MOG-specific FOXP3 reporter T cells were isolated from respective donor mice and transferred alone or together with MOG-specific B cells into OT-II mice followed by immunization with MOG_{p35-55} two days later. Three and four days p.i. different lymph nodes and spleen were analyzed for frequency of GFP^+ (= $FOXP3^+$) cells among the $CD4^+RFP^+$ cells by flow cytometry. One bar represents 1 mouse per time point. Data were generated by Leon Hosang during his 8-week lab rotation.

S3

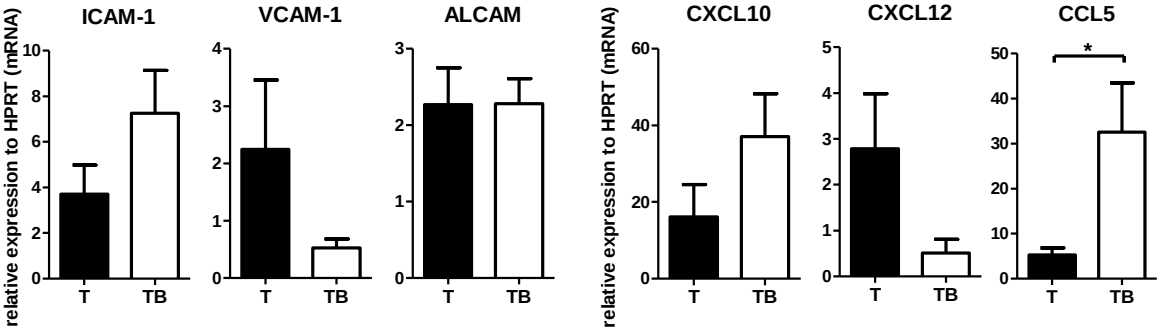

Figure S3: Spinal cord microvessel endothelial cells upregulate chemokine expression upon reactivation of initial infiltrated T cells. Spinal cord microvessels were isolated from T cell-injected or T and B cell-injected OT-II mice between d9-d11 p.i.(without clinical score) and endothelial cells were sorted according to their CD31 expression. Adhesion molecules (ICAM-1, VCAM-1, ALCAM) and chemokine (CXCL10, CXCL12, CCL5) (mRNA) expression analysis was performed by RT-PCR. Data represent 2 independent experiments with 4 animals per group. Data are depicted as mean ± SEM. Significant differences between means are indicated: *p> 0,05, unpaired students *t* test

S4

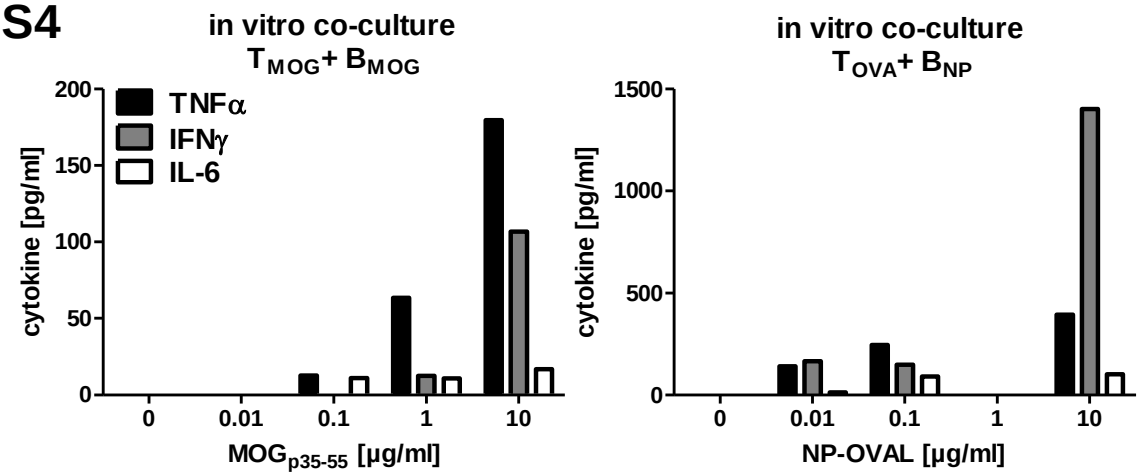

Figure S4: *In vitro* cytokine response of co-cultured antigen-specific lymphocytes. $1x10^6$ MOG-specific T cells were co-cultured with $2x10^6$ MOG-specific B cells with increasing MOG_{p35-55} concentrations (left) or $1x10^6$ OVA-specific T cells were co-cultured with $2x10^6$ NP-specific B cells with increasing NP-OVAL concentrations (right) for 72h. Supernatants were collected and analyzed for IL-10, IL-17A, TNFα, IFNγ, IL-6, IL-4 and IL-2 with cytometric bead array. IL-10, IL-17A, IL-4 and IL-2 were not detectable. Shown is one representative experiment out of two.

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Publications

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Poster presentation

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